

Transformation of NIH 3T3 Cells by HER3 or HER4 Receptors Requires the Presence of HER1 or HER2*

(Received for publication, September 20, 1995, and in revised form, November 8, 1995)

Ke Zhang‡, Jilin Sun, Naili Liu, Duanzhi Wen, David Chang, Arlen Thomason, and Steven K. Yoshinaga

From Amgen Inc., Thousand Oaks, California 91320

Members of the epidermal growth factor receptor (EGFR) subfamily of receptor protein tyrosine kinases have been implicated in the pathogenesis of various malignancies. The ability of one EGFR subfamily member to influence, or function synergistically with, another is likely to be a general feature of these receptors. To assess the role of receptor heterodimerization, we analyzed the ability of Neu differentiation factor (NDF) to induce cell growth and transformation of NIH 3T3 cells transfected with different combinations of the EGFR subfamily of receptors. NDF induced mitogenesis, but not transformation, of cells expressing either HER3 or HER4 alone. However, NDF-induced cell transformation was observed when either HER1 or HER2 was coexpressed with HER3 or HER4. In analogous receptor phosphorylation experiments, NDF-induced transphosphorylation appears to be correlated with synergistic transformation of NIH 3T3 cells. Interestingly, transphosphorylation between HER1 and HER4 can be stimulated by either EGF or NDF.

The epidermal growth factor receptor (EGFR)¹ subfamily of receptor protein tyrosine kinases consists of four known proteins, HER1 (also known as EGFR or ErbB1), HER2 (p185 Neu or ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (1, 2). Members of this subfamily have been implicated in various malignancies. Viral *v-erbB1* was discovered as a potent oncogene and is the determinant for avian erythroblastosis virus-induced neoplasia (3). Another member of this family, p185^{neu}, was first isolated from chemically induced rat neuroblastomas based on its ability to transform NIH 3T3 cells (4, 5). Both the *v-erbB1* and p185^{neu} oncogenes are genetically altered versions of their cellular counterparts (3, 6). The proto-oncogenes of this family are often amplified in various carcinomas, suggesting that the products of the normal genes may also play a role in carcinogenesis (1). This is supported by *in vitro* experiments in which the overexpression of wild type HER2 caused transformation of NIH 3T3 cells (7, 8) even in the absence of its ligand. Overexpression of HER1 also transforms NIH 3T3 cells, but only in the presence of its ligand (9–11). Recent studies show that HER3 enhances the transforming activity of HER2 when coexpressed in NIH 3T3 cells. It is not clear, however, whether the effect requires the presence of NDF, since endogenous NDF

was expressed by the cells (12). Similar studies of HER4 have not been reported.

There are a number of different peptides that bind and activate HER1, including EGF, transforming growth factor α , amphiregulin, heparin-binding EGF, and betacellulin (13, 14). In contrast, NDF (Neu differentiation factor, also called heregulin) is the only known ligand for the other members of the EGFR family. NDF was initially isolated as a ligand for HER2 (15–17). However, recent results demonstrate that HER3 and HER4 are the primary receptors for NDF (18–20) and that HER2 is phosphorylated in a NDF-dependent manner, perhaps by heterodimeric association with either HER3 or HER4 (12, 19, 21, 22). NDF has a higher affinity for heterodimers of HER2 and HER4 than for HER4 homodimers (23). Previous reports also indicated that HER1 and HER2 heterodimerize, transphosphorylate, and transform cells in response to EGF (24–26). It can be concluded that HER2 forms heterodimers with every other identified member of the EGFR subfamily.

Recently, an interaction between HER1 and HER3 has also been demonstrated in several laboratories (27–30). EGF treatment of cells leads to phosphorylation of HER1 and HER3 and to the association of phosphatidylinositol 3-kinase (PI 3-kinase) with HER3, apparently activating a signaling pathway different from HER1 alone. In fact, NDF was also found to stimulate PI3 kinase activity, but only through heterodimers of HER2 and HER3 (31). Therefore, the association of HER3 with either HER1 or HER2 can elicit signaling through the PI3 kinase pathway.

Although it is clear that members of the EGFR subfamily may form heterodimers and transphosphorylate in response to NDF, the role of receptor heterodimerization in cell growth and transformation by NDF is not known. Previous studies of receptor heterodimerization were done in disparate cell lines and under a variety of conditions. Comprehensive studies of the functions of all receptor combinations in a uniform manner were also hampered by the endogenous expression of some of these receptors in the cell lines. To clarify the function of heterodimerization in NDF signaling, we introduced various combinations of EGFR subfamily members into a cell line that lacks detectable endogenous levels of these receptors. We demonstrate that NDF can stimulate mitogenesis in NIH 3T3 cells that express either HER3 or HER4. However, NDF-dependent transformation by HER3 or HER4 relies on the coexpression of either HER1 or HER2. The transforming activities of these receptors are correlated with their ligand-induced transphosphorylation.

MATERIALS AND METHODS

cDNAs—Full-length cDNA molecules encoding individual HER1, HER3, and HER4 receptors were isolated with oligonucleotide probes designed according to the published DNA sequences (2, 32, 33). The libraries used to isolate these cDNAs were a human placenta cDNA

* 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.

‡ To whom correspondence should be addressed: 1840 DeHavilland Dr., Thousand Oaks, CA 91320-1789 Tel.: 805-447-2381; Fax: 805-447-1982; E-mail: kzhang@amgen.com.

¹ The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PI, phosphatidylinositol.

library (EGFR), an SKBR-3 breast tumor cell cDNA library (HER3), and a human fetal brain cDNA library (HER4). The cDNA encoding HER2 was obtained from Dr. Dennis Slamon (University of California, Los Angeles). All cDNAs were subcloned into the mammalian expression vector, pEV7, which was constructed by subcloning the Harvey murine sarcoma virus sequence into the pUC19 vector (data not shown). The resultant plasmids are designated as pEV7-HER1, pEV7-HER2, pEV7-HER3, and pEV7-HER4.

