Heterodimerization of c-erbB2 with Different Epidermal Growth Factor Receptor Mutants Elicits Stimulatory or Inhibitory Responses*

T. Spivak-Kroizman‡, D. Rotin‡, D. Pinchasi‡, A. Ullrich§, J. Schlessinger‡, and I. Lax‡

From the Department of Pharmacology, New York University Medical Center, New York, New York 10016 and the Max-Planck-Institut für Biochemie, 8033 Martinsried bei München, Federal Republic of Germany

Growth factors are polypeptides which stimulate an array of pleiotropic responses culminating in cell growth and differentiation. A large group of growth factors act by binding to surface receptors with intrinsic protein tyrosine kinase activity (reviewed by Ullrich and Schlessinger (1990)). Growth factor receptors with tyrosine kinase activity have different structural features. One of the best characterized family whose natural ligand is still unknown. We show that in response to EGF, wild type EGFR and various EGFR mutants were able to undergo heterodimerization with HER2. Addition of EGF to transfected cells co-expressing HER2 with a kinase negative point mutant of EGFR (K721A) stimulated heterodimer formation, tyrosine phosphorylation of K721A and HER2, and tyrosine phosphorylation of one of their known substrates, phospholipase Cγ. However, the binding of EGF to transfected cells co-expressing HER2 together with another EGFR mutant CD533 (a deletion mutant lacking most of the cytoplasmic domain of EGFR) caused heterodimerization and inhibition of tyrosine kinase activity. It appears therefore that EGF-induced heterodimerization of EGFR and HER2 can promote either stimulatory or inhibitory influences on kinase activity. We propose that the nature of receptor interactions on the cell surface can either activate or inhibit the initiation of growth factor-controlled cellular signaling.

Another member of the same receptor family (subclass I according to Ulrich and Schlessinger, 1990) is the neu proto-oncogene product (Bargmann et al., 1986a, 1986b) and its human counterpart termed either HER2 (Coussens et al., 1985) or c-erbB-2 (Yamamoto et al., 1986). Both EGFR and c-erbB-2 contain an extracellular domain possessing two similar cysteine-rich clusters, a single hydrophobic transmembrane domain, and a cytoplasmic domain containing tyrosine kinase activity and regulatory tyrosine and serine phosphorylation sites (reviewed by Ulrich and Schlessinger (1990)). While several natural ligands of EGFR have been characterized, the natural ligand(s) of HER2 are still poorly characterized.

It has been shown that a chimeric receptor composed of the extracellular ligand binding domain of EGFR and the cytoplasmic domain of HER2 is fully functional (Lee et al., 1989; Lehvaslaiho et al., 1989). The binding of EGF to transfected cells expressing this chimeric receptor stimulates tyrosine kinase activity, various early responses, mitogenesis, and transformation. These results indicate that EGFR and HER2 transduce similar activities in response to ligand binding and that activation of tyrosine kinase activity is mediated by a common mechanism. Receptor dimerization appears to be the universal mechanism underlying ligand-induced activation of growth factor receptors with tyrosine kinase activity (reviewed by Schlessinger (1979, 1988)). Receptor dimerization also sets the stage for autophosphorylation which is mediated by an intermolecular trans-mechanism (Honegger et al., 1989, 1990). Recent studies provide evidence that the negative dominant action of defective EGFR is caused by heterodimerization with wt EGFR leading to formation of inactive heterodimers (Kashles et al., 1991). This may represent the underlying mechanism for the negative dominant function of mutant c-kit in heterozygous W mice (Chabot et al., 1988; Reith et al., 1990; Tan et al., 1990).

Several laboratories have shown that the binding of EGF to EGFR leads both to tyrosine phosphorylation of either wt HER2 (King et al., 1988; Stern and Kamps, 1988; Ariyama et al., 1988) or a kinase negative mutant of neu and to functional interaction between the two receptors (Kokai et al., 1989; Connelly and Stern, 1990). Furthermore, EGF-induced heterodimerization of EGFR and HER2 was detected by utilizing bifunctional cross-linking agents followed by SDS-PAGE analysis (Wada et al., 1990).

