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Herceptin-induced Inhibition of Phosphatidylinositol-3 Kinase and Akt Is Required for Antibody-mediated Effects on p27, Cyclin D1, and Antitumor Action[1]

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ABSTRACT

We have examined whether inhibition of phosphatidylinositol-3 kinase (PI3K) and its target, the serine/threonine kinase Akt, play a role in the antitumor effect of the HER2 antibody Herceptin. Herceptin inhibited colony formation, down-regulated cyclin D1, and increased p27 protein levels in the *HER2* gene-amplified BT-474 and SKBR-3 human breast cancer cells. These effects were temporally associated with the inhibition of PI3K activity *in vitro* as well as Akt function as measured by steady-state levels of phospho-Ser473 Akt and kinase activity against glycogen synthase kinase (GSK)-3β. These responses were not observed in MDA-361 and MDA-453 cells, which do not exhibit *HER2* gene amplification and are relatively resistant to Herceptin. Treatment of BT-474 cells with Herceptin inhibited the constitutive tyrosine phosphorylation of HER3 and disrupted the basal association of HER3 with HER2 and of HER3 with p85α potentially explaining the inhibition of PI3K. Treatment with either Herceptin or the PI3K inhibitor LY294002 increased the levels of p27 in the nucleus>cytosol, thus increasing the ratio of p27:Cdk2 in the nucleus and inhibiting Cdk2 activity and cell proliferation. Antisense p27 oligonucleotides abrogated the increase in p27 induced by Herceptin and prevented the antibody-mediated reduction in S phase. Transduction of BT-474 cells with an adenovirus-encoding active (myristoylated) Akt (Myr-Akt), but not with a β-galactosidase control adenovirus, prevented the Herceptin- or LY294002-induced down-regulation of cyclin D1 and of phosphorylated GSK-3β and prevented the accumulation of p27 in the nucleus and cytosol. In addition, Myr-Akt prevented Herceptin-induced inhibition of the cell proliferation of BT-474 cells and Herceptin-induced apoptosis of SKBR-3 cells. These data suggest that (*a*) changes in cell cycle- and apoptosis-regulatory molecules after HER2 blockade with Herceptin result, at least in part, from the inhibition of Akt; and (*b*) disabling PI3K and Akt is required for the antitumor effect of HER2 inhibitors.

INTRODUCTION

The HER2/*neu* (*erbB2*) proto-oncogene product is a member of the HER (erbB) family of transmembrane receptor tyrosine kinases, which also includes the EGFR[3] (HER1, erbB1), HER3 (erbB3), and HER4 (erbB4). Except for HER2, the binding of receptor-specific ligands to the ectodomain of EGFR, HER3, and HER4 results in the formation of homodimeric and heterodimeric kinase-active complexes into which HER2 is recruited as a preferred partner (1–3). Although HER2 is unable to directly interact with HER-activating ligands, it is well established that its kinase can potentiate signaling by HER2-containing heterodimers and/or increase the binding affinity of ligands to EGFR and HER3 (4–10). Dysregulated signaling by this receptor network has been causally associated with early transformation of mammary epithelial cells (11, 12) as well as with enhanced breast cancer cell proliferation and shorter survival in patients with mammary carcinomas (13, 14). For HER2, the most common change in human breast tumors involves overexpression of HER2 mRNA or protein with or without amplification of the HER2/*neu* locus (15). Recently, trastuzumab (Herceptin), a humanized monoclonal IgG1 that binds the ectodomain of HER2 (16), was shown to induce the regression of HER2-overexpressing breast cancers (17–19), thus confirming the role of HER2 in the progression of some human breast cancers. However, not all tumors expressing high levels of the proto-oncogene responded clinically to the HER2 antibody, which suggests that in some of these advanced cancers, HER2 had become dispensable for tumor progression and survival.