Focus Assay and Isolation of Stable Receptor-expressing Cell Lines—The NIH 3T3-7d cells are a subclone of the NIH 3T3 cell line. The parental NIH 3T3 cells were originally obtained from Dr. Douglas R. Lowy (National Cancer Institute). The cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37 °C and in a 5% CO₂ atmosphere. NIH 3T3-7d was selected from a number of individually isolated clones of NIH 3T3 cells that were analyzed for the lack of expression of the EGFR receptor. The established cell lines expressing different members of the EGFR subfamily were grown in the same medium as the parent cells, except 0.5 mg/ml G418 (Life Technologies, Inc.) was added.

For the focus assay, 300 ng of each receptor expression plasmid was used in single or double transfections. The plasmids were cotransfected with 5 ng of pSV2Noe by the calcium phosphate precipitation method as described previously (34). The cells were grown in normal growth medium for 5 days and then were changed to medium with or without growth factors and cultured for an additional 10 days. The plates were stained with methylene blue, washed with 70% ethanol, and analyzed for the presence of foci. All transfection experiments were repeated on at least three separate occasions. To isolate the cells expressing different receptors, the transfected cells were grown in normal medium containing 0.5 mg/ml G418 for 2 weeks.

Tritiated Thymidine Assay—The transfected cells were plated in six-well plates at a density of 3×10^5 /well and were grown in normal medium containing 10% fetal bovine serum for 26 h. The cells were switched to serum-free medium for 6 h and then changed to serum-free medium with or without growth factors for 14 h. Two microcuries of [³H]thymidine were added to each well, and the cells were incubated for an additional hour. The cells were then harvested, transferred to glass microfiber filters (GF/C, Whatman), and washed with 5% trichloroacetic acid and water. The radioactivity was measured by scintillation counting (Ecocint, National Diagnostics).

Immunoprecipitation and Western Blot Analysis—The anti-phosphotyrosine antibody (anti-Tyr(P), 4G10) was obtained from Upstate Biotechnology, Inc. Monoclonal HER1 antibodies (Ab-3 and Ab-5) and HER2 antibodies (Ab-5) were obtained from Oncogene Science. Polyclonal HER4 antibody (c-18) was obtained from Santa Cruz Biotechnology. Monoclonal HER3 antibodies (S3, S4, and S5) were prepared as follows. Eight-week-old female BALB/c mice were immunized several times with *Escherichia coli*-derived soluble human HER3 at 3-week intervals. The mice were first immunized subcutaneously with 50 µg of soluble HER3 emulsified in Freund's complete adjuvant, which was followed by subcutaneous injections with 25 µg of the same immunogen emulsified in Freund's incomplete adjuvant. Three days after the final immunization, the mice were treated intravenously with 25 µg of soluble HER3 in phosphate-buffered saline. Spleen cells from mice with high antibody titer were fused with SP2/0 myeloma cells at a ratio of 4:1 by a modification of the procedure described by Kohler and Milstein (35). Hybridoma supernatants were screened and characterized by enzyme-linked immunosorbent assay, Western blot, and BIAcore analysis (Pharmacia Biotech Inc.) (36, 37).

For detection of receptor proteins, the cells were grown to near confluence in 100-mm dishes, washed twice with cold phosphate-buffered saline, and lysed in 2 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM dithiothreitol, 0.1 trypsin inhibitor unit/ml of aprotinin, 10 µM phenylmethylsulfonyl fluoride, and 0.4 mM vanadate). The cell lysates were clarified by microcentrifugation at 10,000 rpm for 10 min at 4 °C. In the receptor phosphorylation experiments, the lysates were prepared in the same manner, except that the cells were serum-starved for 20 h and then treated with or without growth factors for 5 min before lysis.

Immunoprecipitation and Western blot analysis were performed essentially as described (38). Equal amounts of protein were used for immunoprecipitations. For immunoprecipitation of HER1, 1 µg each of the two monoclonal antibodies were mixed; for HER2, 1 µg of monoclonal antibody was used; for HER3, 2.5 µg of each of the three monoclonal antibodies were mixed; and for HER4, 2.5 µg of polyclonal antibody was used. The lysates and antibodies were mixed gently at 4 °C for 1 h. The immune complexes were collected with either protein A-Sepharose or protein G-Sepharose (Pharmacia) and were then washed

three times with lysis buffer. Bound proteins were released by boiling for 3 min in an equal volume of 2 × protein sample buffer (0.1 M Tris-HCl, pH 6.8, 40% glycerol, 0.004% bromophenol blue, 2% SDS, and 4% β-mercaptoethanol). The proteins were separated by electrophoresis on a 6% SDS-polyacrylamide gel and were transferred to a nitrocellulose membrane. The filters were blocked overnight with 5% bovine serum albumin and 0.5% Tween 20 in Tris-buffered saline for detecting the receptors or 5% bovine serum albumin, 1% ovalbumin, and 0.2% Tween 20 in Tris-buffered saline for detecting phosphotyrosine and were probed with specific antibodies in the blocking solutions. The signals were visualized with peroxidase-conjugated secondary antibodies and the ECL system (Amersham Corp.).

RESULTS

Biological Functions of HER3 and HER4 in NIH 3T3 Cells—The biological functions of the receptors in the EGFR family were examined in a subclone of the NIH 3T3 cell line, designated NIH 3T3-7d, which, unlike the original NIH 3T3 cell clone (10), does not express detectable levels of any known EGFR subfamily member. With these cells, we can unambiguously assess the interactions of the receptors of the EGFR subfamily without the effects of endogenous family members. To help normalize the efficiency of expression, all cDNAs encoding HER1, HER2, HER3, and HER4 were subcloned into an expression vector, pEV7, in which the expression of the inserted gene is under the control of the Harvey murine sarcoma virus long terminal repeat. The resultant plasmids were designated as pEV7-HER1, pEV7-HER2, pEV7-HER3, and pEV7-HER4, respectively.