In this report we show that soluble extracellular domain of EGFR (sEGFR) and soluble extracellular domain of HER2 (sHER2) undergo EGF-induced heterodimerization. Interestingly, EGF-induced heterodimerization between wt HER2 and an EGFR mutant lacking most of the cytoplasmic domain (CD533) generates an inhibitory influence on kinase activity of HER2. However, EGF is also able to induce autophosphorylation of a kinase negative EGFR point mutant (K721A) in

*This work was supported by grants from RPR central research (to J. S.) and from HFS (to J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; s, soluble extracellular domain; PBS, phosphate-buffered saline; PLCγ, phospholipase Cγ; DS5, disuccinimidyl suberate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

Page 8056

Received for publication, October 9, 1991
cells which co-express wt HER2. It appears therefore that heterodimerization between different EGFR mutants and HER2 may generate either an inhibitory or stimulatory effects on HER2 functions.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cell Lines**

The generation and characterization of expression vectors encoding either wild type human HER2 or EGFR and various receptor mutant receptors were previously described (Livneh et al., 1986; Honegger et al., 1987; Lax et al., 1991). NIH 3T3 cells, expressing wt human HER2 were cotransfected with constructs of various EGFR mutants together with the selectable marker hygromycin B phosphotransferase gene. Colonies resistant to 50 μg/ml hydromycin B were further analyzed.

**Preparation of Soluble Extracellular Ligand Binding Domains of EGFR and HER2**

Detailed description of the structure, expression, and purification of sHER2 and sEGFR have been described elsewhere (Lax et al., 1991).

125I-EGF Binding Experiments

Detailed 125I-EGF binding experiments and data analysis were previously described (Honegger et al., 1987; Kashles et al., 1991).

**[^35]S**Cysteine Labeling

Full details were previously described (Livneh et al., 1986; Honegger et al., 1987; Kashles et al., 1991).

Covalent Cross-linking Experiments

In Intact Cells—Cells grown in fibronectin-coated dishes were labeled overnight with [35S]cysteine, washed twice with Dulbecco's modified Eagle's medium containing 20 mM Hepes (pH 7.5), 0.1% bovine serum albumin, and incubated with or without 160 nM EGF for 1 h at 4 °C. The cells were washed twice with PBS containing 20 mM Hepes (pH 7.5) and treated with 25 mM 1-ethyl-3-[3-dimethylamino)propyl]carbodiimide (EDAC) in PBS/Hepes buffer for 45 min at room temperature. At the end of the reaction the cross-linker was quenched by addition of 10 mM glycine in PBS/Hepes for 10 min at room temperature. The cells were washed twice with cold PBS and lysed in lysis buffer. Immunoprecipitations were performed as described above (Kashles et al., 1991), and the proteins were separated on SDS-polyacrylamide gradient gels (4.5–10% acrylamide) and analyzed by autoradiography.

In Solution—The soluble extracellular domains of EGFR and HER2 (sEGFR and sHER2, respectively) were iodinated using the chloramine-T method to a specific activity of 2 × 10⁸ cpm/mol. Samples containing 5 μg sEGFR (mixed with 125I-labeled sHER2 as a tracer) or 5 μg sHER2 (mixed with 125I-labeled sHER2 as a tracer) individually, or containing both proteins, were incubated in the absence or presence of 10–15 μM EGF in 20 mM Hepes, pH 7.5, 150 mM NaCl, for 1 h at room temperature. The covalent cross-linking agent (0.25 mM) diacessimimidyl suberate (DSS) was incubated for an additional 45 min, followed by immunoprecipitation with either mAb 108 or mAb 4 and 10 for 90 min at room temperature. Samples were washed three times with HNTG, mixed with sample buffer, boiled for 5 min, and analyzed by 4–12% gradient SDS-PAGE and autoradiography.

**Tyrosine Phosphorylation in Living Cells**

Detailed description of analysis of tyrosine phosphorylation in living cells was previously described (Li and Schlessinger, 1991; Li et al., 1991).

In Vitro Autophosphorylation Experiments

Confluent cells were lysed in lysis buffer as described above, and the various receptors and their mutants were immunoprecipitated using mAb 108 against EGFR or mAbs 4 and 10 against HER2. For autophosphorylation, 1 μl of EGFR or 10 μl of HER2 were analyzed by SDS-PAGE and blotted onto nitrocellulose filters. The proteins were blotted with polyclonal antibodies to phosphotyrosine followed by treatment with [γ-32P]ATP and autoradiography.