Studies with breast cancer cell lines and human tumors have demonstrated constitutive phosphorylation of HER2 (20, 21). The biochemical basis for this constitutive activation is not clear, but it is consistent with the reported ability of wild-type neu, the rat homologue of human HER2, to multimerize and become activated when present at high concentrations in cells (22). Although not formally proven, it is likely that this spontaneous dimerization of HER2 also occurs in human tumors with *HER2* gene amplification. Another possible mechanism for activation of the HER2 tyrosine kinase in human breast cancers is the coexpression of ligand-activated EGFR or HER3/4. The recruitment of HER2 by ligand-activated coreceptors markedly potentiates signaling and transformation induced by the HER network. For example, in cells that coexpress HER2, ligand-activated EGFR preferentially recruits HER2 into an EGFR/HER2 heterodimer that exhibits an increased rate of recycling, stability, and signaling potency compared with EGFR homodimers (1, 8, 10). Although neither HER2 nor (kinase-deficient) HER3 alone can be activated by ligand, the HER2/HER3 heterodimer is the most mitogenic and transforming receptor complex within the HER receptor family (7, 20, 23, 24). In addition, inactivation of HER2/neu with single-chain HER2 antibodies or vectors encoding kinase-dead neu has been shown to impair EGFR-mediated transformation and the biochemical effects of HER3/4 ligands (25, 26), further supporting the crucial role of HER2 in the function of the HER network.

Activation of HER2-containing heterodimers results in receptor autophosphorylation on COOH-terminal tyrosine residues, which become the docking sites for a number of signal transducers and adaptor molecules that initiate a plethora of signaling programs leading to cell proliferation, differentiation, migration, adhesion, protection from apoptosis, and transformation, among other effects. Signaling pathways activated by the HER2 network include PLC-γ1, Ras-Raf-MEK-MAPK, PI3K-Akt-ribosomal S6 kinase; Src; the stress-activated protein kinases (SAPKs); PAK-JNNK-JNK; and the signal transducers and activators of transcription (STATs; 2, 3, 27, 28). The specificity and potency of the signaling output by the HER network is highly

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[3] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; PI3K, phosphatidylinositol-3 kinase; PIP_2, phosphatidylinositol 4,5-biphosphate; PIP_3, phosphatidylinositol 3,4,5-triphosphate; MAPK, mitogen-activated protein kinase; IMEM, improved minimal essential medium; Tdt, terminal deoxynucleotide transferase; TUNEL, Tdt dUTP nick end labeling; FACS, fluorescence-activated cell sorting; PMSF, phenylmethylsulfonyl fluoride; HH1, histone H1; TLC, thin layer chromatography; mAb, monoclonal antibody; Myr-Akt, myristoylated Akt; β-gal, β-galactosidase; MOI, multiplicity/multiplicities of infection; FISH, fluorescent *in situ* hybridization; Cdk, cyclin-dependent kinase; MM, mismatch; AS, antisense; P-Tyr, phosphorylated Tyr; P-GSK, phosphorylated GSK; P-Akt, phosphorylated Akt; P-MAPK, phosphorylated MAPK; GSK, glycogen synthase kinase.

dependent on the composition of the heterodimeric receptor complexes. Although kinase-deficient, HER3 is able to directly couple to PI3K (29, 30), a lipid kinase involved in the proliferation, survival, adhesion, and motility of tumor cells (31–33). Because of (*a*) the potent transforming ability of HER2/HER3 heterodimers (*b*) the coexpression and association of HER2 and HER3 in breast cancer cell lines as well as in mouse transgenic and human mammary tumors (20, 34–39), and (*c*) the ability of HER2 to activate PI3K and its downstream targets (36, 40–43), we have examined whether the inhibition of this signaling pathway is required for the antitumor effect of the HER2 blocking antibody Herceptin. The data presented below strongly suggest that the inhibition of PI3K and its target, the serine-threonine kinase Akt, is required for the antitumor action of Herceptin against HER2-overexpressing breast cancer cells.

