Cross-talk between Phorbol Ester-mediated Signaling and Tyrosine Kinase Proto-oncogenes

I. ACTIVATION OF PROTEIN KINASE C STIMULATES TYROSINE PHOSPHORYLATION AND ACTIVATION OF ErbB2 AND ErbB3*

(Received for publication, August 5, 1997, and in revised form, September 23, 1997)

Renee Emkey and C. Ronald Kahn‡
From the Research Division, Joslin Diabetes Center, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

The tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), acutely stimulates the tyrosine phosphorylation of proteins of approximately 190, 120, and 70 kDa in the well differentiated Fao rat hepatoma cell line. This phosphorylation is dependent on protein kinase C (PKC) and is abolished by down-regulation of PKC or pretreatment with a PKC inhibitor. Purification of the 190-kDa tyrosine-phosphorylated protein revealed that it consists of both ErbB2 and ErbB3. Following PMA-induced tyrosine phosphorylation, ErbB2 and ErbB3 were able to associate with the SH2 domains of several signaling proteins including the p85α subunit of phosphatidylinositol 3-kinase, Syp, and Grb2. The 120-kDa protein phosphorylated in response to PMA consists of at least two proteins: focal adhesion kinase that exhibits a minor increase in tyrosine phosphorylation following treatment with PMA, and a major 120-kDa tyrosine-phosphorylated species in PMA-stimulated Fao cells which as yet is unidentified. Similarly, the 70-kDa tyrosine-phosphorylated protein also appears to represent more than one protein, including paxillin and a second protein of similar mobility which appears to be the major tyrosine phosphorylation in response to PMA. Both ErbB2 and paxillin also exhibit reduced migration on SDS-polyacrylamide gel electrophoresis following PMA treatment, suggesting that they are also phosphorylated on serine/threonine residues. The mobility shift of both of these proteins is abolished by treatment with inhibitors of PKC or mitogen-activated protein kinase/extracellular signal-related kinase. The hallmark of this family of proteins includes the presence of two cysteine-rich regions in the extracellular domain, a single transmembrane domain, and, with the exception of ErbB3 (6), an intrinsic tyrosine kinase activity contained in the large intracellular domain (7). In addition to the role of ErbB1 in EGF action, members of the ErB family are often overexpressed in various human tumors (8, 9). Many human breast and ovarian carcinomas contain elevated levels of ErbB2 that result in constitutive activation of its intrinsic tyrosine kinase activity (8, 9–12). This correlates with a poor clinical prognosis (1, 7, 9, 13, 14). Similarly, elevated levels of expression and tyrosine phosphorylation of ErbB3 have been detected in several breast cancer cell lines (3, 15). It is clear that constitutive activation of the intrinsic kinase activity of these receptors is a crucial step in the oncogenic process (16).

Activation of the ErB family of receptors depends on formation of homodimers or heterodimers with other family members (17–22). For example, ErbB2 does not bind epidermal growth factor (EGF) but is phosphorylated in response to EGF in cells expressing both EGFR and ErbB2 due to formation of EGFR/ErbB2 heterodimers (21). Activation of ErbB3 is believed to occur in a similar manner. Heregulin (HRG), an EGF-like ligand, is capable of binding to ErbB3 and ErbB4 but not EGF or ErbB2 (23–30). ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers have an even higher affinity for HRG than ErbB3 or ErbB4 alone (17, 31). Since ErbB3 has a severely impaired intrinsic kinase activity, this heterodimerization is a crucial step for ErbB3-mediated signal transduction (6).

In this report we demonstrate a novel mechanism for activation of ErbB2 and ErbB3 by the tumor promoting phorbol ester phorbol 12-myristate 13-acetate (PMA). The PMA-induced tyrosine phosphorylation of the ErbB receptors is dependent on protein kinase C (PKC). In addition, we present evidence that PMA induces tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin and that ErbB2 and paxillin are also phosphorylated on serine/threonine residues in response to PMA. The existence of cross-talk between activation of the ErbB tyrosine kinase receptors and PKC activation by PMA may provide a pathway for the tumor promoting activity of these agents.

EXPERIMENTAL PROCEDURES

Materials—The following were purchased: phorbol 12-myristate 13-acetate (PMA) from Sigma; PD98059 from New England Biolabs; bisindolylmaleimide (BIM) from Calbiochem; Protein A-Sepharose 6MB and glutathione-Sepharose 4B from Pharmacia Biotech, Inc.; herbimycin A transferase; WGA, wheat germ agglutinin; PI 3-K, phosphatidylinositol 3-kinase; MEK, MAP kinase/extracellular signal-regulated kinase kinase; PAGE, polyacrylamide gel electrophoresis; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase.
PM induced Tyrosine Phosphorylation of ErbB2 and ErbB3

from Life Technologies, Inc.; antibodies against ErbB2, ErbB3, Grb2, and FAK from Santa Cruz Biotechnology; anti-paxillin and anti-phosphotyrosine (PY20) antibodies from Transduction Laboratories; rabbit anti-mouse IgG (H + L) from Jackson ImmunoResearch Laboratories; wheat germ agglutinin coupled to agarose beads from Vector Laboratories; and insulin from Lilly.

GST fusion proteins containing the amino-terminal SH2 domain of phospholipase C-γ, Syr, and the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (p85) and the SH2 domain of Grb2 were kindly provided by Drs. M. White and S. Shoelson (Joslin Diabetes Center; Boston, MA). Antibodies to p85 and GST and a monoclonal anti-phosphotyrosine antibody (4G10) were obtained from Dr. M. White (Joslin Diabetes Center, Boston). Purified recombinant heregulin-β1 (amino acids 177–244, rHRG-β1) (177–244) was the generous gift of Dr. M. X. Sliwkowski (Genentech, Inc., San Francisco, CA).