In order to test if HER3 and HER4 can stimulate cell growth after activation by NDF, cells expressing either HER1, HER3, or HER4 were established as described under "Materials and Methods." These cells, as well as the parental cells, were analyzed for a mitogenic response using a tritiated thymidine incorporation assay. The results are shown in Fig. 1. The untransfected parental cells were not stimulated by either EGF or NDF. The lack of response to these factors indicates that no receptors of the EGFR subfamily are expressed in the parental cells. Cells transfected with the HER1 respond to stimulation by EGF, whereas cells transfected with either HER3 or HER4 responded to stimulation by NDF. Under these conditions, significant stimulation occurred at 1 ng/ml of NDF, with maximal stimulation at a concentration of 10–50 ng/ml. Interestingly, the maximal level of stimulation by EGF in HER1 expressing cells is three times higher than the maximum level induced by NDF in either HER3 or HER4 expressing cells.

Previous studies of HER1 showed that transformation of NIH 3T3 cells by the overexpression of HER1 depends upon the presence of its ligands (9–11). To determine if HER3 and HER4 can also transform NIH 3T3 cells in the same manner, the respective plasmids were individually transfected into cells and assayed for focus formation. The results are shown in Fig. 2. Cells transfected with the vector alone were not transformed. The expression of HER1 elicited significant cell transformation, as revealed by focus formation, only in the presence of EGF. Transfection by either HER3 or HER4 did not cause transformation of NIH 3T3 cells, even in the presence of NDF. In contrast, transfection with HER2 produced a small number of foci, in the presence or absence of NDF.

Synergistic Effect of EGFR Family Members—*In vitro* experiments on several different cell lines have shown that HER2 can form heterodimers with both HER3 and HER4 and that NDF activates HER2 only when it forms heterodimers with either of these two receptors (21, 23). Overexpression of these receptors has been observed in some cancers (39–41). These observations raise the possibility that heterodimers of EGFR family members may play important roles in oncogenesis. To understand the functional difference between heterodimers and homodimers of these receptors, we analyzed the transform-

ing activity of heterodimers in the presence or absence of NDF. Cells cotransfected either with HER2 and HER3, or with HER2 and HER4, were compared with cells transfected with either HER2, HER3, or HER4 alone. The results are shown in Fig. 3. In the absence of NDF, cells transfected with either receptor combination formed about the same number of foci as cells transfected with HER2 alone. However, the number of foci in the cells transfected with HER2 and HER3, or with HER2 and HER4, significantly increased in the presence of NDF. As shown in Fig. 2, NDF had no effect on focus formation in cells transfected by either HER2, HER3, or HER4 alone. In both the single and double transfection experiments, 300 ng of each receptor plasmid was transfected into the cells. The results were similar when 600 ng of receptor plasmid DNA was used in the single transfections and when 300 ng of each receptor

plasmid were used in the double transfections (data not shown), suggesting that the synergy observed was not dependent on the amount of DNA transfected into the cells. In addition, the synergy was not due to an increased affinity of the ligand for the heterodimers, because an excess amount of ligand was used in all the experiments.

Similar synergistic effects were observed when HER1 was coexpressed with either HER3 or HER4 (Fig. 4). Like the single plasmid transfections, cotransfection of either HER1 and HER3, or HER1 and HER4, did not transform NIH 3T3 cells in the absence of ligand. However, addition of NDF induced focus formation in cells transfected with either of the indicated receptor pairs. NDF failed to induce focus formation in cells transfected separately by either HER1, HER3, or HER4.

The transformation results of NIH 3T3 cells transfected with either one or two receptors of the EGFR subfamily, in all possible combinations, are summarized in Table I. There was no synergistic effect of the receptors in absence of the ligands. In the presence of NDF, transformation required either HER1 or HER2 cotransfected with HER3 or HER4. However, there was no synergy when HER3 and HER4 were cotransfected together. In the presence of EGF, more foci were developed by cells cotransfected with HER1 and HER2 than that transfected with HER1 alone. The synergy between HER1 and HER2 was consistent with the results reported previously (42, 43). In contrast, EGF activation of the cells transfected with HER1 together with HER3 or HER4 was not greater than that of the cells transfected with HER1 alone.

Receptor Protein—To confirm the expression of the receptors in the transfected cells, we analyzed the receptor proteins by immunoprecipitation followed by Western blotting. Cells transfected with the individual receptor plasmids, or with combinations of two receptor plasmids, as described above, were analyzed. A Western blot of the receptors from these cells is shown in Fig. 5. The NIH 3T3-7d cells, a subclone of NIH 3T3 cells, used in these assays did not express detectable levels of any of the receptors analyzed. This is consistent with the results of the [³H]thymidine incorporation assay, in which the parental cells did not respond to either EGF or NDF. The expression of the receptors in the transfected cells indicates that every cell line expressed the appropriate genes. The level of a particular receptor did not vary significantly when expressed individually or in combination with another receptor. The weak signal of the HER3 protein on the Western blot (Fig. 5), relative to the other HER proteins, was observed in all transfections. This may be due to the low affinity of the HER3 antibodies used in the experiments or to the low level of HER3 protein expressed in these cells.

