Differential Signaling by an Anti-p185HER2 Antibody and Heregulin

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ABSTRACT

To understand the molecular mechanisms by which anti-p185HER2 antibody and the ligand heregulin inhibit tumor growth, we have investigated several signaling proteins and pathways. We report here that anti-p185HER2 monoclonal antibody ID5 induced tyrosine phosphorylation of HER2 in SKBr3 breast cancer cells that overexpress p185HER2. Heregulin β1 induced phosphorylation of both HER3 and HER2. ID5 produced a greater association of phospholipase C (PLC)γ1 with HER2 than did heregulin. Concordantly, ID5, but not heregulin, increased PLCγ1 activity. However, the G1 cell cycle arrest and inhibition of p27kip1 produced by ID5 were not affected by the inhibition of PLCγ1. ID5 preferentially induced binding of the M9, 46,000 isofrom of SHC to HER2, whereas heregulin preferentially induced binding of the M5, 52,000 isofrom of SHC to HER3. Heregulin, but not ID5, induced the p85 subunit of phosphatidylinositol 3′-kinase (PI3-K) to interact with HER3. Heregulin induced sustained activation of PI3-K signaling, whereas ID5 had only a transient effect. Heregulin, but not ID5, activated the c-Jun-NH2-terminal kinase cascade. Pretreatment of SKBr3 cells with ID5 decreased heregulin-induced association of HER2 with HER3 as well as the activation of c-Jun-NH2-terminal kinase and PI3-K activities. Inhibition of the mitogen-activated protein kinase pathway in SKBr3 cells did not affect heregulin-induced G2-M-phase arrest, apoptosis, and differentiation. Heregulin-induced apoptosis could be blocked by inhibition of p70s6k, but not by inhibition of PI3-K. Heregulin-induced differentiation could be eliminated by inhibition of PI3-K. We conclude that ID5 and heregulin signal via different pathways, although both agents can inhibit the clonal growth of cells that overexpress HER2.

INTRODUCTION

Four receptors have been identified among the human EGF receptor family: (a) HER1 (also called EGFR and erbB1); (b) HER2 (erbB2); (c) HER3 (erbB3); and (d) HER4 (erbB4; Refs. 1 and 2). Members of this family are characterized by the presence of two cysteine-rich extracellular domains, a transmembrane domain, and an intracellular domain with a long COOH-terminal tail that contains multiple sites for autophosphorylation (1). With the exception of HER3, each of these transmembrane receptors has intrinsic tyrosine kinase activity. The ligands that activate these receptors all contain conserved EGF-like domains and are synthesized as transmembrane precursor molecules (1, 2). Binding of ligands or specific antibodies to these receptors induces receptor dimerization, including homodimerization and heterodimerization of multiple members of the HER family. The receptors autophosphorylate and cross-phosphorylate specific tyrosine residues in their cytoplasmic tails. These phosphorylated tyrosines serve to dock SH2 or phosphotyrosine binding domain-containing signal transduction molecules including GRB2, SHC, PLCγ, and the p85 subunit of PI3-K (1–3). The binding and activation of these molecules initiate different intracellular signal transduction pathways, which ultimately lead to a variety of cell responses including cell cycle progression, differentiation, survival, apoptosis, chemotaxis, and cell mobility.

Previous reports, including our own, have demonstrated that anti-p185HER2 antibodies can inhibit breast and ovarian cancer growth in vivo and in vitro (4–10). Anti-p185HER2 antibodies specifically recognize the p185HER2 protein and do not react with the closely related EGFR receptor (7). Heregulin isoforms can also inhibit the growth of breast and ovarian cancer cells that overexpress HER2 (10–14). Heregulins belong to a subfamily of EGF-like growth factors that produce a variety of cellular responses by activating members of the HER family of receptors (1, 2). Unlike anti-p185HER2 antibodies, members of the heregulin family cannot bind to HER-2 alone. Instead, the ligand interacts with homodimers of HER3 or HER4 or with heterodimers formed from HER2 with HER3 or HER4 (15, 16). In an attempt to elucidate the mechanisms by which anti-p185HER2 antibody and heregulin inhibit tumor growth, we have studied the effect of anti-p185HER2 monoclonal antibody ID5 and heregulin β1 on the SKBr3 breast cancer cell line that expresses >106 HER2 receptors/cell. Our recent report indicated that ID5 and heregulin β1 could exert growth-inhibitory effects through different mechanisms (17). ID5 arrested the SKBr3 cells in the G1 phase of the cell cycle associated with the up-regulation of p27kip1, down-regulation of cyclin-dependent kinase 2 and cyclin E kinase activities, and induction of hypophosphorylated Rb protein. In contrast, heregulin promoted G1 to S-phase progression but induced accumulation of cells in the G2-M phase of the cell cycle. Heregulin also initiated programmed cell death and induced cell differentiation (17).

The variety of ligands, receptors, and effectors is thought to contribute to the diversification potential of the HER family signaling network (1, 2). ID5 and heregulin recognize different members of the HER family and are bound to produce different signaling and effectors. The biological effects resulting from ID5 and heregulin treatment as mentioned above are bound to correlate with the different signaling initiated by ID5 and heregulin. To understand the molecular mechanisms by which anti-p185HER2 antibody and heregulin inhibit tumor growth, it is important to investigate the possible involvement of the signaling proteins and signaling pathways in ID5 and heregulin treatment. In this report, we did find significantly different signaling produced by ID5 and heregulin.