**RESULTS**

**Generation of Cell Lines Co-expressing Human HER2 and Various EGFR Mutants—NIH-3T3 cells expressing human HER2 were co-transfected with either one of the following previously described EGF receptors:** 1) wt EGFR (Livneh et al., 1986), 2) a kinase negative point mutant of EGFR, K721A (Honegger et al., 1987; Chen et al., 1987), and 3) a deletion mutant lacking 533 carboxyl-terminal amino acids CD633 (Livneh et al., 1986; Kashles et al., 1991). Hygromycin was used to select resistant cell lines which were further screened for the expression of EGFR mutants utilizing 125I-EGF binding assays. As a control we used transfected cell lines which express EGFR, HER, or CD533 alone. The various cell lines were subjected to immunoprecipitation analysis with either mAb 108 or mAb 4 and 10, which are directed against the extracellular domains of EGFR and HER2 respectively. Fig. 1A shows that the expected proteins were detected after lysis, immunoprecipitation and SDS-PAGE analysis of HER2 (185 kDa), EGFR (170 kDa), or CD633 cells (115 kDa). Both HER2 and EGFR mutants were detected in the double transfants by immunoprecipitation analysis with mAb 108 (against EGFR) or mAb 4 and 10 (against HER2) (Fig. 1A, lanes 7–12). The various cell lines were also subjected to an in vitro phosphorylation assay. In these experiments, the cell lines were solubilized, immunoprecipitated with either monoclonal antibody mAb 108 or 4 and 10, incubated with [γ-32P]ATP and Mn²⁺, and analyzed by SDS-PAGE. Fig. 1B shows that phosphorylated HER2 was detected in all the double transfants. As expected, only wt EGFR was phosphorylated while K721A and CD633 mutants were not.

EGF-induced Heterodimerization of HER2 and EGFR—We have previously used chemical cross-linking agents to reveal EGF-induced dimerization of wt EGFR (Cochet et al., 1988) and various EGFR mutants (Kashles et al., 1991; Lax et al., 1991). Using a similar experimental approach we examined whether EGF is able to induce heterodimerization of HER2 with various EGFR mutants. For this purpose transfected cell lines co-expressing wt HER2 and one of the following three EGFR mutants were studied: HER2 together with wt EGFR, HER2/EGFR cells, HER2 with K721A (HER2/K721A cells), and HER2 with CD633 (HER2/CD633 cells). Trans-
Heterodimerization of c-erbB2 with Different EGFR Mutants

Heterodimerization of c-erbB2 with Different EGFR Mutants

**Fig. 1.** Expression of the various EGFR and HER2 mutants in transfected cell lines. A, transfected cell lines were labeled with [35S]cysteine overnight, lysed, and immunoprecipitated with either mAb 108 against EGFR, or with mAb 4 and 10 against HER2. Samples were subjected to SDS-PAGE analysis followed by autoradiography. Top, middle, and bottom arrows mark HER2, EGFR (ut or K721A), and CD533 mutants respectively (exposure: lanes 1 and 2, 3 h; lanes 5–12, 6 h at -70 °C). B, in vitro autophosphorylation of HER2 and EGFR. Following immunoprecipitation of solubilized cells using either mAb 108 or mAb 4 and 10, the samples were subjected to standard autophosphorylation reaction using [γ-32P]ATP and Mn

**Fig. 2.** Analysis of EGF induced dimerization of HER2, EGFR, and various mutants in living cells using covalent cross-linking agent. Cells were labeled overnight with [35S]cysteine, washed twice, and incubated with or without 60 nM EGF for 1 h at 4 °C. After two washes the cells were incubated for additional 45 min with the covalent cross-linking agent EDAC at room temperature. Following several washes, solubilization, and immunoprecipitation with either mAb 108 or mAb 4 and 10, the samples were analyzed by polyacrylamide gradient gel (4.5–10%) and autoradiography. The EGF-induced band at 355 kDa represents a heterodimer of HER2 and EGFR, at 340 kDa a homodimer of EGFR, at 300 kDa a homodimer of HER2 and CD533, and at 230 kDa a homodimer of CD533. EGF-induced dimerization of CD533 shown in a control experiment is lower than usual. We have previously described detailed analysis of EGF-induced CD533 dimerization (Kashles et al., 1991) (exposure: lanes 1 and 2, 6 h; lanes 3–12, 12 h at -70 °C).