Phosphorylation of Receptors—The mechanism of the synergistic transforming activity of the receptor pairs is not clear. Since HER2 alone has shown ligand-independent transforming

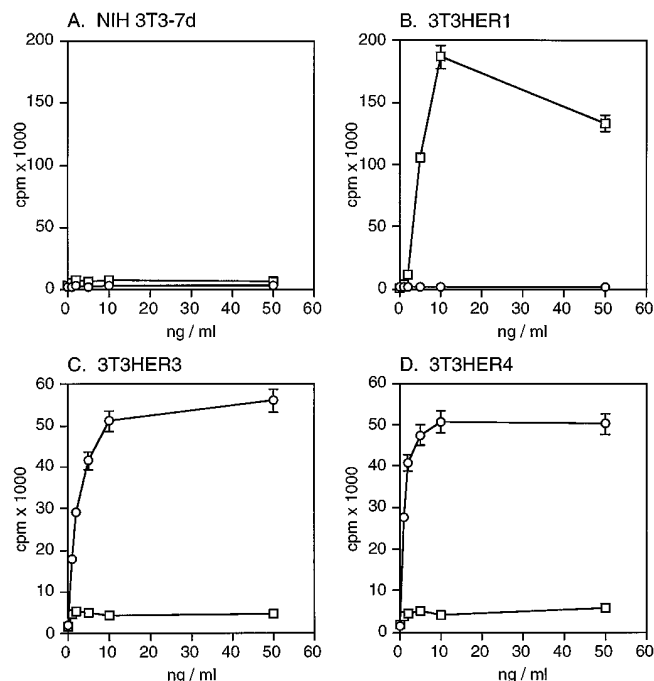


FIG. 1. Stimulation of NIH 3T3 cell growth by EGF and NDF. Cell lines expressing HER1, HER3, and HER4 were established by selection in medium containing 0.5 mg/ml G418 after transfection with the appropriate plasmids. Cells were grown in six-well plates at a density of 5×10^5 cells/well for 24 h in normal medium and then in serum-free medium for 6 h. The cells were changed to serum free medium containing 0, 1, 2, 5, 10, and 50 ng/ml of EGF (squares) or NDFβ1 (circles) and were incubated for an additional 18 h. The cells in each well were labeled with 2 μ Ci of tritiated thymidine for 1 h, then immediately harvested and transferred onto glass filters. The tritiated thymidine incorporation was determined by scintillation counting. The results shown are the average of three experiments.

FIG. 2. Transformation of NIH 3T3 cells by members of the EGFR family. NIH 3T3 cells were transfected with control plasmid, HER1, HER2, HER3, or HER4. For each 35-mm plate of cells, 300 ng of plasmid DNA were transfected. 24 h after transfection, the cells were diluted 1:5 in normal growth medium and split into two 60-mm plates. After 5 days, growth factors were added to one set of cells. For cells transfected with HER1, 20 ng/ml EGF was used. For cells transfected with control plasmid, HER2, HER3, and HER4, 50 ng/ml of NDFβ1 was used. The plates were stained with ethylene blue 15 days after transfection.

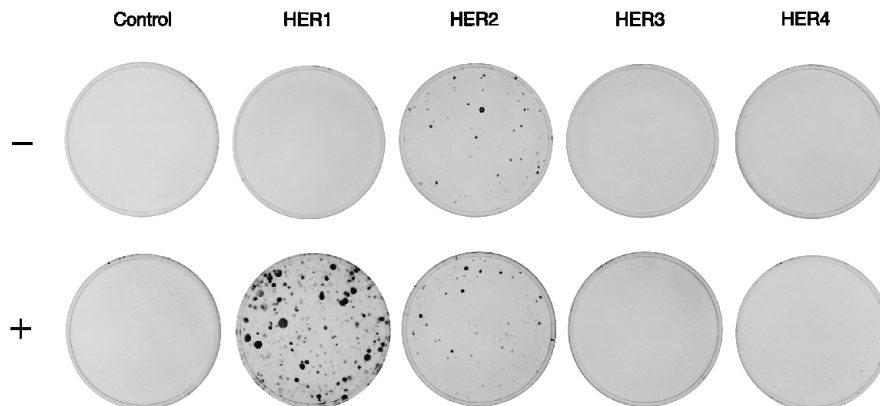


FIG. 3. NDF-induced transformation of NIH 3T3 cells cotransfected by HER2 with either HER3 or HER4. NIH 3T3 cells were transfected with HER2, HER3, HER4 alone, or cotransfected with HER2 and HER3 or HER2 and HER4. The cells were grown in normal medium for 5 days and then changed to either normal medium or normal medium containing 50 ng/ml of NDF for 10 days. The plates were stained with ethylene blue 15 days after transfection.