**Cell Culture, Stimulation, Immunoprecipitation, and Immunoblotting**—Fao cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum at 37 °C, 5% CO2. Cells were grown to 70–80% confluence, washed once with phosphate-buffered saline, and placed in RPMI 1640 lacking serum overnight (~16 h). Cells were stimulated with either 100 nM insulin or 1 μg/ml PMA for 5 and 30 min, respectively, unless indicated otherwise. Alternatively, cells were pretreated with either 10 μM BIM for 90 min or 25–100 μM PD98059 for 60 min prior to stimulation with PMA. Cells were washed twice with phosphate-buffered saline and lysed in 50 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM Na3P2O7, 100 mM NaF, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM benzamidine. Lysates were cleared by centrifugation for 10 min in a microcentrifuge at 4 °C. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). An equal concentration of protein from each lysate was immunoprecipitated with the indicated antibody by incubating the lysate with the antibody for 2 h at 4 °C with mixing. Protein A-Sepharose was added to each sample and incubated an additional hour at 4 °C with mixing. The beads were washed three times in lysis buffer, boiled, subjected to SDS-PAGE, transferred to nitrocellulose, and visualized by autoradiography. Alternatively, blots were exposed in a phosphorimager cassette, scanned on Molecular Dynamics PhosphorImager, and quantitated using ImageQuant software.

**For Westerns with GST-SH2 Fusion Proteins**—Cells were stimulated and lysed as described above. Lysates were immunoprecipitated with the 4G10 monoclonal anti-phosphotyrosine antibody, separated by SDS-PAGE on a 6% acrylamide gel, transferred to nitrocellulose, and blocked as described above. The blots were incubated with the indicated GST-SH2 fusion protein at a concentration of 2.5 μg/ml in blocking solution overnight at 4 °C. The blots were extensively washed with TBS-Tween, incubated with an anti-GST antibody for 90 min, washed with TBS-Tween, incubated with 125I-protein A for 1 h, washed with TBS-Tween, and visualized by autoradiography. Alternatively, blots were exposed in a phosphorimager cassette, scanned on Molecular Dynamics PhosphorImager, and quantitated using ImageQuant software.

**Cell Fractionation**—Cells were stimulated as described above then collected in phosphate-buffered saline and resuspended in hypotonic lysis buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, 1 mM EDTA, 10 mM Na3P2O7, 100 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM benzamidine) and subjected to Dounce homogenization. The crude lysate was clarified by centrifugation in a microcentrifuge. Total membrane and cytosolic fractions were prepared by centrifugation of the lysate at 100,000 × g for 1 h at 4 °C. The pellet (the total membrane fraction) was resuspended in Triton X-100-containing lysis buffer described above and centrifuged again at 100,000 × g for 1 h at 4 °C. The supernatant from this spin was called Triton-solubilized membranes. The fractions were immunoprecipitated and immunoblotted as described above.

**WGA Binding**—Wheat germ agglutinin (WGA) coupled to agarose beads was washed with 0.01% SDS, water, and finally lysis buffer described above. The beads were resuspended with lysis buffer to make a 5% slurry. Cells were stimulated with either insulin or PMA and lysed in Triton X-100-containing buffer as described above. Equal concentrations of lysates were incubated with 100 μl of a 50% slurry of WGA coupled to agarose beads for 2 h at 4 °C with mixing. The beads were pelleted and washed three times with 50 mM HEPES, pH 7.4, 0.1% Triton X-100, and 100 mM NaCl. The beads were resuspended in Laemmli sample buffer, boiled, subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted as described above. Purification of p190 (ErbB2 and ErbB3)—Fao cells were grown in 24.5 × 24.5-cm dishes to near confluence. Fifty-three dishes were used for this purification. Cells were serum-starved overnight and stimulated with 1 μg/ml PMA for 30 min as described above. Cells were lysed in hypotonic lysis buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, 1 mM EDTA, 100 mM NaF, 2 mM phenylmethlysulfonyl fluoride, 10 μg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml benzamidine; 5 ml/dish) by Dounce homogenization. The crude lysate was subjected to low speed centrifugation for 5 min. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The pellet was resuspended in 20 ml of Triton X-100-containing lysis buffer described above and centrifuged again at 100,000 × g for 1 h at 4 °C. The supernatant from this centrifugation step, Triton-solubilized membranes, was incubated with GST bound to glutathione-Sepharose beads (5 ml of Sepharose beads) overnight at 4 °C with mixing. The slurry was poured into a disposable 10-ml column and allowed to flow by gravity. The column was washed extensively with Triton X-100-containing lysis buffer. Proteins remaining bound to the column were eluted with 100 mM Tris, pH 8, 20 mM glutathione, 120 mM NaCl, 2 mM Na2VO4, and 10 mM benzamidine (10 ml); 20 ml of Triton X-100-containing lysis buffer was added to the eluate and incubated overnight at 4 °C with mixing with an anti-phosphotyrosine immunoadfinity column (2 ml) that consisted of 4 mg of PY20 anti-phosphotyrosine antibody cross-linked to protein A-Sepharose as described (32). The slurry was poured into a 10-ml disposable column, washed extensively with lysis buffer, and eluted with 10 ml of 100 mM glycine, pH 2.5. The pH of the eluate was immediately adjusted to pH 7.5.

**RESULTS**

PMA Induces Protein Tyrosine Phosphorylation in Fao Cells—Fig. 1A compares the pattern of tyrosine phosphoryla-
tion in the well differentiated rat hepatoma cell line Fao after treatment with the tumor-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA, 1 μg/ml) or insulin (100 nM). In the absence of stimulation, Fao cells contained a major tyrosine-phosphorylated band at 120 kDa and a minor phosphorylated band at 70 kDa. As described previously (33), insulin treatment resulted in enhanced tyrosine phosphorylation of a broad band at ~185 kDa consisting of IRS-1 and IRS-2 and a band at 95 kDa representing the β-subunit of the insulin receptor. PMA also stimulated tyrosine phosphorylation of multiple proteins in these cells. The most marked increase was in the tyrosine phosphorylation of a ~190-kDa protein(s) that migrated above IRS-1 and IRS-2 (Fig. 1A). PMA also stimulated increases in tyrosine phosphorylation of 70- and 120-kDa proteins. Quantitation by scanning densitometry revealed a nearly 4-fold increase in the tyrosine phosphorylation of p190 and 50–70% increases in the phosphorylation of p120 and p70, respectively (Fig. 1B). The elevated tyrosine phosphorylation of the 70-kDa protein was accompanied by a decreased mobility on SDS-PAGE suggesting that this protein may also be phosphorylated on serine/threonine residues.