The various cell lines were metabolically labeled with [35S]cysteine, incubated in the presence or absence of EGF, subjected to covalent cross-linking reacting with EDAC, lysed, immunoprecipitated with either mAb 108 or mAb 4 and 10, and analyzed by SDS-PAGE and autoradiography (Fig. 2). In cells not treated with EGF only monomeric forms of the various receptors were detected (Fig. 2, lanes 1, 3, 5, 7, 9, and 11). As previously reported, EGF induced the formation of homodimers of ut EGFR (340 kDa) or homodimers of the CD533 mutant (230 kDa) in cells which express each of the receptors alone (Kashles et al., 1991). However, under the same experimental conditions EGF-induced dimerization of HER2 was not observed. It is noteworthy that a small amount of HER2 homodimers were observed in the absence or presence of EGF (data not shown). This suggests that HER2 cells produce the so far unidentified HER2 ligand or that HER2 tends to dimerize even in the absence of ligand binding. These interpretations are also consistent with the fact that the tyrosine kinase activity of HER2 appears to be higher than the tyrosine kinase activity of EGFR in the absence of EGF (King et al., 1988).

A different pattern of cross-linked proteins was observed when similar experiments were performed with cells which co-express HER2 together with various EGFR mutants. The addition of EGF to HER2/EGFR cells led to formation of homodimers of ut EGFR (340 kDa) and heterodimers of HER2 with EGFR (355 kDa). The 340-kDa species was only recognized by mAb 108 (against EGFR) while the 355-kDa species was recognized by either mAb 108 or mAb 4 and 10 (against HER2) (Fig. 2 and data not shown). A similar pattern was observed in HER2/K721A cells. Namely, EGF induced homodimers of K721A mutant (340 kDa) and heterodimers of HER2 and K721A (355 kDa). The addition of EGF to HER2/CD533 cells led to the formation of two cross-linked higher molecular mass species; a 230-kDa complex which represents homodimers of CD533 and a 300-kDa complex which is recognized by both mAb 108 and mAb 4 and 10 thus representing heterodimers of HER2 and CD533. It is noteworthy that the efficiency of the covalent cross-linking reaction with EDAC is not more than 5–10% (Cochet et al., 1988).

The relative ratio of the various homodimeric and heterodimeric receptors does not reflect the actual stoichiometry of the products of the dimerization process. Therefore the results presented in Figs. 2 and 3 demonstrate HER2 and EGFR heterodimerization but do not provide adequate quantitative information concerning the stoichiometry of this process (Cochet et al., 1988; Kashles et al., 1991).
domains involved in the association between HER2 and EGFR: we have previously shown that in response to EGF, dimers of EGFR (340 kDa) and homodimers of K721A mutant EGFR (210 kDa), trimers (315 kDa), and high oligomerization states (Kd = (0.1-1) x 10^-10 M) were detected. Moreover, a heterodimeric complex of low affinity receptors was found to be phosphorylated constitutively (Fig. 4, lanes 6). Contents of lanes are as follows: EGF plus sHER2 (lane 1); EGF plus sEGFR and sHER2 (lanes 2 and 4); sEGFR plus sHER2 (lanes 3 and 5); EGF plus sHER2 (lane 6).

Domains of HER2 and EGFR—In order to delineate the regions involved in the association between HER2 and EGFR, we have used recombinant sEGFR and sHER2. The sEGFR and sHER2 were produced by transfected Chinese hamster ovary cells, secreted into the conditioned medium, and purified by affinity chromatography with monoclonal anti-receptor antibodies followed by ion exchange chromatography (Lax et al., 1991). We have recently shown that binding of EGF to sEGFR causes dimerization indicating that the extracellular domain of EGFR is endowed with at least two functions: specific ligand recognition and a capacity to undergo ligand dependent dimerization (Lax et al., 1991). Hence, a similar approach was used to determine whether sEGFR and sHER2 undergo EGF-dependent heterodimerization. In these experiments sEGFR and sHER2 were incubated with EGF, treated with the covalent cross-linking agent disuccinimidyl suberate (DSS), immunoprecipitated with specific antibodies, and analyzed by 4–10% SDS-PAGE. Because sEGFR and sHER2 have a similar apparent molecular mass of 105 kDa, it is impossible to resolve between homodimers and potential heterodimers by SDS-PAGE analysis. Therefore, [125I]-labeled sEGF was added to a mixture of sEGFR and sHER2 and monoclonal antibodies against HER2 (mAb 4 and 10) were utilized for the immunoprecipitation analysis. Similarly, [125I]-labeled sHER2 was added to a mixture of sEGFR and sHER2 and monoclonal antibodies against EGFR (mAb 108) were used for the immunoprecipitation analysis. This approach enabled unequivocal determination of EGFR-induced heterodimerization of sEGFR and sHER2 (Fig. 3).