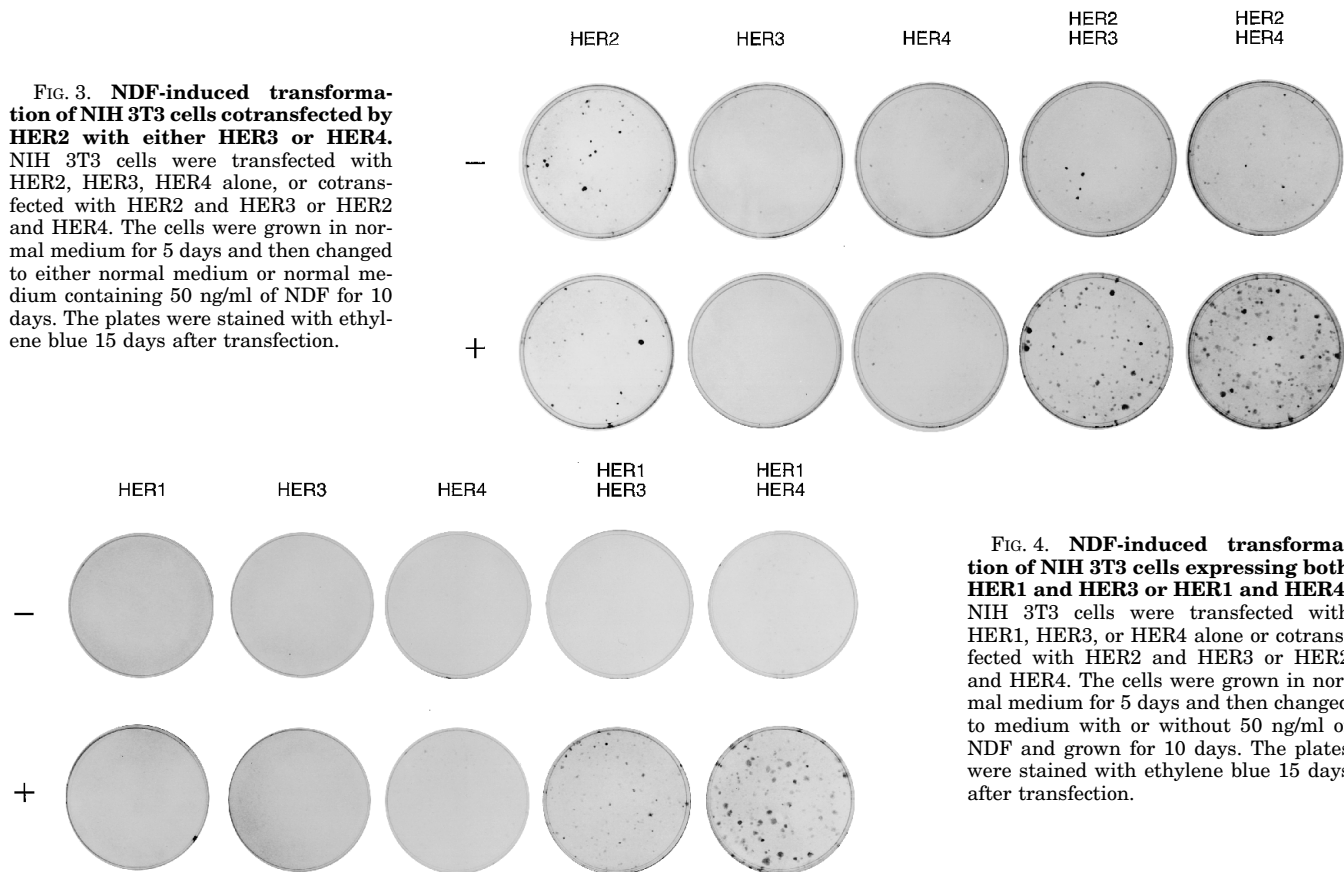


FIG. 4. NDF-induced transformation of NIH 3T3 cells expressing both HER1 and HER3 or HER1 and HER4. NIH 3T3 cells were transfected with HER1, HER3, or HER4 alone or cotransfected with HER2 and HER3 or HER2 and HER4. The cells were grown in normal medium for 5 days and then changed to medium with or without 50 ng/ml of NDF and grown for 10 days. The plates were stained with ethylene blue 15 days after transfection.

activity, the synergy of the HER2/HER3 and HER2/HER4 heterodimers may be simply due to the addition of HER3 and HER4 activity to the basal HER2 activity. Alternatively, the activity of HER2 can be increased through transphosphorylation by the other receptors. Consistent with the latter hypothesis, previous studies of other cell lines have revealed that HER2 can be phosphorylated by either HER3 or HER4 after stimulation by NDF (21, 23). To determine whether the transphosphorylation correlates with the synergistic effect of HER2 and HER3 or HER4, we examined the phosphorylation of HER2 in these cells before and after addition of NDF. Cells expressing HER2 alone, HER2 and HER3, or HER2 and HER4 were lysed after treatment. The HER2 protein in the lysates was immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis. Phosphorylated tyrosine moieties on the receptors were detected by Western blot analysis with an anti-Tyr(P) antibody. As shown in Fig. 6, there was a slight amount of background phosphorylation of HER2 in the untreated cells. The phosphorylation of HER2 increased significantly only when HER2 was coexpressed with either HER3 or HER4, and the cells were treated with NDF. The increased phosphorylation signal was not due to more HER2 protein in the doubly transfected cells, since Western blotting of the same membrane with an anti-HER2 antibody showed similar levels of HER2 protein in either the presence or absence of NDF. In fact, in cells coexpressing HER2 and HER3, NDF treatment may have caused receptor down-regulation or internalization, thus decreasing the signal. These results correlate the synergistic effect on transformation with transphosphorylation. This is consistent with a model that NDF stimulates transformation by activating HER2 through HER3 or HER4, perhaps as HER2/HER3 or HER2/HER4 heterodimers.

A similar analysis of transphosphorylation was performed for cells transfected with HER1 and either HER3 or HER4. Fig.

TABLE I
Transformation of NIH 3T3 cells by members of the EGFR subfamily

NIH 3T3 cells were transfected with members of the EGFR subfamily, as indicated. The cells were untreated (control) or treated with either EGF or NDF, as described under "Materials and Methods." The numbers shown in the table are the focus forming units per plate, and each number represents the average of three separate experiments, with the standard deviation.