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line SKBr3 (American Type Culture Collection, Manassas, VA) was grown in complete medium containing RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in humidified air with 5% CO2.
at 37°C. For all experiments, cells were detached with 0.25% trypsin-0.02% EDTA. For cell culture, 2 × 10^3 exponentially growing cells were plated into 100-mm tissue culture dishes. After culture for 24 h in complete medium, cells were treated (if applicable) with either the phosphatidylinositol-PLC-specific inhibitor U73122 (4 μM), the PI3-K inhibitors LY294002 (10 μM) or wortmannin (2 μM), the ERK1/2 MAP kinase inhibitor PD98059 (50 μM), the p38 MAP kinase inhibitor SB203580 (2 μM), or the FRAP/mTOR/p70S6k inhibitor rapamycin (55 nM) in complete medium overnight. Cells were then treated with ID5 (65 nm) or heregulin β1 (0.3–1 nm) in medium with 2% dialyzed fetal bovine serum (Life Technologies, Inc.) at 37°C for the indicated time intervals.

**Preparation and Purification of Anti-HER2 Antibodies.** The anti-HER2 murine monoclonal antibody ID5 was obtained from Applied BioTechnology/Oncogene Science (Cambridge, MA [18]). Hybridoma cells specific for ID5 were used to produce ascites fluid, and the immunoglobulin was purified as reported previously (10).

**Reagents.** Chemical inhibitors U73122, LY294002, and PD98059 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). SB203580, wortmannin, and rapamycin were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Recombinant human heregulin β1 (referred to hereafter as heregulin) was obtained from NeoMarkers (Freemont, CA). Antibodies reactive with HER2 (for Western blotting) and p27^Kip1 were purchased from Oncogene Research Products (Cambridge, MA). Antibodies reactive with HER2 (for immunoprecipitation) were obtained from NeoMarkers, Inc. Antibodies to HER3 (for Western blotting and immunoprecipitation), SHC, phospho-JNK, control JNK, and rabbit immunoglobulin conjugated with biotin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Streptavidin-agarose and antibodies to phosphotyrosine (4G10), PI3-K p85β, and PLC-γ1 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Antibody to GRB2 was purchased from Transduction Laboratories (Lexington, KY). Antibodies to phospho-p38, control p38, phospho-AKT, control AKT, phospho-p70S6k (Ser^411), and control p70S6k were obtained from New England BioLabs, Inc. (Beverly, MA). Antibodies to phospho-ERK1/2 and control ERK1/2 were obtained from Promega Corp. (Madison, WI).

**Preparation of Total Cell Lysate and Western Immunoblot Analysis.** The procedures for preparation of total protein and Western immunoblot analysis were performed as described previously (19).

**Immunoprecipitation.** Aliquots of total cell lysates containing equal amounts of protein in lysis buffer [137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM DTT, 1% NP40, 10% glycerol, and protease inhibitors] were precleared with 2 μg of normal mouse or rabbit IgG (Santa Cruz Biotechnology, Inc.) together with 20 μl of protein A/G-agarose conjugate. Lysates were then immunoprecipitated overnight at 4°C with 2 μg of each antibody reactive with a mouse anti-HER2 antibody and 20 μl of protein A/G-agarose conjugate. After washing four times with lysis buffer, the agarse beads were boiled in Laemmli SDS loading buffer for 5 min, and samples were resolved by 6–10% SDS-PAGE and subjected to Western blotting. To avoid the problems of indirect association, a streptavidin-biotin system and a rabbit anti-HER2 antibody were used in HER3 immunoprecipitation. Rabbit anti-HER3 antibody and protein A-agarose conjugate were used to immunoprecipitate SHC protein in a manner similar to that described for immunoprecipitation of HER2.

**PI3-K Activity Assay.** A PI3-K activity assay was carried out as described previously (20) using immunoprecipitation with an anti-phosphotyrosine antibody. Briefly, cell lysates (1 mg of protein) were immunoprecipitated with anti-phosphotyrosine antibody and subjected to an in vitro kinase reaction in 50 μl of kinase buffer containing 0.2 mg/ml phosphatidylinositol (Sigma), 20 μCi of [γ-32P]ATP, and 20 mM MgCl2. The reaction products were separated on TLC plates using chloroform:methanol:ammonium hydroxide:water (86:76:10:14).

**JNK Activity Assay.** JNK activity was assayed using a pull-down kinase assay kit for stress-activated protein kinase/JNK (New England Biolabs, Inc.). Equal amounts of protein (200 μg) from cells treated with different reagents were incubated overnight with 2 μg of c-Jun-glutathione S-transferase fusion three times (25 mM Tris, 5 mM β-glycerophosphate, 2 mM DTT, 10 mM MgCl2, and 0.1 mM NaVO3). Beads were resuspended in 30 μl of kinase buffer containing 5 μCi of [γ-32P]ATP and incubated for 30 min at 30°C. The reaction was terminated by the addition of SDS buffer, and beads were separated on 10% SDS-PAGE and analyzed with a phosphorimagery.