We have previously shown that in response to EGF, dimers (210 kDa), trimers (315 kDa), and high oligomerization states of sEGFR were detected (Lax et al., 1991). Similar species were also detected in sEGFR and sHER2 mixtures in response to EGF. Parallel immunoprecipitation analyses with mAb 108 and mAb 4 and 10 indicated that both homo-oligomeric and hetero-oligomeric forms of sHER2 and sEGFR were formed. Because of the low efficiency of covalent cross-linking reaction it is impossible to quantitate the degree and stoichiometry of EGF-induced oligomerization process. It is clear however, that the extracellular domains of HER2 and HER2 play crucial roles in facilitating the process of EGF-induced heterodimerization.

Presence of HER2 Prevents the Display of High Affinity EGF-binding Sites—Quantitative binding experiments of [125I]-EGF to cells expressing either wt or kinase negative EGF receptor mutant K721A revealed the display 5–10% high affinity EGF receptors (K_D = (0.1–1) x 10^-10 M) and 90–95% of low affinity receptors (K_D = (5–1) x 10^-7 M) (Shoyab et al., 1979; King and Cuatrecasas, 1982). Similar [125I]-EGF binding experiments with transfected cell lines expressing CD533 revealed only low affinity binding sites (Livneh et al., 1986; Kashles et al., 1991). Moreover, the presence of CD533 in cells expressing wt EGFR eliminated the display of high affinity binding sites, suggesting that the dimeric state of wt EGFR receptors is responsible for generation of high affinity binding sites (Kashles et al., 1991). We have therefore performed [125I]-EGF binding experiments to cells which co-express HER2 with EGFR mutants to examine the influence of HER2 presence on binding characteristics. Table I shows that cell lines expressing either wt EGFR or K721A alone display typical high and low affinity binding sites for [125I]-EGF (Honegger et al., 1987). However, cells expressing CD533 display only low affinity binding sites (Livneh et al., 1986). Interestingly, only low affinity binding sites for EGF were detected in the double transfectants HER2/EGFR cells and HER2/K721A cells (Table I). This result is consistent with the idea that the dimeric state of EGFR is responsible for the display of high affinity EGF-binding sites (Schlessinger, 1988; Kashles et al., 1991). Our results suggest that formation of heterodimers of HER2 with EGFR diminishes the amount of EGFR homodimers which are responsible for high affinity binding.

Phosphorylation of EGFR and HER2 in Living Cells—Cells co-expressing HER2 and one of EGFR mutants (wt EGFR, K721A, or CD533) and cells expressing each receptor alone were labeled with [35S]cysteine and incubated in the absence of presence of EGF for 1 h at 4°C. After cross-linking with EGF the samples were lysed and immunoprecipitated with monoclonal antibodies against phosphotyrosine covalently attached to Sepharose beads. The tyrosine-phosphorylated proteins were eluted from the beads using phenyl phosphate and further immunoprecipitated with either anti-EGFR mAb 108 (Fig. 4, lanes 3–12) or anti-HER2 mAb 4 and 10 (Fig. 4, lanes 1 and 2). The samples were analyzed by 4–12% SDS-PAGE and autoradiography. Fig. 4 shows that tyrosine-phosphorylated proteins were detected only in EGF-treated cells, except HER2 cells in which monomeric tyrosine-phosphorylated species (185 kDa) and dimeric (370 kDa) species were found to be phosphorylated constitutively (Fig. 4, lanes 1 and 2). As mentioned before these results are consistent with the fact that the transfected cells may produce a HER2 ligand or that the HER2 receptors dimerize even in the absence of ligand binding.

In response to EGF, cells expressing wt EGFR alone contained both tyrosine phosphorylated monomers (170 kDa) and dimers (340 kDa) (Fig. 4, lanes 3 and 4) (see also Kashles et al. (1991)). An interesting pattern of phosphorylated proteins was observed in double transfectants HER2/EGFR cells and HER2/K721A cells. In addition to the monomeric tyrosine-phosphorylated species, tyrosine-phosphorylated homodimers of EGFR (340 kDa) and homodimers of K721A mutant (340 kDa) were detected. Moreover, a heterodimeric complex