Receptor	Control	EGF	NDF
HER1	0	83 ± 12	0
HER2	18 ± 3	16 ± 3	17 ± 3
HER3	0	0	0
HER4	0	0	0
HER1 + HER2	16 ± 5	123 ± 20	13 ± 5
HER1 + HER3	0	69 ± 6	34 ± 4
HER1 + HER4	0	74 ± 10	69 ± 3
HER2 + HER3	15 ± 1	18 ± 1	68 ± 6
HER2 + HER4	17 ± 2	20 ± 1	78 ± 8
HER3 + HER4	0	0	0

7 shows the phosphorylation analysis of either HER1 or HER4, alone or in combination, in the presence or absence of NDF or EGF. Consistent with the results of the transformation experiments, when HER1 was expressed alone, it was phosphorylated in response to EGF, but not to NDF. However, when HER4 was coexpressed with HER1, NDF also activated the phosphorylation of HER1. Conversely, in cells expressing only HER4, the receptor was phosphorylated only in the presence of NDF, but not EGF. When HER1 was coexpressed with HER4, EGF also activated the phosphorylation of HER4. Therefore, when HER1 and HER4 were coexpressed, NDF or EGF induced the phosphorylation of HER1 and HER4. It was determined that the antibodies were specific to their respective receptors and do not cross-react between the receptor types, since cell lysates containing either HER1 or HER4 alone are precipitated only by

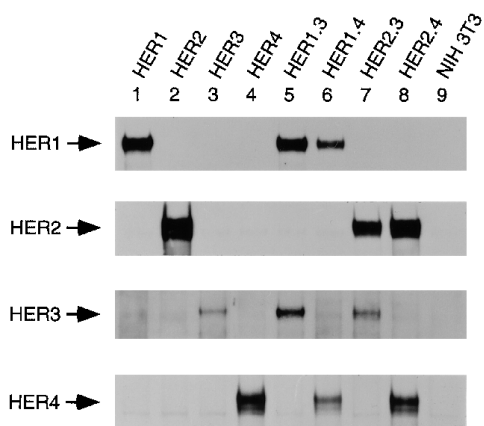


FIG. 5. Detection of receptor proteins in the transfected cell lines. Cell lysates were prepared from each cell line, as described under "Materials and Methods." Receptor proteins were immunoprecipitated, separated by electrophoresis on a 6% polyacrylamide-SDS gel, and transferred onto nitrocellulose. The proteins were probed with their respective antibodies and detected with the ECL system. For each Western blot, only the region of specific receptor bands is shown. *Lanes 1-4* are from cells transfected with HER1, HER2, HER3, and HER4, respectively. *Lane 5-8* are from cells cotransfected with HER1 and HER3, HER1 and HER4, HER2 and HER3, and HER2 and HER4, respectively. *Lane 9* shows untransfected NIH 3T3 cells.

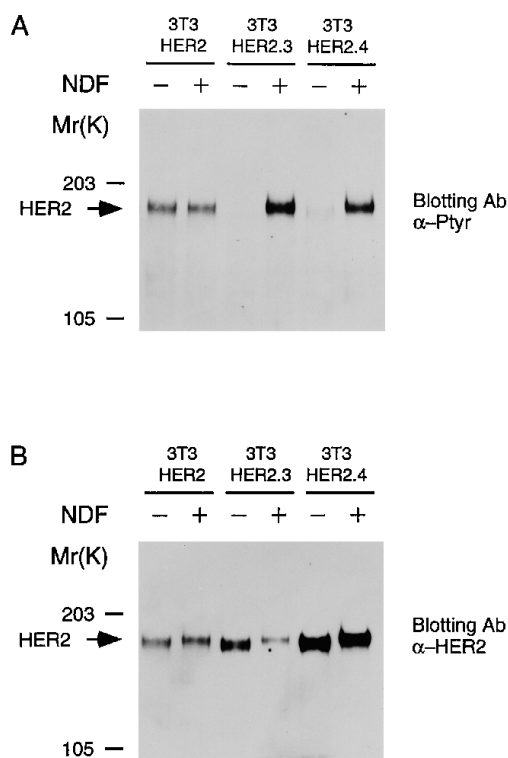


FIG. 6. NDF-induced phosphorylation of HER2. Cells were transfected with HER2, HER2 and HER3, or HER2 and HER4, as described under "Materials and Methods." Lysates were prepared from cells that were either untreated or treated with 50 ng/ml NDF for 5 min before lysis. HER2 receptors were immunoprecipitated, separated by electrophoresis on a 6% polyacrylamide-SDS gel, and transferred onto a nitrocellulose filter. The filter was probed with an anti-phosphotyrosine antibody (A). The antibodies were then removed, and the filter was re-probed with anti-HER2 antibody (B).

the relevant antibody. In addition, when the appropriate membranes were re-probed with the HER1 and HER4 antibodies, similar levels of the receptor proteins were detected in samples with or without ligand treatment (data not shown). These data strongly suggest that both NDF and EGF activate the HER1/

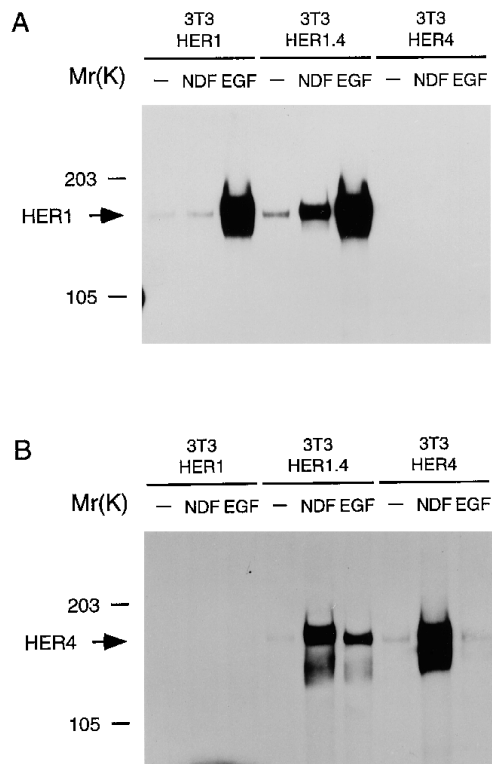


FIG. 7. Ligand-induced transphosphorylation of HER1 and HER4. Cells were transfected with HER1, HER1 and HER4, or HER4 as described under "Materials and Methods." Lysates were prepared from cells that were either untreated or treated with 20 ng/ml EGF or 50 ng/ml NDF for 5 min before lysis. HER1 (A) and HER4 (B) were first immunoprecipitated, separated by electrophoresis on a 6% polyacrylamide-SDS gel, and transferred onto a nitrocellulose filter. Both filters were probed with anti-phosphotyrosine antibody.