**PLC-γ1 Enzymatic Activity Assay.** Inositol phosphate formation was used to measure PLC-γ1 activity according to a previous report (21). Briefly, cells grown in 6-well plates were labeled for 24 h in myo-inositol-free RPMI 1640 (Life Technologies, Inc.) supplemented with myo-[3H]inositol (1 μCi/ml, 25 mCi/mmol; New England Nuclear). The cells were rinsed twice with DMEM containing 20 mM HEPES (pH 7.5), 20 mM LiCl, and BSA (1 mg/ml) and incubated in the same solution at 37°C for 45 min. The cells were stimulated with ID5 or heregulin at 37°C for 20 min, rinsed twice with PBS, and treated with ice-cold 5% perchloric acid. Cells were scraped into Eppendorf tubes, and the cellular debris was pelleted by centrifugation. The pellets were dissolved in 1 N NaOH and used for the determination of protein concentration with the use of BCA solution (Pierce Chemical Co., Rockford, IL). The supernatants were diluted with water and applied to a Bio-Rad AG1-X2 ion exchange column to separate total inositol phosphates from inositol. The radioactive eluates from triplicate samples were measured on a Beta counter (Beckman), and the average number of cpm was calculated. The 1H radioactivity in total inositol phosphates was determined per milligram of protein.

**Cell Cycle Analysis.** Cell cycle distribution was analyzed by flow cytometry. Cells treated with antibodies or heregulin for 24 or 72 h were trypsinized, washed once with PBS, and fixed overnight in 70% ethanol. Fixed cells were centrifuged at 300 × g for 10 min and washed with PBS. Cell pellets were resuspended in PBS containing 50 μg/ml RNase A and 50 μg/ml propidium iodide and incubated for 20 min at 37°C with gentle shaking. Stained cells were filtered through nylon mesh (41 μm) and analyzed on a Coulter XL-MCL flow cytometer (Coulter Corporation, Miami, FL) for relative DNA content based on red fluorescence levels. Doublets and cell debris were excluded from the DNA histograms. The percentages of the sub-G1 cell population were determined based on the relative DNA content. The percentages of cells in different cell cycle compartments were determined using the MULTICYCLE software program (Phoenix Flow Systems, San Diego, CA).

**Oil Red O Staining.** A modified Oil Red O in propylene glycol method was used to visualize neutral lipids as reported previously (12). Briefly, SKBr3 cells were cytopsin to slides after different treatments and fixed in 6% paraformaldehyde in PBS. After dehydration in absolute propylene glycol for 5 min, the slides were stained with 0.5% Oil Red O in propylene glycol for 1 h. The cells were then differentiated in 85% propylene glycol for 2 min, rinsed in distilled water, counterstained with Harris hematoxylin for 2 min, washed in PBS, and mounted in glycerin.

**RESULTS**

**Tyrosine Phosphorylation and Interaction of HER2 and HER3 after ID5 and Heregulin Treatment.** Previous studies have indicated that a number of anti-HER2 antibodies and heregulin can suppress the tumor growth of HER2-overexpressing breast and ovarian cancer cells in vitro and in vivo (4–7). Our previous data have also demonstrated that ID5 and heregulin both can inhibit the anchorage-dependent and anchorage-independent growth of SKBr3 cells (8–10). Our recent data indicated, however, that ID5 and heregulin could exert their growth-inhibitory effects through different mechanism (17). To further explore the mechanisms used by ID5 and heregulin to inhibit the growth of breast cancer cells that overexpress HER2, we have studied signal transduction after treatment with the antibody or ligand.

**Tyrosine phosphorylation of HER2 and HER3 after treatment with ID5 or Heregulin.** Our preliminary data indicated that HER2 phosphorylation could be detected as early as 5 min after treatment with ID5 or heregulin. The phosphorylation peaked at 20 min and lasted for at least 2 h (data not shown). SKBr3 cells were therefore treated with ID5, heregulin, or medium for 20 min, and cell lysates were immunoprecipitated with either murine anti-HER2 or rabbit anti-HER3 antibodies as detailed in “Materials and Methods.” The immunoprecipitates were then analyzed by Western blotting with an anti-phosphotyrosine antibody. As shown in Fig. 1, ID5 markedly increased tyrosine phosphorylation of HER2 with a concomitant de-
immunoprecipitated. This experiment is representative of three independent experiments.

-blue signaling proteins and blotted with the indicated antibodies. Finally, the blots were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Filters were cut horizontally into strips of appropriate ranges for detecting associated complexes. The immunoprecipitates were then analyzed by Western blotting with anti-HER2 or anti-HER3 antibody to reveal the levels of HER2 or HER3 immunoprecipitated. This experiment is representative of three independent experiments.

crease in basal HER3 phosphorylation. In contrast, heregulin induced a marked increase in HER3 phosphorylation with a modest increase in HER2 phosphorylation. These studies also permitted evaluation of the interaction between HER2 and HER3. In untreated SKBr3 cells, HER3 could be coimmunoprecipitated with HER2 (Fig. 2A, Lane 1). Treatment with ID5 induced intense tyrosine phosphorylation of HER2 but did not recruit additional HER3 to the HER2 when compared with the untreated control [1.2 versus 1 (Fig. 2A, Lane 2)]. In contrast, heregulin treatment induced weak HER2 tyrosine phosphorylation but resulted in much greater association of HER3 with HER2 [2.3 versus 1 (Fig. 2A, Lane 3)]. Importantly, pretreatment of SKBr3 cells with ID5 decreased the association of HER2 with HER3 from 2.8- to 1.5-fold (Fig. 2A, Lane 4). These results indicate that ID5, which binds HER2 but not HER3 (21), induced HER2 but not HER3 tyrosine phosphorylation and did not significantly increase HER2-HER3 association in SKBr3 cells. Heregulin, which binds HER3 and HER4 (15, 16), markedly increased HER3 tyrosine phosphorylation and HER2-HER3 association with a modest HER2 tyrosine phosphorylation.