Since several 120–130-kDa tyrosine-phosphorylated proteins have been identified in many cell types stimulated by a variety of agents, it seemed likely that the 120-kDa tyrosine-phosphorylated polypeptide in PMA-treated hepatoma cells consisted of more than one protein. In fact, anti-phosphotyrosine blots of total solubilized membranes revealed a doublet at ~120 kDa following PMA stimulation (Fig. 2A, lane 2). Immunoprecipitation of the extract with an antibody directed against focal adhesion kinase (FAK) demonstrated that the lower component of the 120-kDa band was FAK (Fig. 2A). In Fao cells the basal level of tyrosine-phosphorylated FAK was high, and treatment with PMA produced a small (30–50%), but significant, increase in the tyrosine phosphorylation (Fig. 2B). The second protein present in the 120–130-kDa range exhibited a slightly retarded mobility on SDS-PAGE compared with FAK (Fig. 2A). The tyrosine phosphorylation of this slower migrating species (p120/130) was undetectable in unstimulated cells and was markedly enhanced by PMA. This p120/130 protein was not recognized by the FAK antibody. Possible candidates for p120/130 include a 120-kDa liver-specific plasma membrane glycoprotein (pp120/HA4) that is a substrate for the insulin receptor tyrosine kinase (34, 35), p130Cas (36), a cytosolic calcium-dependent tyrosine kinase (37), and a 120-kDa Syp/SH2 binding protein (38).

Studies performed with Swiss 3T3 cells have demonstrated that PMA treatment increases tyrosine, as well as serine/threonine, phosphorylation of paxillin, a 70-kDa protein (39). The ~70-kDa protein observed in Fao cells after stimulation with PMA possessed similar characteristics with an increase in tyrosine phosphorylation and a decreased mobility on SDS-PAGE. Immunoprecipitation of lysates prepared from PMA-stimulated cells with anti-FAK antibody and immunoblotted (IB) with an antibody against anti-phosphotyrosine. B and C, cell lysates prepared from unstimulated (C) and PMA-treated (P) cells were subjected to immunoprecipitation with either an anti-FAK (B) or anti-paxillin (C) antibody and analyzed by anti-phosphotyrosine (PY) immuno blot. Alternatively, whole cell lysates prepared from control (C) and PMA-stimulated (P) cells using 1% Triton X-100 buffer were immunoprecipitated (IP) with an anti-FAK antibody and immunoblotted (IB) with an antibody against anti-phosphotyrosine. B and C, cell lysates prepared from unstimulated (C) and PMA-treated (P) cells were subjected to immunoprecipitation with either an anti-FAK (B) or anti-paxillin (C) antibody and analyzed by anti-phosphotyrosine (B and C), FAK (B), or paxillin (C) immunoblot. 100 μg of cell lysate was also subjected to anti-FAK (B) or anti-paxillin (C) immunoblot analysis.

The third PMA-stimulated tyrosine phosphorylation was of a band at ~190 kDa (p190). As noted above, on SDS-PAGE p190 migrated slightly above the insulin-induced phosphorylated proteins IRS-1 and IRS-2 (Fig. 1A). Immunoprecipitation of lysates prepared from PMA-stimulated cells with anti-IRS-1 and anti-IRS-2 antibodies failed to reveal any tyrosine-phosphorylated proteins confirming that p190 was not IRS-1 or IRS-2 (data not shown). The phosphorylation of p190 showed a similar time course to that of paxillin and p120 but was of much greater magnitude, reaching 4- to 5-fold above basal levels by 10 min after PMA treatment (Fig. 3, A and B). Tyrosine phosphorylation of p190 was apparent as little as 0.01 μg/ml PMA and was maximal with 0.5 μg/ml (Fig. 3, C and D). The tyrosine phosphorylation of FAK/p120/130 and p70/paxillin showed similar dose responses. To assure maximal stimulation, all subsequent studies were performed with 1 μg/ml PMA.

PMA-induced Tyrosine Phosphorylation of ErbB2 and ErbB3

Fig. 2. PMA stimulates tyrosine phosphorylation of FAK, paxillin, and an unidentified 120-kDa protein. Fao cells were stimulated as described in Fig. 1. A, unstimulated (C) or PMA-treated (P) cells were lysed in hypotonic buffer and separated into cytosolic and total membrane fractions as described under “Experimental Procedures.” The membrane fraction was solubilized with 1% Triton X-100 to obtain Triton-solubilized membranes (TSM). Equal concentrations of cytosol and Triton-solubilized membranes were analyzed by anti-phosphotyrosine (PY) immunoblot. Alternatively, whole cell lysates prepared from control (C) and PMA-stimulated (P) cells using 1% Triton X-100 buffer were immunoprecipitated (IP) with an anti-FAK antibody and immunoblotted (IB) with an antibody against anti-phosphotyrosine. B and C, cell lysates prepared from unstimulated (C) and PMA-treated (P) cells were subjected to immunoprecipitation with either an anti-FAK (B) or anti-paxillin (C) antibody and analyzed by anti-phosphotyrosine (B and C), FAK (B), or paxillin (C) immunoblot. 100 μg of cell lysate was also subjected to anti-FAK (B) or anti-paxillin (C) immunoblot analysis.
Tyrosine phosphorylation has been shown to play an important role in signal transduction, primarily due to the ability of tyrosine-phosphorylated proteins to bind SH2 domains present in various signaling proteins (40). Immunoprecipitation of the 85-kDa regulatory subunit of PI 3-kinase (p85) and Grb2, both of which contain SH2 domains, from PMA-treated Fao cells followed by anti-phosphotyrosine immunoblot analysis revealed the presence of tyrosine-phosphorylated p190 (Fig. 4A). Thus, following PMA stimulation, p85 and Grb2 were in a complex with p190.