MATERIALS AND METHODS

Cell Lines, Kinase Inhibitors, and Antibodies. The human breast cancer cell lines BT-474, SKBR-3, MDA-453, and MDA-361 were obtained from the American Type Tissue Culture Collection (Manassas, VA) and maintained in IMEM (Life Technologies, Inc. Rockville, MD) supplemented with 10% FCS. Herceptin was purchased from the Vanderbilt University Hospital Pharmacy (Nashville, TN). LY294002, a specific inhibitor of the p110 catalytic subunit of PI3K (44), was from BIOMOL (Plymouth Meeting, PA). For immunoprecipitations and/or immunoblot analysis, the following antibodies were used: GSK-3β and p27 (Transduction Laboratories, Lexington, KY); HER2/neu and HER3 (Neomarkers, Freemont, CA); P-Tyr (Upstate Biotechnology, Lake Placid, NY); cyclin D1 (PharMingen, San Diego, CA); MAPK, P-GSK-3β, Akt and Ser473 P-Akt (New England Biolabs, Beverly, MA); P-MAPK (Promega, Madison, WI); p85α, Cdk2, and c-jun (Santa Cruz Biotechnology, Santa Cruz, CA). Protein content in cell lysates was measured by the BCA method (Pierce, Rockford, IL).

Monolayer Growth, Colony Formation, and TUNEL Assays. Cells were seeded in 6-well plates in IMEM/10% FCS at a density of 3×10^4 cells/well; Herceptin or LY294002 was added the next day. Medium and inhibitors were replaced with fresh medium and inhibitors every other day until cells were harvested by trypsinization and counted with a Zeiss Coulter Counter (Beckman Coulter, Miami, FL). Colony-forming assays were performed as described previously (45) in the presence or absence of Herceptin. Tumor cell colonies measuring $\geq$50 μm were counted using an Omnicon 3800 Colony Counter an Tumor Colony Analyzer V2.IIA software (Imaging Products International, Inc.). To measure apoptosis, adherent cells in IMEM/10% FCS were treated with Herceptin for 72 h, harvested by scraping, and pooled with floating cells. TUNEL assay was performed using the APO-BrdU kit (Phoenix Flow Systems, San Diego, CA). Flow cytometric detection of FITC-positive cells was performed using a FACS/Calibur Flow Cytometer (Becton Dickinson, Mansfield, MA).

Flow Cytometric Analysis. Cells were trypsinized and labeled with propidium iodide as described previously (42). A total of 10,000 labeled nuclei were analyzed in a FACS/Calibur Flow Cytometer. DNA histograms were analyzed off-line using CELLQuest software (Becton Dickinson).

Cell Fractionation. Nuclear and cytoplasmic fractions were prepared as described previously (46). Cells were harvested by trypsinization, washed with PBS, and incubated in hypotonic buffer [10 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin and aprotinin, 200 μM Na_3VO_4, 1 mM PMSF, and 10 mM NaF] for 30 min at 4°C. The swollen cells were next homogenized in a Dounce homogenizer with 30 strokes and centrifuged at 12,000 $\times$ *g* for 10 min at 4°C. The supernatant was removed and labeled as "cytoplasmic" fraction The nuclear pellet was washed once in hypotonic buffer, lysed with Triton X lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 μg/ml leupeptin and aprotinin, 200 μM Na_3VO_4, 1 mM PMSF, and 10 mM NaF], sonicated for 1 min in a water bath sonicator (Fisher Scientific), and incubated for 30 min at 4°C. The lysate was centrifuged at 12,000 $\times$ *g* for 10 min at 4°C; the supernatant was labeled as "nuclear" fraction.

Immunoblot Analysis and Immunoprecipitation. After washes, cells were lysed in NP40 lysis buffer [0.5% NP40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 200 μM Na_3VO_4, 1 mM PMSF, and 2 μg/ml leupeptin and aprotinin]. For immunoblot analysis, total protein from cell lysates was resolved by SDS-PAGE followed by transfer to nitrocellulose. Immunoblot analysis was performed as described previously (45) using the primary antibodies mentioned above and horseradish peroxidase-linked IgG (Amersham Pharmacia) followed by enhanced chemiluminescence (Roche Molecular Biochemicals, Indianapolis, IN). For immunoprecipitations, 0.5–1 mg of protein from either cell fractions or whole cell lysates was incubated overnight with primary antibody at 4°C; protein A-Sepharose (Sigma) or protein G Sepharose (Pharmacia) was then added for 2 h at 4°C while rocking. The precipitates were washed four times with ice-cold PBS, resuspended in 6$\times$ Laemmli sample buffer, and resolved using SDS-PAGE followed by immunoblot analysis.