**GRB2 and SHC Association with HER2 and HER3.** Ligand-induced receptor phosphorylation in homodimers and heterodimers provides phosphotyrosine docking sites for downstream signal transduction molecules including GRB2, SHC, PLC-γ, and the p85 subunit of PI3-K (22). To examine the association of the signal transduction proteins GRB2 and SHC with HER2 and HER3, SKBr3 cells were treated with ID5, heregulin, or medium for 20 min, and cell lysates were immunoprecipitated by either anti-HER2 or anti-HER3 antibodies. The immunoprecipitates were then analyzed by Western blotting with anti-GRB2 or anti-SHC antibodies. GRB2 coimmunoprecipitated with HER2 and HER3 in SKBr3 cells (Fig. 1). Both heregulin and ID5 treatment increased GRB2 binding to HER2 and HER3 (Fig. 1). Interestingly, association of GRB2 with HER2 was more marked after ID5 treatment than after heregulin treatment, whereas association of GRB2 with HER3 was similar in heregulin-treated cells and ID5-treated cells (Fig. 1). Two different isoforms of the SHC protein, the M*, 52,000 and M, 46,000 isoforms, were detected in SKBr3 cells (Fig. 2B). These two isoforms all bound to the HER2 (Fig. 1). ID5 treatment preferentially increased binding of the M*, 46,000 isoform of SHC to HER2. Only the M*, 52,000 isoform of SHC was found to bind HER3 (Fig. 1). Both ID5 and heregulin dramatically increased tyrosine phosphorylation of SHC isoforms (Fig. 2C). These data suggest that both ID5 and heregulin increased the association of GRB2 and SHC protein with HER2 and HER3 and increased tyrosine phosphorylation of SHC.

**Activation of ERK1/2, p38, and JNK Kinases by ID5 and Heregulin.** GRB2 constitutively binds the SOS guanine nucleotide exchange proteins through its two SH3 domains (22). After binding directly to phosphorylated HER2 and HER3 receptors, the adaptor protein SHC can be phosphorylated on tyrosine residues by the receptors. This phosphorylation recruits additional SH2 proteins to SHC, such as GRB2. The GRB2-SOS and/or SHC-GRB2-SOS complexes are thought to promote nucleotide exchange by Ras and subsequently activate signal pathways through MAP kinases (3, 22). To examine the activation of ERK1/2, p38, and JNK kinases, SKBr3 cells were treated with ID5 or heregulin for different intervals (shown in Figs. 3 and 4). Total cell lysates were obtained and analyzed by Western blotting with anti-ERK1/2, p38, or JNK antibodies. As illustrated in Fig. 3, ID5 induced a transient activation of both ERK1/2 and p38 MAP kinase detected by phospho-ERK1/2 and phospho-p38 antibodies (Fig. 3, A and B). Heregulin induced a more prolonged activation of both ERK1/2 and p38 MAP kinases (Fig. 3). Heregulin was shown to activate the JNK pathway by using phospho-JNK antibody (Fig. 4A) and a JNK kinase assay (Fig. 4B, Lane 2). This activation was sustained for at least 2 h after heregulin stimulation.
In contrast, ID5 did not increase phosphorylation or kinase activity of JNK (Fig. 4, A and B, Lane 3). Furthermore, heregulin-induced JNK activation was reduced by about 46% in cells that were preincubated with ID5 for 30 min (Fig. 4B, Lane 4). These results suggest that although ID5 and heregulin can both activate ERK1/2 and p38 MAP kinase signaling pathways, heregulin produces a more prolonged signal. Heregulin activates the JNK signaling pathway, whereas ID5 does not.

**PI3-K Association with HER2 and HER3.** PI3-K is a heterodimer consisting of a Mr 85,000 regulatory component and a Mr 110,000 catalytic component. The regulatory subunit possesses one SH3 domain, two SH2 domains, and one Rho-GAP domain. To examine the association of the regulatory subunit of PI3-K with HER2 and HER3, SKBr3 cells were treated with ID5, heregulin, or medium for 20 min, and cell lysates were immunoprecipitated with either anti-HER2 or anti-HER3 antibodies. The immunoprecipitates were then analyzed by Western blotting with anti-PI3-K p85 antibody. Consistent with previous data (23, 24), only trace amounts of the p85 subunit of PI3-K were associated with HER2 in untreated SKBr3 cells, whereas a significant amount of the p85 subunit was associated with the HER3 (Fig. 1). Heregulin induced a marked increase in the association of p85 with HER3 (Fig. 1). The amount of p85 associated with HER3 in ID5-treated cells was similar to that in untreated cells, indicating that ID5 treatment did not recruit p85 to HER3, consistent with a failure of ID5 treatment to increase tyrosine phosphorylation of HER3. These data suggest that heregulin increases the association of PI3-K with HER3, whereas ID5 treatment does not.