HER4 heterodimers.

HER1 and HER3 transphosphorylation was examined in an experiment analogous to the HER1 and HER4 study (data not shown). The transphosphorylation between HER1 and HER3 was much weaker than the transphosphorylation between HER1 and HER4. Transphosphorylation of HER3 by HER1 in response to EGF was observed, but to a lower extent than the HER1/HER4 transphosphorylation. However, the transphosphorylation of HER1 by HER3 was not detected, presumably due to the lack of intrinsic kinase activity of the HER3 receptor (22, 44).

DISCUSSION

Using a subclone of the NIH 3T3 cell line that does not endogenously express known members of the EGFR subfamily, we compared the abilities of HER3 and HER4 to induce cell growth and transformation of NIH 3T3 cells, when they are expressed either individually or coexpressed with HER1 and HER2. The results show that when expressed alone, either HER3 or HER4 can stimulate cell growth in response to activation by NDF; however, signaling through either of these receptors fails to induce morphological transformation. Therefore, NDF elicits cell growth, but not transformation, in cells expressing either HER3 or HER4. This is in contrast to HER1 and HER2 which cause both cell growth and transformation. These results imply that HER3 and HER4 may activate different signaling pathways from that activated by HER1 or HER2. On the other hand, activation of growth may be necessary, but not sufficient, for cell transformation. An additional pathway may be needed for transformation.

Receptor phosphorylation experiments indicate that there

may be differences in the signaling pathways between HER3 and HER4. When HER4 was expressed alone, it was strongly phosphorylated after NDF stimulation. This is consistent with a previous report that HER4 homodimers constitute a functional NDF receptor (21). In contrast, the phosphorylation of HER3 in response to NDF was very weak. Previous studies have shown that several amino acids usually conserved in the protein kinase catalytic domain are altered in HER3, and HER3 expressed in insect cells has impaired intrinsic kinase activity as compared with that of HER1 (44). Our results are consistent with these observations. However, HER3 induction of robust cell growth in response to NDF activation implies that HER3 may synergize with proteins outside of the EGFR family. A recent report indicates that HER3 can bind to and activate PI 3-kinase (27, 28, 31). In the experiments described here, we have not determined which proteins are activated by HER3. Alternatively, it is possible that the low level of phosphorylation of HER3 induced by NDF is sufficient to initiate cell growth. The differential levels of receptor phosphorylation imply that the signaling pathways activated by HER3 and HER4 are different. However, there is no synergistic effect on the transformation of NIH 3T3 cells when these two receptors are cotransfected (Table I).

The synergistic activity of the EGFR subfamily members on cell transformation was evident when particular combinations of receptor plasmids were cotransfected. HER2 has been previously shown to cause transformation when overexpressed (7, 8). The small number of ligand-independent foci formed in cells transfected by HER2 alone, or by HER2 in combination with HER3 or HER4, is presumably due to a minor population of cells that express high levels of HER2. Coexpression of either HER3 or HER4 had no effect on the ligand-independent transforming activity of HER2. However, the synergistic effects of those receptor combinations were apparent in the presence of NDF, as focus production was significantly increased as compared with HER2 alone. The ligand-dependent formation of foci in the HER2/HER3 or HER2/HER4 cotransfections are likely due to the activation of HER2 by either HER3 or HER4, perhaps through receptor heterodimerization. This explanation is supported by the observations that NDF promotes increases in foci formation and HER2 phosphorylation only in cells coexpressing HER2 and HER3 or HER4. Another less likely possibility is that signaling through HER3 or HER4 homodimers simply adds to the level of transformation by HER2.

The transformation of the NIH 3T3 cells by coexpressing HER1 with HER3 or HER4 is particularly intriguing. Heterodimer formation between HER1 and either HER3 or HER4 has not been extensively analyzed. Unlike HER2, HER1 does not transform NIH 3T3 cells in the absence of ligand. Since HER1 alone is not stimulated by NDF, the mechanism of synergistic action may involve the activation of HER1 through heterodimerization with HER3 or HER4. Our results show that phosphorylation of both HER1 and HER4 can be induced by either NDF or EGF. When transphosphorylation between HER1 and HER3 was examined, NDF induced phosphorylation only of HER3, but not HER1, whereas EGF induced phosphorylation of both receptors. The same phenomenon was also observed in A431 cells by Soltoff *et al.* (28). The phosphorylation of HER3 upon activation by EGF suggests that heterodimers of HER1 and HER3 may be functionally active. It is also worth noting that the relative transforming activities of the heterodimers containing either HER1 or HER2 are always stronger with HER4 than with HER3. The weaker transforming activity of the HER3 heterodimers may be due to the dysfunctional intrinsic kinase domain in HER3 (44) or the requirement for additional proteins such as PI 3-kinase

(27–31).

By showing that coexpression of either HER1 or HER2 with HER3 or HER4 can result in the ligand-dependent transformation of NIH 3T3 cells, we suggest that NDF may play an important role in the development of human cancer. In cells that express particular combinations of these receptors, induction of NDF expression may be an important step in the transformation of cells in some cancers. This is supported by the observation that *ras*-transformed rat cells express higher levels of NDF than nontransformed cells (17). Our results also suggest that members of the EGFR subfamily may have distinct functions. Furthermore, NDF may have distinct biological functions that are dependent on the combination of receptors expressed on the cells of a particular tissue. To advance our understanding of the EGFR subfamily members and their ligands, it will be necessary to determine the signaling pathways that respond to different receptors and receptor heterodimers. Particularly important are the signaling pathways involved in the morphological transformation of cancer cells.