In an attempt to demonstrate better a direct interaction between p190 and various SH2 domain containing proteins, in vitro binding studies were performed. Anti-phosphotyrosine immunoprecipitates from unstimulated Fao cells or cells treated with insulin or PMA were separated on SDS-PAGE and transferred to nitrocellulose which was then incubated with GST fusion proteins containing SH2 domains from various signaling proteins. The membrane was extensively washed, and the fusion proteins remaining bound were visualized by immunoblotting with an anti-GST antibody. Under these conditions, the GST-SH2 fusion proteins of p85\(\alpha\), Grb2, Syp, and PLC\(\gamma\) bound to tyrosine-phosphorylated p190 from PMA-treated cells (Fig. 4B). This interaction was specific and was mediated by the various SH2 domains, since GST alone did not selectively bind to any proteins from stimulated cells. Thus, tyrosine-phosphorylated p190 was able to directly interact with several signaling proteins via their SH2 domain. Using the same technique, we could also observe p85\(\alpha\) binding to the insulin receptor \(\beta\)-subunit in an insulin-dependent fashion and IRS-1/IRS-2 binding to the SH2 domains of p85\(\alpha\), Grb2, Syp, and PLC\(\gamma\) in insulin-stimulated lysates (Fig. 4B). Again these interactions were specific and not observed in unstimulated cells. The binding of PLC\(\gamma\) to IRS-1/IRS-2 was somewhat surprising since this interaction does not occur to a significant extent in most intact cells. However, IRS-1 and IRS-2 contain potential binding sites for PLC\(\gamma\) (IRS-1, 1172YIYLD; IRS-2, 1166YIYLD) (41) that are apparently phosphorylated following insulin stimulation (42).

Cellular localization of p190 was examined. Cytosol and total membrane fractions were prepared from control unstimulated Fao cells or cells treated with PMA. The isolated membranes were solubilized in buffer containing 1% Triton X-100. Tyrosine-phosphorylated proteins were immunoprecipitated from both the cytosolic and the solubilized membrane fractions, separated on SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibodies. As shown in Fig. 5A, tyrosine-phosphorylated p190 was found exclusively in the solubilized membrane fraction prepared from PMA-stimulated cells and was undetectable in the cytosolic fraction of these cells.

Many membrane-associated proteins are glycosylated and are capable of binding to a variety of lectins, including WGA (43). To determine whether p190 was glycosylated, the ability of p190 to bind WGA coupled to agarose beads was assessed (Fig. 5B). As expected, anti-phosphotyrosine immunoblot analysis of proteins from insulin-stimulated Fao cells that bound to WGA-agarose revealed a band at 95 kDa representing the \(\beta\)-subunit of the insulin receptor. Incubation of WGA-agarose with PMA-treated cell lysates bound only one tyrosine-phosphorylated protein, p190. Taken together, these data indicate that p190 is a glycosylated membrane protein whose tyrosine phosphorylation is stimulated by PMA and can bind various SH2 domain containing proteins.

p190 Consists of ErbB2 and ErbB3—In an attempt to purify and identify p190, the Triton-solubilized membrane fraction of PMA-stimulated Fao cells was subjected to two affinity columns as follows: first, a column composed of a GST fusion protein consisting of the SH2 domain of p85 immobilized on gluthatione-Sepharose; and second, an anti-phosphotyrosine immunoblot analysis column. These two columns were sufficient to obtain a single polypeptide in the 190-kDa range as determined by silver staining (Fig. 6A). Anti-phosphotyrosine immunoblot analysis also revealed only one tyrosine-phosphorylated protein that migrated at 190 kDa (Fig. 6B). Based on the size, membrane association, and glycosylation of p190, several candidates were considered for p190. Antibodies directed against either the epidermal growth factor (EGF) or platelet-derived growth factor receptor did not recognize purified p190 in immunoblot analysis (data not shown). In addition, EGF and platelet-derived growth factor receptor antibodies were unable to detect any proteins contained in anti-phosphotyrosine immunoprecipitates from PMA-treated cells (data not shown).

The purified 190-kDa polypeptide did, however, react with antibodies directed against both ErbB2 and ErbB3 (Fig. 6, C and D). The purified 190-kDa polypeptide did, however, react with antibodies directed against both ErbB2 and ErbB3 (Fig. 6, C and D). Since the level of cross-reaction of each of these ErbB...
antibodies with other ErbB proteins is minimal (see Fig. 7D), it appeared that this single band contained both proteins.

To confirm the identity of p190 as ErbB2 and ErbB3, anti-phosphotyrosine immunoblot analysis was performed on ErbB2 or ErbB3 immunoprecipitates from PMA-treated Fao cells. As shown in Fig. 7, PMA-induced the tyrosine phosphorylation of both of these proteins. By scanning densitometry, the increase in tyrosine phosphorylation of ErbB2 and ErbB3 was 3.5- and 4.2-fold, respectively. Thus, p190 is composed of both ErbB2 and ErbB3, and PMA treatment induces the tyrosine phosphorylation of these proteins in Fao cells.

FIG. 4. Tyrosine-phosphorylated p190 associates with the SH2 domain of several signaling proteins. Fao cells were maintained in serum-free medium overnight. Cells were stimulated with 1 μg/ml PMA for 30 min (P), 100 nm insulin for 5 min (I), or unstimulated (C). A, equal concentrations of cell lysates were immunoprecipitated with an antibody against either phosphotyrosine (PY), the 85-kDa regulatory subunit of PI 3-kinase (p85), or Grb2. Immunoprecipitated proteins were separated on 6% SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody. B, cells were stimulated as in A. Lysates were immunoprecipitated with an anti-phosphotyrosine antibody, separated on 6% SDS-PAGE, transferred to nitrocellulose, and incubated with 2.5 μg/ml of the indicated GST-SH2 fusion protein overnight at 4 °C as described under “Experimental Procedures.” The blots were analyzed by immunoblotting (IB) with either anti-phosphotyrosine (PY) or anti-GST antibodies. The blots shown here are representative of at least three experiments.