**Activation of PI3-K Signaling Pathways by ID5 and Heregulin.** Binding of the p85 subunit of PI3-K to the tyrosine-phosphorylated HER3 receptor results in PI3-K activation. When PI3-K catalytic activity was assessed (Fig. 5A), cells were treated with ID5 and (Fig. 4A). In contrast, ID5 did not increase phosphorylation or kinase activity of JNK (Fig. 4, A and B, Lane 3). Furthermore, heregulin-induced JNK activation was reduced by about 46% in cells that were preincubated with ID5 for 30 min (Fig. 4B, Lane 4). These results suggest that although ID5 and heregulin can both activate ERK1/2 and p38 MAP kinase signaling pathways, heregulin produces a more prolonged signal. Heregulin activates the JNK signaling pathway, whereas ID5 does not.
heregulin for 10 min. Both ID5 and heregulin increased the production of phosphatidylinositol 3-phosphate, indicating the activation of PI3-K. Both ID5 and heregulin activated PI3-K (Fig. 5A), but ID5-induced PI3-K activation returned to an undetectable level 30 min after the addition of antibody (Fig. 5B). Heregulin-induced PI3-K activation was sustained for at least 60 min (data not shown). To assess the effect of pretreatment with ID5 on heregulin-induced PI3-K activation, SKBr3 cells were treated with ID5 for 30 min and then subjected to heregulin treatment for an additional 10 min (at this time point, ID5-induced activation would no longer exist). Fig. 5A indicated that pretreatment of ID5 completely blocked subsequent activation of PI3-K by heregulin (Lane 4).

AKT and p70s6k were also investigated because AKT and p70s6k are downstream targets of PI3-K. Total SKBr3 cell lysates incubated with and without ID5 or heregulin were obtained and then analyzed by Western blotting with anti-AKT and p70s6k antibodies. As shown in Fig. 5C, ID5 induced a transient activation of AKT. In sharp contrast, heregulin induced a more prolonged activation of AKT (Fig. 5C). Similarly, ID5 induced a transient activation of p70s6k, whereas heregulin induced sustained activation of p70s6k (Fig. 5D). These data indicated that ID5 had only a transient effect on the PI3-K signaling pathways, whereas heregulin induced sustained activation of the PI3-K signaling pathways.

**PLC-γ1 Association with HER2 and HER3.** PLC-γ1, a member of the PLC isozyme family, has been shown to be a physiological substrate for tyrosine phosphorylation by EGF and is selectively phosphorylated on specific tyrosine residues. To examine the association of PLC-γ1 protein with HER2 and HER3, the immunoprecipitates (as described in Fig. 1) were then analyzed by Western blotting with anti-PLC-γ1 antibody. As illustrated in Fig. 1, PLC-γ1 protein coimmunoprecipitated with HER2 in SKBr3 cells. When compared with the untreated control, more PLC-γ1 was associated with HER2 in ID5-treated cells (Fig. 1). Heregulin treatment resulted in a decrease in the amount of PLC-γ1 associated with HER2 (Fig. 1). Consistent with a previous report (24), no PLC-γ1 protein was detected in HER3 immunoprecipitates (Fig. 1). These data suggest that changes in tyrosine phosphorylation induced by ID5 recruit PLC-γ1 to HER2. The reason for the decrease in association of PLC-γ1 with HER2 after heregulin treatment is not known.

**Activation of PLC-γ1 by ID5 and Effect of Blocking PLC-γ1 Activity on ID5-induced G1 Cell Cycle Arrest and p27kip1 Expression.** From the above-mentioned data, there is an obvious difference in the recruitment of PLC-γ1 protein to HER2 after treatment with ID5 and with heregulin (Fig. 1). To determine the effect of ID5 and heregulin on PLC-γ1 enzymatic activity, inositol phosphate production was examined as described in “Materials and Methods.” As demonstrated in Fig. 6A, ID5 increased inositol phosphate production and indicated increased PLC-γ1 enzymatic activity, whereas heregulin did not.

Based on our studies and those of others (17, 25–28), several anti-p185HER2 monoclonal antibodies induced G1 cell cycle arrest and p27kip1 expression in HER2-overexpressing breast cancer cells. To

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**Fig. 6. Effect of ID5 on PLC-γ1 activity.** In A, an inositol phosphate formation assay was used to determine PLC-γ1 activity according to a previous report (36). *P < 0.05 compared with the control. In B, cell cycle distribution was analyzed by flow cytometry after cells were treated with ID5 for 24 h in the presence or absence of the PLC-γ1 inhibitor U73122 (4 μM). In C, cells were pretreated with or without U73122 overnight and then treated with ID5 for 2 h. Total lysates were prepared and analyzed by Western blotting with an anti-p27kip1 antibody. In D, cells were pretreated with or without U73122 overnight and then subjected to the inositol phosphate formation assay as described in “Materials and Methods.” *P < 0.05 compared with the control.
address the possibility that increased PLC-γ1 enzymatic activity might contribute to the G₁ arrest and p27Kip1 expression observed after treatment with ID5, a phosphatidylinositol-PLC-specific inhibitor, U73122 (29), was used to pretreat SKBr3 cells before the addition of ID5. ID5-induced G₁ arrest (Fig. 6B) and p27Kip1 expression (Fig. 6C) were not altered by pretreatment with U73122, although PLC-γ1 enzymatic activity was inhibited (Fig. 6D). Thus, PLC-γ1 signaling does not appear to be required for ID5-induced G₁ arrest and p27Kip1 expression.