---

8 A. Hudziak and A. Ullrich, submitted for publication.
Heterodimerization of c-erbB2 with Different EGFR Mutants

Parameters of 125I-labeled-EGF binding results to EGFR expressed on transfected cells analyzed according to the method of Scatchard (1949). $K_i$ and $N_i$ are dissociation constants of low and high affinity binding sites, respectively. $N_i$ and $N_o$ are numbers of low and high affinity binding sites per cell, respectively. Presented are average values ± standard deviations for three different binding experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$K_i$ (M$^{-1}$)</th>
<th>$N_i$ (receptor/cell)</th>
<th>$K_o$ (M$^{-1}$)</th>
<th>$N_o$ (receptor/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2</td>
<td>(6 ± 0.4) x 10^{-9}</td>
<td>(2.0 ± 0.4) x 10^{10}</td>
<td>(0.7 ± 0.2) x 10^{-11}</td>
<td>(1.8 ± 4) x 10^{10}</td>
</tr>
<tr>
<td>EGFR</td>
<td>(5 ± 1) x 10^{-9}</td>
<td>(3.0 ± 0.4) x 10^{10}</td>
<td>(0.6 ± 0.3) x 10^{-11}</td>
<td>(9 ± 3) x 10^{10}</td>
</tr>
<tr>
<td>K721A</td>
<td>(7 ± 4) x 10^{-9}</td>
<td>(10 ± 2) x 10^{10}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD533</td>
<td>(4 ± 2) x 10^{-9}</td>
<td>(3.5 ± 1.5) x 10^{10}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2/EGFR</td>
<td>(3.5 ± 1.3) x 10^{-9}</td>
<td>(3.2 ± 1.3) x 10^{10}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2/K721A</td>
<td>(5 ± 2) x 10^{-9}</td>
<td>(3 ± 1) x 10^{10}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2/CD533</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results show that binding of EGF leads to heterodimerization between HER2 and K721A. This process probably activates the kinase of HER2, which in turn phosphorylates phosphorylated homodimers of K721A which are trapped by the action of the covalent cross-linking agent.

An interesting result was also obtained for HER2/CD533 cells subjected to the same analysis. However, in this experiment no tyrosine-phosphorylated proteins were detected using the same anti-phosphotyrosine affinity purification approach and the same experimental protocol (Fig. 4, lane 5, 6, 11 and 12). Immunoprecipitation of the same lysates with mAb 108 depicted the presence of 110-kDa monomeric and the 230-kDa EGF-induced dimers in CD533 cells. Treatment with EGF of HER2/CD533 dimers led to the appearance of 230-kDa homodimer and the 300-kDa heterodimer. However, these proteins were not phosphorylated indicating that the formation of heteromers with CD533 prevented activation of the tyrosine kinase of HER2. Similar negative dominant influence of CD533 was observed in cells which express wt EGFR (Kashles et al., 1991).

Our results show that EGF-induced heterodimerization with K721A has positive influence on HER2 activity leading to tyrosine phosphorylation of K721A. However, EGF-induced heterodimerization between HER2 and CD533 has negative influence on HER2 activity preventing tyrosine kinase activity. In this case heterodimerization with EGFR deletion mutant functions as a dominant negative mutation, suppressing the activation and response of HER2.