FIG. 5. p190 is a glycosylated membrane-associated protein. Fao cells were maintained in serum-free medium overnight. Cells were unstimulated (C) or treated with PMA (P) as described in Fig. 4. A, cells were lysed in hypotonic buffer and lysates were separated into cytosol and total membrane fractions as described under “Experimental Procedures.” The total membrane fraction was solubilized in 1% Triton X-100 to prepare Triton-solubilized membranes (TSM). Tyrosine-phosphorylated proteins were immunoprecipitated with an anti-phosphotyrosine antibody, separated on 6% SDS-PAGE, and immunoblotted with the anti-phosphotyrosine antibody. B, cells were stimulated with PMA (P), insulin (I), or unstimulated (C) as described in Fig. 4. Cell lysates were allowed to bind to wheat germ agglutinin (WGA) coupled to agarose beads as described under “Experimental Procedures.” The beads were extensively washed and subjected to SDS-PAGE on a 6% gel which was immunoblotted with an anti-phosphotyrosine antibody. Alternatively, anti-phosphotyrosine immunoprecipitates were prepared from the lysates and analyzed by anti-phosphotyrosine immunoblotting. These blots are representative of at least two experiments.

FIG. 6. p190 consists of ErbB2 and ErbB3. p190 was purified from PMA-stimulated Fao cells as described under “Experimental Procedures.” An aliquot of the purified protein was subjected to SDS-PAGE on a 6% gel and silver-stained (A) or immunoblotted (IB) with an anti-phosphotyrosine (PY) (B), anti-ErbB2 (C), or anti-ErbB3 (D) antibody.

ErbB2 or ErbB3 immunoprecipitates from PMA-treated Fao cells were found to contain a tyrosine-phosphorylated protein that migrated at ⩽120 kDa. In an attempt to determine whether this 120-kDa protein was actually FAK, the ErbB2 and ErbB3 immunoprecipitates were subjected to Western blot analysis using an anti-FAK antibody. The 120-kDa tyrosine-phosphorylated protein was not recognized by the FAK antibody indicating that this protein is not FAK (data not shown). An alternative possibility is that this 120-kDa protein that co-immunoprecipitates with ErbB2 and ErbB3 represents a proteolytic fragment of ErbB4 (p180). However, ErbB4 immunoblot analysis of Fao cell lysates from control unstimulated and PMA-treated cells recognized a single band that migrated well below the 120-kDa tyrosine-phosphorylated protein observed in ErbB2/ErbB3 immunoprecipitates (data not shown). In addition, ErbB4 immunoprecipitated from PMA-treated
Therefore, we examined whether PMA was able to promote the tyrosine phosphorylation of ErbB2 and ErbB3, a crucial step in activation of the kinase-deficient ErbB3. Treatment with heregulin, a ligand for ErbB3 (44, 45), resulted in the reduced mobility of ErbB2 on SDS-PAGE when analyzed by immunoprecipitation and blotting (Fig. 7C). Indeed, following treatment with PMA, immunoprecipitated ErbB2 exhibited a slower migration on SDS-PAGE than control samples (data not shown). Since the kinase inhibitors used here had no effect on ligand-induced phosphorylation of ErbB2 and ErbB3, it remained possible that the residues responsible for the lower mobility are the PMA-induced phosphorylation sites for the SH2 domains of several signaling proteins (46–52). Although PMA stimulates tyrosine phosphorylation of ErbB2 and ErbB3, it remained possible that the low level of heterodimerization observed after PMA treatment is sufficient for transphosphorylation between these two receptors.

FIG. 7. PMA stimulates the tyrosine phosphorylation of ErbB2 and ErbB3 in Fao cells. Fao cells were maintained overnight in serum-free medium. Cells were either kept untreated (C) or stimulated with PMA (P) as described in Fig. 4. Equal concentrations of cell lysates were immunoprecipitated with either anti-phosphotyrosine (PY), anti-ErbB2, or anti-ErbB3 antibody as indicated. The immunoprecipitates (IP) were separated by SDS-PAGE on a 6% gel and immunoblotted (IB) with the indicated antibody. These blots are representative of at least three experiments.

The involvement of a protein tyrosine kinase in the PMA-induced phosphorylation of the ErbB proteins was examined using herbimycin A, an inhibitor of protein tyrosine kinases. Fao cells were maintained in serum-free medium overnight in the presence or absence of 2 μM herbimycin A. The cells were then stimulated with either 1 μg/ml PMA (30 min), 100 nM insulin (5 min), or 50 nM heregulin (8 min), a ligand for ErbB3. Lysates were immunoprecipitated and immunoblotted with an anti-phosphotyrosine antibody (Fig. 8). Pretreatment with herbimycin A inhibited insulin-induced tyrosine phosphorylation of IRS-1/IRS-2 and the insulin receptor by 49 and 51%, respectively. However, under these conditions, there was no observable effect of herbimycin A on the PMA-induced tyrosine phosphorylation of ErbB2 and ErbB3. Interestingly, herbimycin A also had no effect on heregulin-induced phosphorylation of these proteins. Similar results were obtained by treating the cells with genistein, another protein tyrosine kinase inhibitor at a concentration of 100 μM for 3 h (data not shown). Since the kinase inhibitors used here had no effect on ligand-induced phosphorylation of ErbB2 and ErbB3, we are unable to interpret the lack of an effect on the PMA-induced phosphorylation. These results suggest that perhaps the conditions used in these studies are not optimal for action of these inhibitors on these cells or that the ErbB2 kinase is particularly resistant to inhibition by herbimycin A or genistein.

In addition to stimulating the tyrosine phosphorylation of ErbB2, PMA resulted in the reduced mobility of ErbB2 on SDS-PAGE when analyzed by immunoprecipitation and blotting (Fig. 7C). Indeed, following treatment with PMA, immunoprecipitated ErbB2 exhibited a slower migration on SDS-PAGE which resulted in it co-migrating with the larger ErbB3. Such a reduction in migration on SDS-PAGE suggests the presence of serine/threonine phosphorylation and explains why the two proteins resulted in a single band on the silver-stained gel. Alkaline phosphatase treatment of ErbB2 from PMA-treated cells resulted in increasing the migration of ErbB2 on SDS-PAGE to a position identical to that of ErbB2 isolated from unstimulated cells (data not shown).