Effect of Blocking PI3-K and MAP Kinase Signaling Pathways on ID5-induced Cell Cycle G₁ Arrest. We also examined the effect of blocking PI3-K and MAP kinase signaling pathways on ID5-induced G₁ arrest. As described in “Materials and Methods,” cells were pretreated with either the ERK1/2 MAP kinase-specific inhibitor PD98059, the p38 MAP kinase-specific inhibitor SB203580, the PI3-K-specific inhibitors LY294002 or wortmannin, or the p70s6k-specific inhibitor rapamycin. Cells were then subjected to ID5 treatment for 24 h. Results shown in Fig. 7 indicated that blocking of the ERK1/2 and p38 MAP kinase signaling pathways did not alter ID5-induced G₁ cell cycle arrest. Similarly, blocking of the ERK1/2 and p38 MAP kinase signaling pathways did not alter ID5-induced p27Kip1 expression (Fig. 8A). The efficacy of PD98059 and SB203580 has been confirmed by their ability to inhibit heregulin-induced ERK1/2 (Fig. 8B) and p38 (Fig. 8C) activation, respectively. Blocking of p70s6k signaling with rapamycin resulted in a more profound G₁ arrest. Rapamycin itself caused a sustained G₁ arrest in SKBr3 cells at 16–72 h after the addition of the inhibitor; however, this arrest was increased by the addition of ID5 (Fig. 7; data not shown). Interestingly, inhibition of PI3-K activity by LY294002 and wortmannin
caused a marked but transient G1 arrest (>90%) in SKBr3 cells 16–24 h after addition of the inhibitors (data not shown). LY294002 or wortmannin treatment of SKBr3 cells for 24 h also significantly increased p27Kip1 expression (data not shown). Thus, inhibition of the PI3-K signaling mimicked the activity of ID5. These data suggest that ID5-induced G1 arrest in SKBr3 cells is not dependent on signaling via MAP kinases.

Effect of Blocking PI3-K and MAP Kinase Signaling Pathways on Heregulin-induced Cell Cycle G2-M-phase Arrest. Heregulin has been shown to inhibit the growth of HER2-overexpressing breast cancer cells (10–14) through induction of G2-M-phase arrest, apoptosis, and cell differentiation (11–13, 17). Multiple reports (30–37) including the current study have found that heregulin activates the MAP kinase and PI3-K signaling pathways. As shown in Fig. 9, pretreatment with PD98059 and SB203580 did not alter heregulin-induced G2-M-phase arrest, whereas pretreatment with LY294002, wortmannin, or rapamycin impaired heregulin-induced G2-M-phase arrest. The effect of LY294002, wortmannin, or rapamycin on G2-M phase could be due to the ability of these inhibitors to arrest SKBr3 cells in the G1 phase (Fig. 7; data not shown). These data suggest that MAP kinase pathways play no role in heregulin-induced G2-M-phase arrest.

Effect of Blocking the PI3-K and MAP Kinase Signaling Pathways on Heregulin-induced Apoptosis. To examine the effect of blocking the PI3-K and MAP kinase signaling pathways on heregulin-induced apoptosis, cells were pretreated with the individual inhibitors. Cells were then subjected to heregulin treatment for 72 h. Treatment with either PD98059, SB203580, LY294002, wortmannin, LY294002 plus PD98059, or LY294002 plus SB203580 did not block heregulin-induced apoptosis (Fig. 10). However, treatment with the p70s6k inhibitor rapamycin completely prevented heregulin-induced apoptosis, as shown in Fig. 10. The efficacy of LY294002 (Fig. 11A), wortmannin (Fig. 11B), and rapamycin (Fig. 11C) used in this study has been confirmed by the ability of these inhibitors to inhibit heregulin-induced AKT (Fig. 11, A and B) and p70s6k (Fig. 11C) activation, respectively. These results indicate that a rapamycin-sensitive signaling pathway is involved in heregulin-induced apoptosis. Although PI3-K inhibition altered heregulin-induced G2-M-phase arrest (Fig. 9), it did not alter heregulin-induced apoptosis (Fig. 10).

Effect of Blocking PI3-K and MAP Kinase Signaling Pathways on Heregulin-induced Cell Differentiation. To examine the effect of blocking the MAP kinase and PI3-K signaling pathways on heregulin-induced differentiation, cells were again pretreated with the individual inhibitors as described above and then subjected to heregulin treatment for 72 h. Cell differentiation was assessed by Oil Red O staining as described in “Materials and Methods.” As shown in Fig. 12, LY294002 or wortmannin treatment completely abolished heregulin-induced cell differentiation, whereas PD98059 or SB203580 treatment did not affect this process. Simultaneous inhibition of both MAP kinase and PI3-K pathways by LY294002 plus PD98059 or LY294002 plus SB203580 further confirmed that inhibition of PI3-K was sufficient to block heregulin-induced cell differentiation. Interestingly, rapamycin itself induced dramatic cell differentiation in
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SKBr3 cells (Fig. 12). Heregulin plus rapamycin produced a degree of cell differentiation similar to that observed with rapamycin alone (Fig. 12). These results indicated that the LY294002- and Wortmannin-sensitive PI3-K pathway, but not the MAP kinase pathways, played an important role in heregulin-induced differentiation.

DISCUSSION

Overexpression of the HER2 proto-oncogene is found in 30% of human breast cancers and 15–30% of human ovarian cancers (38). The extent of overexpression correlates inversely with survival in node-positive breast cancer and advanced ovarian cancer patients. An adverse prognosis may relate more to increased invasive potential than to an increased rate of proliferation (10). New data suggest that HER2 may be useful not only as a prognostic factor but also as a predictive marker for response to certain cytotoxic drugs, antiestrogens, and anti-p185HER2 antibodies (39).