EGF-induced Association between HER2 and PLCγ in Double Transfected Cells—We have examined the possibility whether enhanced tyrosine phosphorylation of EGFR and HER2 caused by heterodimerization with K721A mutant is able to modulate interactions with a known cellular substrate. It is well established that activation of EGFR leads to association of PLCγ with phosphorylated EGFR and to tyrosine phosphorylation of PLCγ (Margolis et al., 1989, 1990a, 1990b; Meisenhelder et al., 1989; Wahl et al., 1989). The SH2 domain of PLCγ and other signaling molecules serve as binding sites for activated tyrosine-phosphorylated receptors (reviewed by Koch et al., 1991). Moreover, the tyrosine-phosphorylated carboxyl-terminal tail of EGFR represents the binding site for the SH2 domain of PLCγ and other SH2-containing signaling molecules (Margolis et al., 1990b; Skolnik et al., 1991). We have therefore examined the capacity of PLCγ to become tyrosine-phosphorylated by and associated with HER2 and EGFR in the various cell lines described in this report. For this purpose the various cells were treated with EGF, lysed, subjected to immunoprecipitation with anti-PLCγ antibodies or with mAb 108 and mAb 4 and 10, and subsequently immunoblotted with antibodies against phosphotyrosine. As expected the CD533 mutant or the K721A mutant did not become associated with PLCγ, while wt EGFR was co-immunoprecipitated by anti-PLCγ antibodies after exposure to EGF (Fig. 5A). Similar experiments were done with the double transfecteds. Either HER2/EGFR or HER2/K721A cells were treated with EGF, lysed, and subjected to immunoprecipitation with anti-PLCγ (Fig. 5A, lanes 1, 2, 5 and 6). In response to EGF, wt EGFR and K721A were not detected by co-immunoprecipitation with anti-PLCγ antibodies. In spite of the fact that tyrosine phosphorylation of EGFR and K721A was observed, only tyrosine-phosphorylated
HER2 was detected by co-immunoprecipitation with anti-PLCγ antibodies. We have previously shown that 1–2% of EGFR molecules were co-immunoprecipitated with PLCγ antibodies (Margolis et al., 1989 and 1990). It is possible that PLCγ binds with higher affinity to tyrosine-phosphorylated HER2 than to tyrosine-phosphorylated EGFR. Moreover, the double transfectants express approximately 2-fold more HER2 than EGFR. The limited amount of available PLCγ molecules may bind to the activated HER2 rather than EGFR molecules. In order to examine this possibility, similar experiments were performed under in vitro conditions using a higher concentration of recombinant SH2 domain of PLCγ not limited by the level of endogenous cellular PLCγ. A recombinant fragment containing two SH2 domains and one SH3 domain of PLCγ was expressed in Escherichia coli as glutathione S-transferase fusion protein and purified using glutathione-agarose beads (Rotin et al., 1992). Our results show that EGF-induced tyrosine-phosphorylated HER2, EGFR, and K721A, which were phosphorylated in the context of double transfected cells, bind to SH2 domain containing recombinant protein immobilized on beads (Fig. 6). It is clear that the binding of EGF to cells co-expressing K721A and HER2 leads to tyrosine phosphorylation of PLCγ (Fig. 6). Since tyrosine phosphorylation of PLCγ is essential for its activation (Kim et al., 1991) it is possible that heterodimerization could provide a stimulatory role for intracellular signaling pathways.

**DISCUSSION**

Numerous studies provide evidence that receptors with tyrosine kinase activity are activated by ligand-induced dimerization initiated by ligand binding to the extracellular domain (Schlessinger, 1978, 1988). Dimerization of adjacent extracellular domains brings about interaction between juxtaposed cytoplasmic domains. These interactions caused activation of the tyrosine kinase by an intramolecular mechanism followed by rapid trans-autophosphorylation (Honegger et al., 1988, 1989). Since receptor dimerization is essential for activation, formation of heterodimers between inactive EGFR mutants prevented the activation of normal EGFR expressed in the same cells (Kashles et al., 1991) and inhibited transformation caused by overexpression of EGFR (Redemann et al., 1992). Similarly, it has been shown that inactive insulin receptors or inactive c-kit receptors have a negative dominant influence on normal insulin receptor or c-kit in cell culture and in the living W mouse, respectively (Chou et al., 1987; Chabot et al., 1988; Reith et al., 1990; Tan et al., 1990).

In this report we describe the analysis of interactions between EGFR and HER2, two members of the same receptor subfamily (reviewed by Ullrich and Schlessinger (1990)). We have used transfected cell lines expressing mixed populations of wt HER2 and one of the following EGFRs: wt EGFR, kinase negative point mutant K721A, and deletion mutant CD533. Using this experimental system we explored the structural requirements and functional significance of EGF-induced heterodimerization of these two similar, yet distinct receptor molecules.

Our data show that in response to EGF, soluble, recombinant extracellular ligand bindings domains shER2 and sEGFR undergo heterodimerization. The heterodimerization between HER2 and EGFR prevents the display of high affinity binding sites for EGF. This provides further support for the hypothesis that heterodimers of EGFR are responsible for the generation of high affinity EGFR receptors (Schlessinger, 1988). Our results are in conflict with a report by Wada et al. (1990) which described enhanced binding affinity towards 125I-EGF in cells co-expressing EGFR and HER2. The reason
for the discrepancy is not clear.