ErbB2 and ErbB3 have been shown to heterodimerize following treatment with heregulin, a ligand for ErbB3 (44, 45). This is a crucial step in activation of the kinase-deficient ErbB3. Therefore, we examined whether PMA was able to promote the heterodimerization of ErbB2 and ErbB3 in Fao cells. This was accomplished by subjecting anti-ErbB2 immunoprecipitates from control or PMA-treated cells to anti-ErbB3 immunoblot analysis and vice versa. As shown in Fig. 7D, there was a low amount of association between ErbB2 and ErbB3 in unstimulated cells. Following treatment with PMA, both ErbB2 and ErbB3 were tyrosine-phosphorylated; however, there was no increase in the amount of ErbB2/ErbB3 heterodimerization. Thus, it appears that PMA induces the phosphorylation and activation of ErbB3 in an ErbB2-independent manner. Alternatively, it remains possible that the low level of heterodimerization between ErbB2 and ErbB3 observed after PMA treatment is sufficient for transphosphorylation between these two receptors.

FIG. 8. Effect of herbimycin A, a protein tyrosine kinase inhibitor, on PMA-induced tyrosine phosphorylation of ErbB2 and ErbB3. Fao cells were maintained in serum-free medium overnight in the presence or absence of 2 μM herbimycin A (HA). The cells were then stimulated with either 1 μg/ml PMA for 30 min, 100 nM insulin (Ins) for 5 min, or 50 nM heregulin (HRG; rHRG-β1177–244) for 8 min as indicated. Lysates were immunoprecipitated with an anti-phosphotyrosine antibody, subjected to SDS-PAGE on a 6% gel, transferred to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody.
phosphorylated following PMA treatment were different than those phosphorylated in response to the natural ligand for ErbB3, heregulin. This difference would result in the recruitment of distinct signaling proteins and, therefore, activation of various signaling pathways depending on the stimulant. Therefore, we were interested in comparing the SH2 domain containing proteins that bound to ErbB2 and ErbB3 following treatment with either PMA or heregulin.

ErbB2 and ErbB3 were immunoprecipitated from either unstimulated, PMA, or heregulin-treated cells, subjected to SDS-PAGE, and transferred to nitrocellulose. The blots were incubated overnight at 4 °C with GST fusion protein containing the indicated SH2 domain (2.5 μg/ml). The blots were extensively washed and immunoblotted (IB) with the indicated antibody. PY, anti-phosphotyrosine.

**FIG. 9.** PMA-induced tyrosine-phosphorylated ErbB2 and ErbB3 associate with various SH2 domains. Fao cells were serum-starved overnight followed by stimulation with either 50 nM recombinant heregulin-β1 (amino acids 177–244) for 8 min or 1 μg/ml PMA for 30 min. Cell lysates were immunoprecipitated (IP) with either anti-ErbB2 or anti-ErbB3 antibodies as indicated. The immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. The blots were incubated overnight at 4 °C with a GST fusion protein containing the indicated SH2 domain (2.5 μg/ml). The blots were extensively washed and immunoblotted (IB) with the indicated antibody. PY, anti-phosphotyrosine.

**FIG. 10.** PMA-induced tyrosine phosphorylation of ErbB2 and ErbB3 is dependent on PKC. Fao cells were maintained overnight in serum-free medium. Cells were either untreated (C), stimulated with PMA (P) as described in Fig. 4, or treated with 10 μM bisindolylmaleimide (BIM) for 90 min or the indicated concentration of PD98059 for 60 min prior to stimulation with PMA. Equal concentrations of cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine (PY) (A), anti-ErbB2 (B and C), or anti-ErbB3 (D and E) antibodies. Proteins were separated by SDS-PAGE on a 6% gel and immunoblotted (IB) with anti-phosphotyrosine (A, B, and D), anti-ErbB2 (C), or anti-ErbB3 (E) antibodies. These blots are representative of at least three experiments.

**The Role of PKC and MAP Kinase in PMA-induced Phosphorylation of ErbB2 and ErbB3**—To determine whether the PMA-induced tyrosine phosphorylation of ErbB2 and ErbB3 was dependent on activation of PKC, the PKC inhibitor bisindolylmaleimide (BIM) was used to inhibit the serine/threonine kinase activity of PKC. Immunoprecipitation of ErbB2 or ErbB3 followed by anti-phosphotyrosine immunoblot analysis revealed that pretreatment with BIM prevented the tyrosine phosphorylation of both ErbB proteins in response to PMA (Fig. 10). The decreased mobility of ErbB2 on SDS-PAGE was also abolished by BIM pretreatment (Fig. 10). Down-regulation of PKC by prolonged treatment with PMA also inhibited the ability of phorbol ester to stimulate tyrosine phosphorylation of ErbB2 and ErbB3 (data not shown). Thus, both PMA-induced tyrosine phosphorylation of ErbB2 and ErbB3 and the mobility shift of ErbB2 are dependent on PKC. Attempts to phosphorylate ErbB2 in vitro with preparations containing a mixture of PKCo and PKCβ, however, have thus far been unsuccessful, suggesting that there is at least one step between PKC activation and ErbB2/ErbB3 phosphorylation.

Despite the fact that all of the PMA-induced effects in Fao
cells with respect to tyrosine phosphorylation and apparent serine/threonine phosphorylation were abolished by either down-regulation of PKC with phorbol esters or treatment with an inhibitor of PKC (BIM), it remained possible that these effects were due to PMA-induced secretion of heregulin. However, this does not appear to be the case since the medium from PMA-treated cells was unable to induce tyrosine phosphorylation of ErbB2, ErbB3, p70/paxillin, or p120/FAK when added to Fao cells pretreated with BIM (data not shown). Instead, the media from heregulin-treated cells was able to stimulate tyrosine phosphorylation in BIM-pretreated cells to the same extent as cells treated with heregulin (data not shown). Taken together, these results indicate that the PMA-induced tyrosine phosphorylation of ErbB2 and ErbB3 is a direct effect of activation of PKC.

Since PMA has been shown to result in activation of mitogen-activated protein kinase (MAP kinases) in cells (53, 54) which might theoretically be involved in the serine/threonine or tyrosine phosphorylation of ErbB2 and ErbB3, Fao cells were treated with the MEK1 inhibitor PD98059 prior to stimulation with PMA. As shown in Fig. 10, PD98059 had no effect on tyrosine phosphorylation at either 25 or 50 μM and only slightly (24%) inhibited tyrosine phosphorylation of the ErbB proteins at 100 μM. This suggests that MEK1/MEK2, and proteins downstream of MEK in the MAP kinase cascade, are not necessary for tyrosine phosphorylation of the ErbB proteins in response to phorbol ester but may play a direct or indirect role in serine/threonine phosphorylation. Interestingly, however, the MEK inhibitor completely abolished the PMA-induced mobility shift of ErbB2 at doses as low as 50 μM (Fig. 10). Attempts at phosphorylating ErbB2 directly with ERK2 in vitro, however, were unsuccessful.