***In Vitro* Kinase Reactions.** Cells were lysed in NP40 lysis buffer as described above; 100–300 μg of protein from whole cell lysates or 50–100 μg of protein from cytoplasmic and nuclear fractions were precipitated overnight at 4°C with immobilized-Akt 1G1 monoclonal IgG2a (New England BioLabs) or a Cdk2 antibody, respectively. The immunoprecipitates were washed extensively with NP40 lysis buffer followed by washings in the respective kinase buffer. Cdk2 activity against HH1 was measured as described by Lenferink *et al.* (42). For Akt activity, reactions were performed at 37°C for 30 min in a total volume of 30 μl containing kinase buffer [50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin and aprotinen], 10 μCi [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol; Amersham Pharmacia), and 0.04 μg GSK-3β (Sigma). All of the reactions were terminated with the addition of 6$\times$ Laemmli buffer and heating. Kinase products were subjected to 12% SDS-PAGE followed by autoradiography.

For the determination of PI3K activity, cells were seeded at a density of 5×10^5 cells/100-mm dish 24 h prior to a treatment with Herceptin or LY294002. After treatment, the cells were washed twice with 137 mM NaCl, 20 mM Tris (pH 7.5), 1 mM $CaCl_2$, and 1 mM $MgCl_2$, and were lysed in the wash buffer supplemented with 10% glycerol, 1% NP40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM NaF, and 200 μM Na_3VO_4. Cell extracts were precleared by centrifugation and then were precipitated overnight with a P-Tyr mAb (Upstate Biotechnology) and protein A-Sepharose. Immune complexes were washed three times with 1% NP40 in PBS; 2 $\times$ with 100 mM Tris (pH 7.5) and 0.5 M LiCl; twice with 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA; and twice with kinase assay buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 0.5 mM EGTA]. All of the wash buffers contained 200 μM Na_3VO_4. The beads were suspended in 40 μl of kinase buffer followed by the addition of 10 μCi [γ-^{32}P]ATP and sonication in 0.2 mg/ml PIP2 (Avanti). The kinase reaction proceeded for 10 min at room temperature and was terminated with stop buffer (1:1, methanol:HCl) followed by extraction with chloroform. The reaction products were separated by TLC on 1% oxalate-pretreated TLC with chloroform:methanol:acetone:glacial acetic acid:water (60:20:23:18:11) and were detected by autoradiography.

Immunofluorescent Localization of p27^{KIP1}. BT-474 cells were seeded on coverslips in 6-well plates at a density of 4×10^4 cells/well. After an 8–24 h incubation with Herceptin or LY294002, the cells were washed with PBS, fixed in 4% paraformaldehyde/PBS for 10 min, washed, and stored overnight at 4°C. Cells were then permeabilized with 0.1% Triton X-100/PBS for 15 min, washed, and then incubated for 1 h with a p27 mAb (Transduction Laboratories) diluted 1:250 in 0.05% Triton X-100/PBS. After three washes with 0.05% Triton X-100/PBS, the cells were incubated for 45 min with antimouse Cy3 IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:500 in 0.05% Triton X-100/PBS. The cells were washed six times, stained with 1 mg/ml Hoechst, and mounted in AquaPoly Mount (PolySciences, Inc.). Cy3 immunofluorescence was recorded with a Princeton Instruments cooled digital CCD camera on a Zeiss Axiophot upright microscope.