Acknowledgments—We thank Drs. D. Lowy and Dennis Slamon for plasmids and cell lines. We thank Drs. Barry Ratzkin and William J. Boyle for critical reading of this manuscript and helpful suggestions throughout the course of this work. We also thank Dr. Brenda Guthrie for critical reading of this manuscript and helpful advice for the phosphorylation experiments.

REFERENCES

- Di Fiore, P. P., and Kraus, M. H. (1992) *Cancer Treat. Res.* **61**, 139–160
- Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1746–1750
- Frykberg, L., Palmieri, S., Beug, H., Graf, T., Hayman, M. J., and Vennstrom, B. (1983) *Cell* **32**, 227–238
- Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I., and Weinberg, R. A. (1984) *Nature* **312**, 513–516
- Drebin, J. A., Stern, D. F., Link, V. C., Weinberg, R. A., and Greene, M. I. (1984) *Nature* **312**, 545–548
- Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) *Cell* **45**, 649–657
- Hudziak, R. M., Schlessinger, J., and Ullrich, A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7159–7163
- Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. (1987) *Science* **237**, 178–182
- Riedel, H., Massoglia, S., Schlessinger, J., and Ullrich, A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1477–1481
- Di Fiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J., and Aaronson, S. A. (1987) *Cell* **51**, 1063–1070
- Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I., and Lowy, D. R. (1987) *Science* **238**, 1408–1410
- Alimandi, M., Romano, A., Curia, M. C., Muraro, R., Fedi, P., Aaronson, S. A., Di Fiore, P. P., and Kraus, M. H. (1995) *Oncogene* **10**, 1813–1821
- Prigent, S. A., and Lemoine, N. R. (1992) *Proc. Growth Factor Res.* **4**, 1–24
- Groenen, L. C., Nice, E. C., and Burgess, A. W. (1994) *Growth Factors* **11**, 235–257
- Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W.-J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. (1992) *Science* **256**, 1205–1210
- Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Ben Levy, R., Koski, R. A., Lu, H. S., and Yarden, Y. (1992) *Cell* **69**, 559–572
- Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben Levy, R., and Yarden, Y. (1992) *Cell* **69**, 205–216
- Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994) *J. Biol. Chem.* **269**, 25226–25233
- Kita, Y. A., Barff, J., Luo, Y., Wen, D., Brankow, D., Hu, S., Liu, N., Prigent, S. A., Gullick, W. J., and Nicolson, M. (1994) *FEBS Lett.* **349**, 139–143
- Carraway, K. L., III, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994) *J. Biol. Chem.* **269**, 14303–14306
- Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) *Nature* **366**, 473–475
- Carraway, K. L., III, and Cantley, L. C. (1994) *Cell* **78**, 5–8
- Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) *J. Biol. Chem.* **269**, 14661–14665
- Wada, T., Qian, X., and Greene, M. I. (1990) *Cell* **61**, 1339–1347
- Spivak Kroizman, T., Rotin, D., Pinchasi, D., Ullrich, A., Schlessinger, J., and Lax, I. (1992) *J. Biol. Chem.* **267**, 8056–8063
- Goldman, R., Levy, R. B., Peles, E., and Yarden, Y. (1990) *Biochemistry* **29**, 11024–11028
- Kim, H. H., Sierke, S. L., and Koland, J. G. (1994) *J. Biol. Chem.* **269**, 24747–24755

28. Soltoff, S. P., Carraway, K. L., Prigent, S. A., Gullick, W. J., and Cantley, L. C. (1994) *Mol. Cell. Biol.* **14**, 3550–3558
29. Prigent, S. A., and Gullick, W. J. (1994) *EMBO J.* **13**, 2831–2841
30. Fedi, P., Pierce, J. H., Di Fiore, P. P., and Kraus, M. H. (1994) *Mol. Cell. Biol.* **14**, 492–500
31. Carraway, K. L., III, Soltoff, S. P., Diamonti, A. J., and Cantley, L. C. (1995) *J. Biol. Chem.* **270**, 7111–7116
32. Lin, C. R., Chen, W. S., Krueger, W., Stolarsky, L. S., Weber, W., Evans, R. M., Verma, I. M., Gill, G. N., and Rosenfeld, M. G. (1984) *Science* **224**, 843–848
33. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C., and Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 9193–9197
34. Velu, T. J., Vass, W. C., Lowy, D. R., and Tambourin, P. E. (1989) *J. Virol.* **63**, 1384–1392
35. Kohler, G., and Milstein, C. (1992) *Bio/Technology* **24**, 524–526
36. Malmberg, A. C., Michaelsson, A., Ohlin, M., Jansson, B., and Borrebaeck, C. A. (1992) *Scand. J. Immunol.* **35**, 643–650
37. Faegerstam, L. G., Frostell, A., Karlsson, R., Kullman, M., Larsson, A., Malmqvist, M., and Butt, H. (1990) *J. Mol. Recognit.* **3**, 208–214
38. Harlow, E., and Lane, D. (1988) *Plainview*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
39. Rajkumar, T., and Gullick, W. J. (1994) *Breast Cancer Res. Treat.* **29**, 3–9
40. Lemoine, N. R., Barnes, D. M., Hollywood, D. P., Hughes, C. M., Smith, P., Dublin, E., Prigent, S. A., Gullick, W. J., and Hurst, H. C. (1992) *Br. J. Cancer* **66**, 1116–1121
41. Rajkumar, T., and Gullick, W. J. (1994) *Br. J. Cancer* **70**, 459–465
42. Dougall, W. C., Qian, X., Peterson, N. C., Miller, M. J., Samanta, A., and Greene, M. I. (1994) *Oncogene* **9**, 2109–2123
43. Kokai, Y., Meyers, J. N., Wada, T., Brown, V. I., LeVea, C. M., Davis, J. G., Dobashi, K., and Greene, M. I. (1989) *Cell* **58**, 287–292
44. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8132–8136