The Role of PKC and MAP Kinase in PMA-induced Tyrosine Phosphorylation and Mobility Shift of Paxillin—As discussed earlier, in addition to enhancing the tyrosine phosphorylation of ErbB2 and ErbB3, PMA resulted in the reduced migration of paxillin on SDS-PAGE presumably due to phosphorylation on serine/threonine residues. Alkaline phosphatase treatment of paxillin from PMA-treated cells resulted in restoring the mobility of paxillin to the same position as that of paxillin from control cells (data not shown). This supports the hypothesis that the mobility shift of paxillin following stimulation with PMA is due to phosphorylation. As with ErbB2, pretreatment of cells with BIM inhibited the PMA-induced mobility shift of paxillin (Fig. 11) indicating that this effect of PMA is dependent on activation of PKC. The MEK inhibitor PD98059 was able to prevent the mobility shift of paxillin at doses as low as 25 μM (Fig. 11). These results suggest that the MAP kinase cascade is necessary for the PMA-induced mobility shift of paxillin.

PMA is a well known activator of the phospholipid- and calcium-dependent family of serine/threonine kinases known as protein kinase C (PKC) (55, 56). In addition, PMA has recently been shown to stimulate the tyrosine phosphorylation of some proteins in various cell lines suggesting that PMA is also capable of activating a tyrosine kinase (39, 57–59). In the present study we have demonstrated several proteins in Fao cells that are tyrosine-phosphorylated in response to stimulation with PMA and identified four of these as proteins that might play a role in the tumor promoting activity of this class of compounds.

Two of the proteins in Fao cells whose tyrosine phosphorylation is slightly increased following PMA treatment are localized to the focal adhesion complex; these are paxillin and focal adhesion kinase (FAK). PMA treatment of Swiss 3T3 cells also results in enhanced tyrosine phosphorylation of FAK and paxillin (39, 59) suggesting that this may be a general, rather than cell-specific, response. This PMA-induced phosphorylation is clearly dependent on PKC, since down-regulation of PKC by prolonged treatment with phorbol ester abolishes the tyrosine phosphorylation. Our data suggest that an unidentified protein co-migrates with paxillin on SDS-PAGE. It appears as though this unidentified protein, rather than paxillin, is the major 70-kDa tyrosine-phosphorylated protein in PMA-stimulated cells. However, it remains possible that this observation may be attributed to a decreased ability of the anti-paxillin antibody to immunoprecipitate tyrosine-phosphorylated paxillin from PMA-stimulated cells. Further study is required to distinguish between these two possibilities.

PMA-treated Fao cells also exhibit enhanced tyrosine phosphorylation of a 120–130-kDa protein which is immunologically distinct from FAK. Several tyrosine-phosphorylated proteins of this size have been identified in cells following exposure to a variety of agents including insulin but not by treatment with phorbol ester. These proteins include pp120/HA4 (34, 35), a Syp-SH2 binding protein (38), a calcium-dependent tyrosine kinase (37), and p130cas (36). pp120/HA4 is a plasma membrane-associated glycoprotein that is a substrate for the insulin receptor tyrosine kinase and was subsequently identified in liver as an ecto-ATPase (60–63). Insulin also induces the tyrosine phosphorylation of a 120-kDa protein in NIH3T3 cells which associates with the SH2 domain of the protein tyrosine phosphatase Syp (38). Treatment of rat liver cells with angiotensin II results in activation of a potentially novel cytosolic 115–120-kDa calcium-regulated tyrosine kinase (37). Finally, an excellent candidate for the p120/130 observed here is p130cas, a ~130-kDa tyrosine kinase substrate localized to cell-cell junctions (64–66) that is tyrosine-phosphorylated in v-ErbB-transformed fibroblasts (67). Further studies are needed to determine whether the p120/130 described here is one of the above or another novel tyrosine kinase substrate.

The most pronounced effect of PMA in Fao cells is the 4-fold increase in the tyrosine phosphorylation of a 190-kDa band on anti-phosphotyrosine immunoblots. We have shown that the components of the 190-kDa band are membrane-associated glycoproteins that are capable of binding the SH2 domains of several signaling proteins. Purification of this 190-kDa phosphorylated species allowed us to identify the two proteins comprising this 190-kDa band as the tyrosine kinase receptors ErbB2 and ErbB3. PMA-induced phosphorylation of these receptors was inhibited by pretreatment with an inhibitor of PKC, BIM, and by down-regulation of PKC. This indicates that PMA induces phosphorylation of ErbB2 and ErbB3 in a PKC-dependent manner.

Many isoforms of PKC have been identified; therefore, we
were interested in trying to determine which isoform(s) of PKC were responsible for the PMA-induced tyrosine phosphorylation of ErbB2 and ErbB3. Pretreatment of Fao cells with 100 nM G66976 had no effect on the ability of PMA to induce the tyrosine phosphorylation of ErbB2 and ErbB3. This suggests that PKCα and -β are not involved in this effect of PMA since the IC₅₀ of G66976 for these isoforms are 2.3 and 6.2 nM, respectively (68–71). In contrast, pretreatment of cells with 60 μM rottlerin resulted in a dramatic decrease in PMA-induced tyrosine phosphorylation of the ErbB proteins with complete inhibition of phosphorylation with 200 μM rottlerin (data not shown). The IC₅₀ of rottlerin for the PKC isoforms range from 30 to 42 μM (α, β, and γ) and 80–100 μM (ε, ξ, and η) (72). Although it is presently unclear which PKC isoform(s) are responsible for the PMA-induced tyrosine phosphorylation of the ErbB proteins, additional studies using other specific PKC inhibitors should clarify this question.