Studies with AS p27 Oligonucleotides. The sequences of the 15-mer p27 and MM phosphorothioates (provided by M. Flanagan, Gilead Sciences, Foster City, CA) were reported previously (47). BT-474 cells in the presence or absence of Herceptin were treated with 30 nM oligonucleotides for 5 h as described by Lenferink *et al.* (42). To control for nonspecific effects of cytofectin, cells treated with neither AS p27 nor MM oligonucleotides were, nevertheless, treated with 2 μg/ml cytofectin GS3815 (Gilead Sciences). Forty-eight h after treatment with oligonucleotides, the cells were either trypsinized and subjected to cell cycle analysis by flow cytometry or were lysed and subjected to p27 and HER2 immunoblot procedures as indicated above.

Myr-Akt and β-gal Adenoviral Infection. Adenoviral vectors were provided by Dr. W. Ogawa (Kobe University, Kobe, Japan) and have been described elsewhere (48). BT-474 cells were seeded at a density of 3×10^5 cells/60-mm dish 24 h before transduction with adenoviral vectors at 80 plaque-forming units/cell (MOI) as described by Sakaue *et al.* (49). More than 90% of BT-474 cells infected at a similar MOI with a β-gal adenovirus exhibited blue staining. Infections were conducted for 5 h in serum-free medium followed by a 48-h incubation in IMEM/10% FCS before the addition of antibodies or kinase inhibitors.

HER2/neu FISH. Adherent tumor cells were trypsinized, washed, and then swollen in (hypotonic) 0.075 M KCl and fixed in 3:1 methanol:acetic acid prior to seeding onto slides. After an overnight incubation at room temperature, the slides were denatured in 2× SSC/70% formamide at 73°C for 5 min. Denatured slides were dehydrated in 70, 85, and 100% ethanol for 1 min each. The slides were next dried and 10 μl of the Vysis LSI HER2 SpectrumOrange and CEP 17 SpectrumGreen probe mixture (Downers Grove, IL) were applied to them. Slides were sealed with rubber cement, incubated overnight in a humidified chamber at 37°C, washed in 0.4× SSC/0.3% NP40 for 2 min at 73°C, and then rinsed in 2× SSC/0.1% NP40 at room temperature. After adding DAPI II counterstain (10 μl) to each hybridization area on the slides, coverslips were applied onto them. Signal enumeration was performed at ×400 magnification in a fluorescence microscope equipped with a dual-pass filter (Chromatechnology, Brattlebro, VT) for the simultaneous detection of SpectrumOrange and DAPI and a single-pass filter for the detection of SpectrumAqua. Enumeration of HER2 and CEP 17 signals was performed on 25 consecutive cells. Images of representative cells were captured at ×630 with a single-bandpass filter for the detection of SpectrumOrange, SpectrumAqua, or DAPI using IP Labs imaging software package (Scanalytics, Inc., Fairfax VA).

RESULTS

Herceptin Inhibits HER2 Signaling in *HER2* Gene-amplified Breast Cancer Cells. We initially examined the effect of the humanized IgG1 Herceptin against BT-474, SKBR-3, MDA-361, and MDA-453 human breast cancer cells, which are known to overexpress HER2 (20, 50). In a colony-forming assay, Herceptin inhibited BT-474 and SKBR-3 cells with an IC_{50} of ~0.2 μg/ml. However, doses as high as 20 μg/ml did not inhibit MDA-361 and MDA-453 colony formation (Fig. 1*A*). The degree of gene amplification measured by DNA dot-blot analysis (45) as well as the levels of HER2 protein measured by HER2 antibody binding or immunohistochemical studies (51, 52) are reportedly different among these four cell lines. Thus, we next deter-