Experimental studies suggest that anti-p185HER2 antibodies can enhance the efficacy of treatment for women with breast cancers that overexpress HER2 (4–10). Clinical trials have shown that a humanized anti-p185HER2 antibody, trastuzumab (Herceptin), can produce a 12–20% overall response rate as a single agent in patients whose cancers overexpress the proto-oncogene. Trastuzumab, in combination with chemotherapy, significantly enhanced the activity of cytotoxic chemotherapy against recurrent metastatic breast cancer, as assessed by response rate (62.0% versus 36.2%) and time to disease progression [8.8 versus 5.5 months (40)]. The mechanisms by which the antibody inhibits cancer growth and potentiates chemotherapy are not clear. Recently, several reports, including our own, have demonstrated that anti-p185HER2 antibodies can induce cell cycle Gi arrest by up-regulation of p27Kip1, hypophosphorylation of the RB protein, and down-regulation of G1-related cyclins and cyclin-dependent kinases (17, 25–28). Inhibition of anchorage-independent growth is irreversibly determined within 6 h of antibody treatment (17); therefore, early signaling events are likely to be important.

Whereas anti-p185HER2 antibodies bind to the receptor alone, no specific ligand has been found that binds only to HER2. Instead, HER2 appears to be the preferred partner for heterodimerization with the other three HER members (41). The relative expression of different receptors appears to be an important determinant of ligand activity. Heregulin can stimulate anchorage-independent growth of cells that express low levels of HER2 and HER3 but inhibits the growth of cells with high levels of HER2 (42). Again, inhibition of clonogenic growth is determined irreversibly by 24 h after treatment with heregulin (17). In a previous report, we have shown that heregulin induces G0-M-phase arrest, apoptosis, and differentiation in SKBr3 cells that overexpress HER2 and also express low levels of HER3 (17). The anti-p185HER2 antibody ID5 did not exert these effects. Consequently, signaling through HER2/HER3 with heregulin and signaling through HER2 alone with antibody appeared to have very different outcomes. The present study was undertaken to examine the differences in signaling induced by these two probes in greater depth.

Both heregulin and the anti-p185HER2 antibodies have been reported to act as agonists to induce tyrosine phosphorylation of HER2 (5, 6, 43). In this study, we have found that ID5 induced tyrosine phosphorylation of HER2 but not HER3 in SKBr3 cells. In contrast, heregulin induced phosphorylation of both HER3 and HER2 (Fig. 1). Heregulin-induced tyrosine phosphorylation of HER3 is thought to be mediated by the HER2 kinase because HER3 lacks intrinsic kinase activity (23, 44). The difference in tyrosine phosphorylation of HER family receptors undoubtedly contributes to the difference in subsequent signal transduction.

Tyrosine phosphorylation of HER2 produces docking sites for GRB2 and PLC-γ. As shown in Figs. 1 and 6, ID5 produced a greater association of PLC-γ1 with phosphorylated HER2 than did heregulin. Moreover, ID5, but not heregulin, increased PLC-γ1 activity. In previous reports, PLC-γ1 has been shown to associate with activated HER2 (45), whereas PLC-γ1 does not associate with HER3 (24). Our data in Fig. 1 confirmed that HER3 did not interact with PLC-γ1. As shown in Fig. 1, ID5 was able to induce the association of phosphorylated HER2 with GRB2. Activation of ERK1/2 and p38 MAP kinase induced by ID5 was transient and weak (Fig. 3). We speculated that ID5 might induce GRB2 to couple to and activate PLC-γ1 because GRB2 has already been reported to associate with PLC-γ1 (46). Our data (shown in Fig. 6) indicated that ID5 increased PLC-γ1 enzymatic activity, which also agreed with the results of another report showing that several anti-p185HER2 monoclonal antibodies stimulated PLC-γ1 enzymatic activity in SKBr3 cells (43).

We have found that both ID5 and heregulin induced association of SHC and GRB2 with HER2. However, ID5 preferentially induced binding of the M5, 46,000 isoform of SHC to HER2, whereas heregulin preferentially induced binding of the M7, 52,000 isoform of SHC to HER3 (Fig. 1). GRB2 can dock to growth factor receptors by at least two different mechanisms: (a) directly through the SH2 domain of GRB2; and (b) indirectly through docking to tyrosine-phosphorylated SHC, which is already docked to phosphorylated tyrosines in the receptors through its own SH2 domain (22). It has been reported that GRB2 and SHC are constitutively associated with HER2 and HER3 receptors (24, 33). Our data suggest that ID5-dependent SHC signaling may occur mainly via the M5, 46,000 SHC isoform, whereas heregulin signaling preferentially uses the M7, 52,000 SHC isoform. Similarly, Okada et al. (47) reported that insulin-induced signaling in Chinese hamster ovary cells uses only the M5, 52,000 SHC isoform, whereas EGF-dependent SHC signaling involves both the M7, 52,000 and M4, 46,000 SHC species. Crovello et al. (48) also reported that in MDA-MB-468 breast cancer cells, heregulin induced association of only the M7, 52,000 SHC isoform with HER3.