We have also demonstrated that the PMA-induced tyrosine phosphorylation of ErbB2/ErbB3 results in the recruitment of several signaling proteins via their SH2 domains. In fact, of the SH2 domains examined here, there was no difference between the association of these domains with ErbB proteins isolated from either PMA- or ligand-stimulated cells. This indicates that PMA results in activation of at least some of the signaling pathways activated by heregulin, a natural ligand for ErbB3.

Phorbol diesters, such as PMA, are tumor promoters and are capable of potentiating the mitogenic response of growth factors such as EGF (73, 74). The mechanism of tumor promotion by these agents remains a mystery but has generally been ascribed to activation of the serine/threonine kinase PKC. However, our data along with other studies are now producing a significant amount of evidence suggesting that PMA also activates a tyrosine kinase(s). PMA has been shown to induce the tyrosine phosphorylation of several proteins in a variety of cells (39, 57–59). These include the tyrosine phosphorylation of mitogen-activated protein kinase (MAP kinase) (50), the 60-kDa substrate of the insulin receptor which associates with the Ras GTPase activating protein (Ras-GAP) (58), as well as paxillin (39) and FAK (59). Treatment of human monocyte-like U-937 cells with PMA also results in the enhanced tyrosine phosphorylation of a ~140-kDa protein that has not been identified, as well as several other endogenous proteins (75). Although the ability of PMA to induce the tyrosine phosphorylation of these proteins has been shown to be dependent on PKC in most of these systems, PMA will stimulate the tyrosine phosphorylation of EGF receptors in membranes prepared from A431 cells that lack PKC (76), suggesting that in some cases, PMA may be able to activate a tyrosine kinase in a PKC-independent manner. Finally, as we have reported here, PMA stimulates tyrosine phosphorylation of several proteins in Fao cells including the proto-oncogene tyrosine kinase receptors ErbB2 and ErbB3 in a PKC-dependent manner. It appears as though this phosphorylation is due to activation of a protein tyrosine kinase rather than inactivation of a protein tyrosine phosphatase, since the phosphatase inhibitor sodium orthovanadate was unable to mimic the PMA-induced phosphorylation. The ability of PMA to induce the tyrosine phosphorylation, and presumably activation, of ErbB2 and ErbB3, in combination with the correlation between ErB2 activation and the occurrence of tumor formation (8, 10–12), suggests an interesting mechanism for the tumor promoting activity of PMA. The mechanism of PMA-induced tyrosine phosphorylation of the ErbB proteins is examined in the accompanying paper (77) and appears to involve activation of two tyrosine kinases. Furthermore, the hypothetical kinase(s) responsible for phosphorylating ErbB2 is negatively regulated by insulin.

In any case, the PMA-activated tyrosine kinase(s) likely plays a significant role in the tumor promoting activity of phorbol esters.

In addition to stimulating the tyrosine phosphorylation of the ErbB2 and ErbB3 proto-oncogenes, p70/paxillin, and p120/FAK, PMA appears to result in the serine/threonine phosphorylation of ErbB2 and paxillin as suggested by a retarded migration of these proteins on SDS-PAGE following PMA treatment. Such a mobility shift is characteristic of serine/threonine phosphorylation. The shift was dependent on PKC since pretreatment with BIM restored the proteins’ migration to the same as that from control cells. The MEK inhibitor, PD98059, also abolished the mobility shift of these proteins. Taken together, these results implicate both PKC and the MAP kinase pathway in the apparent serine/threonine phosphorylation of ErbB2 and paxillin. However, we have been unable to phosphorylate either of these proteins in vitro by PCK or ERK2. This may be due to the fact that ErbB2 and paxillin are present as immune complexes and that the antibody interferes with the phosphorylation of these proteins, or that the isoforms of PKC and ERK responsible for phosphorylation of ErbB2 or paxillin are different than those used. It is also possible that the role of PKC and ERK2 are as upstream components of a signaling pathway and that the final kinase (or kinases) involved are something downstream of these two components.

In summary, the present study demonstrates a novel mechanism for stimulating the tyrosine phosphorylation, and presumably activation, of ErbB2 and ErbB3 initiated by the tumor promoting phorbol ester, PMA. The activation of these receptors by PMA is qualitatively and quantitatively indistinguishable from that produced by the natural ligand heregulin. This cross-talk between serine/threonine kinases and tyrosine kinases provides a potential mechanism of PMA-induced activation of ErbB2 and ErbB3 which could contribute to its tumor promoting activity.

Acknowledgments—We thank M. X. Sliwkowski for providing recombinant heregulin-α1. We also thank M. F. White for antibodies against GST and p85 and T. L. Bellman for excellent secretarial assistance.

REFERENCES
1. Beerli, R. R., Graus Porta, D., Woods-Cook, K., Chen, X., Yarden, Y., and Hynes, N. E. (1996) Mol. Cell. Biol. 15, 6496–6505
2. Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J., and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4905–4909
3. Kraus, M. H., Ising, W., Miki, T., Popescu, N. C., and Aaronson, S. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9193–9197
4. Plowman, G. D., Colussou, 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
5. Yamamoto, T., Iwakura, S., Akita, T., Sato, N., Miyazaki, N., Saito, T., and Toyoshima, K. (1986) Nature 319, 230–234
6. Guy, P. M., Platko, J. V., Cannizzaro, G. C., Cerione, R. A., and Carraway, K. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8132–8136
7. Wen, D., Peles, E., Cupples, R., Sugge, S. V., Bacs, S. S., Luo, Y., Trail, G., Hu, S., Silhberger, S. M., Levy, R. B., et al. (1992) Cell 70, 579–572
8. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ulrich, A., et al. (1989) Science 244, 707–712
9. Holmes, W. E., Sliwkowski, M. X., Aikita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., et al. (1992) Science 256, 1205–1210
10. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ulrich, A., and McGuire, W. L. (1987) Science 235, 177–182
11. King, C. R., Kraus, M. H., and Aaronson, S. A. (1985) Science 229, 974–976
12. Kraus, M. H., Popescu, N. C., Amshauer, S. C., and King, C. R. (1987) EMBO J. 6, 605–610
13. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Muller, W. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10578–10582
14. Plowman, G. D., Colussou, J. M., Kalin, C. W., Carlson, G. W., Rothwell,