The binding of EGF to its own receptor triggers rapid tyrosine phosphorylation of either wt or kinase negative mutant of HER2 (Connelly and Stern, 1990). Our study shows that tyrosine phosphorylation of HER2 is mediated not only by cross-phosphorylation of kinase negative new by wt EGFR (Connelly and Stern, 1990) but also by heterodimer formation between the kinase negative mutant of EGFR (K721A) and wt HER2. The binding of EGF to cells co-expressing K721A and HER2 caused elevation of tyrosine autophosphorylation of HER2 and K721A and stimulation of tyrosine phosphorylation of (and association with) PLCγ. We propose the following interpretation for these results. The binding of EGF to the extracellular domain of K721A stimulates heterodimerization with the extracellular domain of HER2. This process juxtaposes the cytoplasmic domains of K721A and HER2 which in turn results in cross-tyrosine phosphorylation of K721A. Since dimerization is a reversible process, some of the heterodimers will dissociate to form HER2 and K721A monomers. In the presence of EGF some of the monomeric-tyrosine phosphorylated K721A molecules will dimerize to form homodimers of tyrosine-phosphorylated K721A. Our results suggest that following activation, HER2 can tyrosine-phosphorylate other HER2 molecules by a trans-membrane interaction between the extracellular domains of HER2 molecules will interact with the SH2 domain of PLCγ enabling tyrosine phosphorylation of PLCγ. We have recently shown that the interaction between tyrosine-phosphorylated carboxyl-terminal tail of EGFR and the SH2 domain of PLCγ is the limiting factor for phosphorylation of PLCγ. Moreover, tyrosine phosphorylation of PLCγ is essential for its activation (Kim et al., 1991). It is therefore likely that the EGF-induced heterodimerization of HER2 and K721A may lead to stimulation of phospholipase C activity.

We reason that the relative concentration of K721A and HER2 could lead to either stimulatory or inhibitory effects. At low concentrations of K721A (K721A ≪ [HER2]) significant amount of heterodimers will be formed and stimulation of kinase activity of HER2 by EGF will not be detected. When [K721A] ≳ [HER2], approximately 50% of dimers will be heterodimers enabling stimulation of HER2 kinase activity in response to EGF. However, at high concentration of K721A ([K721A] ≳ [HER2]), mainly homodimers of K721A will be formed preventing the activation of HER2 leading to a negative dominant influence similar to the effect of CD553 on kinase response of wt EGFR (Kashles et al., 1991; Redemann et al., 1992). Indeed, we have previously shown that high concentration of K721A was able to decrease the mitogenic response of wt EGFR expressed in the same cells (Honegger et al., 1990). Similarly, excess of kinase negative mutant of insulin receptor suppressed the action of wt insulin receptors co-expressed in the same cells (Chou et al., 1987; McClain et al., 1987).

In addition to the quantitative influence of receptor concentration the nature of the neighboring receptor can lead to either negative or positive influences. Our results show that the kinase domain of HER2 is active in the context of a heterodimer. If the neighboring cytoplasmic domain is minimally mutated and it contains autophosphorylation sites such as K721A, trans-phosphorylation could take place. However, if the neighboring molecule lacks a cytoplasmic domain such as CD553, an inactive heterodimer will be formed and tyrosine phosphorylation will be suppressed. These results are also in accord with our earlier studies with EGFR demonstrating that autophosphorylation is mediated by an intermolecular mechanism (Honegger et al., 1988, 1990). It has been proposed that the regulatory response is mediated by heterodimerization proportional to $K_a/K_d$ where $K_a$ is the dissociation constant of the dimeric receptor and $K_d$ is the dissociation constant of the monomeric form towards their ligand (Schlessinger, 1988).

However, when only one of the constituents of the heterodimer is occupied, such as in the case of HER2/K721A heterodimers or HER2/CD553 heterodimers, the regulatory response is proportional to $K_d/K_a$ (Schlessinger, 1988). Hence, homodimeric interactions mediated by a single ligand molecule produce a stronger regulatory signal than heterodimeric interactions.

All these studies indicate that interactions at the cell surface between growth factors and their receptors are complex and do not necessarily involve a simple binding and interaction between one ligand and one receptor. Freedom of motion within the two-dimensional lipid bilayer facilitated by rapid translational and rotational diffusion (Schlessinger, 1979; Schlessinger and Elson, 1982) enables efficient lateral interactions and communication between surface receptors. This will provide important regulatory role which will extend and amplify initial interactions occurring at the cell surface.

REFERENCES


Livneh, E., Frywes, R., Kashles, O., Reiss, N., Sasson, I., Mor, Y., submitted for publication.
Heterodimerization of c-erbB2 with Different EGFR Mutants

Heterodimerization of c-erbB2 with different epidermal growth factor receptor mutants elicits stimulatory or inhibitory responses.
T Spivak-Kroizman, D Rotin, D Pinchasi, A Ullrich, J Schlessinger and I Lax


Access the most updated version of this article at http://www.jbc.org/content/267/12/8056

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/267/12/8056.full.html#ref-list-1