Heregulin has been shown to activate both the MAP kinase and PI3-K signaling pathways (30–37, 49). The critical role of PI3-K activity has been well documented in heregulin-induced signaling pathways (30–32, 49). Heregulin-induced activation of PI3-K has been reported to be mainly through HER2-HER3 heterodimers (36).
Data presented in Fig. 2A indicated that heregulin, but not ID5, induced HER2-HER3 association in SKBr3 cells. ID5 was capable of interrupting heregulin-induced HER3-HER2 interaction (Fig. 2A). ID5 also inhibited heregulin-induced JNK kinase activity (Fig. 3D) and PI3-K activity (Fig. 5A). These findings were supported by reports that heregulin binding to HER3 and HER4 was inhibited with a HER2 monoclonal antibody (50) and that a monoclonal antibody to HER2 inhibited the ability of heregulin to activate PI3-K-dependent AKT in the MCF-7 cells (49). Heregulin, but not ID5, was found to induce association of the p85 of PI3-K with HER3 (Fig. 1). Sustained activation of PI3-K signaling was induced by heregulin, whereas ID5 had only a transient effect (Fig. 5B). Our observations are consistent with previous reports that HER3, but not HER2, associates with the p85 subunit of PI3-K and initiates signal transduction through the PI3-K pathway (23, 24, 48). ID5 did not generate tyrosine phosphorylation of HER3 and HER2-HER3 heterodimers (Figs. 1 and 2A). Thus, ID5 would be unable to activate PI3-K signaling. The transient activation of PI3-K signaling observed by ID5 (Fig. 5) likely derived from other than PI3-K signaling such as Ras-MAP kinase pathways.

PI3-K signaling can modulate G1 cell cycle progression. PTEN, a tumor suppressor that possesses intrinsic phosphatase activity, was shown to increase p27Kip1 expression, decrease G1 cyclin-dependent kinase activities, and block cell cycle progression in G1 phase (51). Inhibition of PI3-K by LY294002 or wortmannin led to up-regulation of p27Kip1 and a transient G1 arrest in SKBr3 cells (data not shown). Thus, inhibition of PI3-K, to some extent, mimicked the effect of anti-p185HER2 antibody in SKBr3 cells. Indeed, ID5 activated only HER2 and produced little, if any, signal to the PI3-K pathway as shown in Figs. 1 and 5. Furthermore, ID5 was able to suppress heregulin-induced PI3-K activation (Fig. 5A). In contrast, heregulin produced strong and sustained PI3-K signaling through HER2-HER3 heterodimerization and association of HER3 with p85 (Figs. 1 and 5). As presented in Fig. 9, heregulin induced G1-M-phase cell cycle arrest in SKBr3 cells, rather than G1 arrest.

It is known that Ras functions as a molecular switch for reentry into the cell cycle at the border between G0 and G1 by transducing extracellular growth stimuli into early G1 mitogenic signals. Inactivation of Ras function with dominant negative Ras mutant and farnesyl-protein transferase inhibitor prevents down-regulation of p27Kip1 (52, 53), leads to failure of MAP kinase to phosphorylate p27Kip1 [the phosphorylated p27Kip1 cannot bind to and inhibit cyclin-dependent kinase 2 (54)], and down-regulates cyclin D1 expression (55). On the other hand, sustained activation of Ras function will promote G1-S-phase progression (52–55). ID5, which induces G1 arrest in SKBr3 cells, generated only transient signaling through the Ras-MAP pathway (Figs. 3 and 4). Heregulin, which induces G2-M-phase arrest, generated more prolonged or sustained signals via Ras-MAP pathways (Figs. 3 and 4). Daly et al. (30) reported that the inhibition of p38 MAP kinase at a late stage (day 2–3) could prevent heregulin-induced apoptosis, whereas our data (presented in Fig. 10) showed that inhibition of p38 MAP kinase did not prevent heregulin-induced apoptosis. No effect on heregulin-induced apoptosis of SKBr3 cells was observed when p38 MAP kinase inhibitor was added on day 2 or day 3 in addition to the initiation of culture (data not shown). Also, Daly et al. (30) showed that the PI3-K inhibitor LY294002 inhibited heregulin-induced apoptosis, whereas our data in Fig. 11 indicated that neither LY294002 nor wortmannin prevents heregulin-induced apoptosis. At present, we do not have an explanation for the different outcome of the two studies. Apparently, the biological effect and the underlying signal transduction of heregulin vary remarkably among different cell types. In support of this contention, in AU565 human breast cancer cells, sustained activation of the ERK1/2 pathway is both essential and sufficient for heregulin-induced differentiation (32). In T-47D human breast cancer cells, ERK1/2 activation is critical to heregulin-induced cell cycle G1 to S-phase progression, whereas the PI3-K pathway plays a minor role (34). In SKBr3 human breast cancer cells, ERK1/2 has been shown to play a limited role in heregulin-induced cell cycle arrest (Ref. 30 and this report). Differences in signaling between cell types may reflect different HER receptor profiles (36).

In a previous report, we have demonstrated that heregulin inhibits the growth of SKBr3 cells through induction of G2-M-phase arrest, apoptosis, and cell differentiation (17). Data in Figs. 9, 10, and 12 of this study showed that heregulin-induced G2-M-phase cell cycle arrest, apoptosis, and differentiation could not be affected by inhibition of MAP kinase. Heregulin-induced apoptosis could be blocked by inhibition of p70s6k, but not by inhibition of PI3-K. Heregulin-induced differentiation could be eliminated by inhibition of PI3-K. These results suggest that the three events (G2-M-phase cell cycle arrest, apoptosis, and cell differentiation) induced by heregulin in SKBr3 cells use different signaling pathways.

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Differential Signaling by an Anti-p185^HER2^ Antibody and Heregulin

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