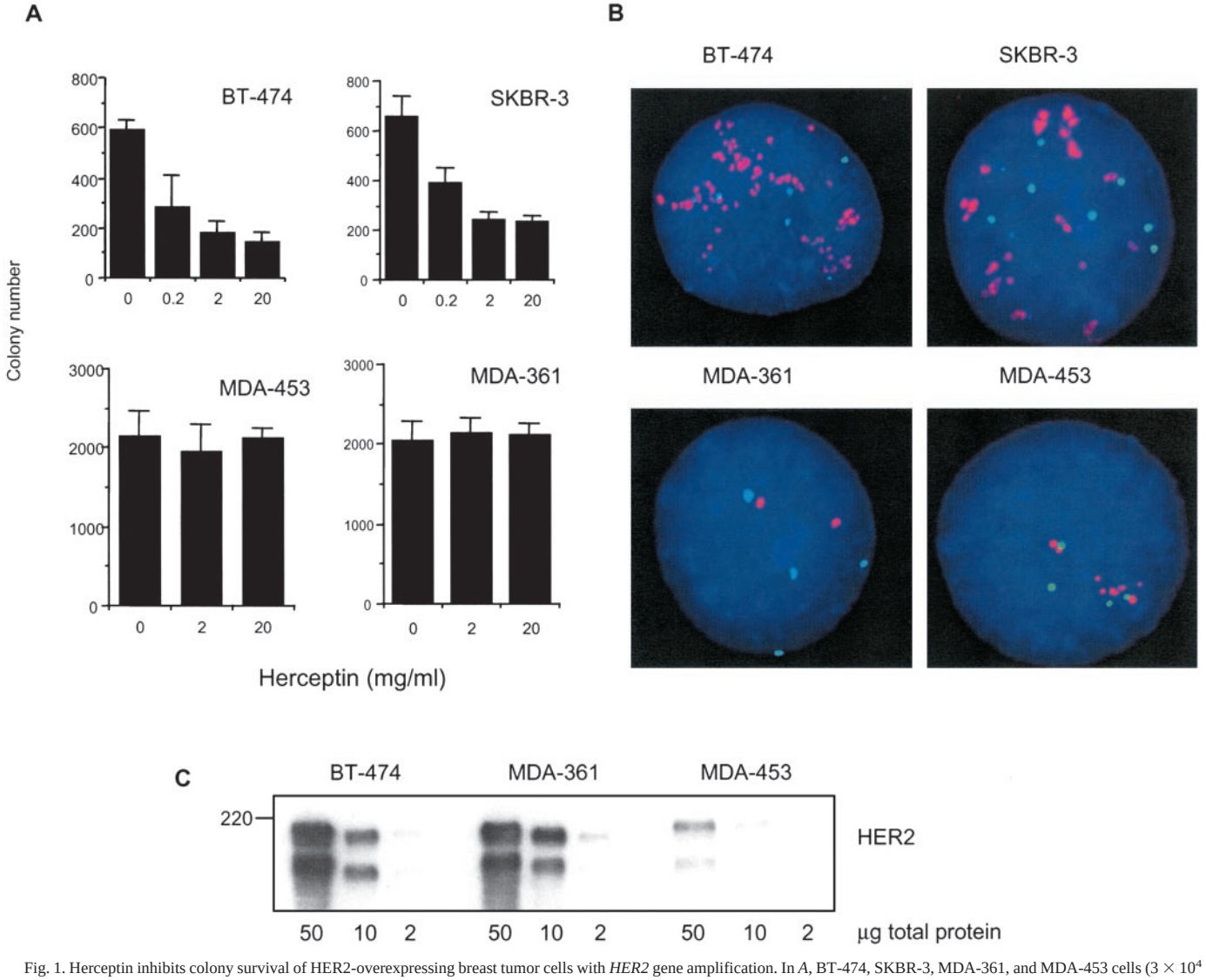

Fig. 1. Herceptin inhibits colony survival of HER2-overexpressing breast tumor cells with *HER2* gene amplification. In *A*, BT-474, SKBR-3, MDA-361, and MDA-453 cells (3×10^4 cells) were plated in 35-mm dishes in IMEM/10% FCS, 0.8% agarose, and 10 mM HEPES in the absence or presence of 0.2–20 μg/ml Herceptin. After 7 days, colonies measuring ≥50 μm were counted as indicated in "Materials and Methods." *Each bar*, the mean ± SD of three dishes. In *B*, cell suspensions of all four cell lines were fixed and subjected to FISH analysis as described in "Materials and Methods." In *C*, monolayers of the indicated cell lines were washed and solubilized in NP40 lysis buffer. The indicated amounts of protein were subjected to immunoblot analysis for HER2.

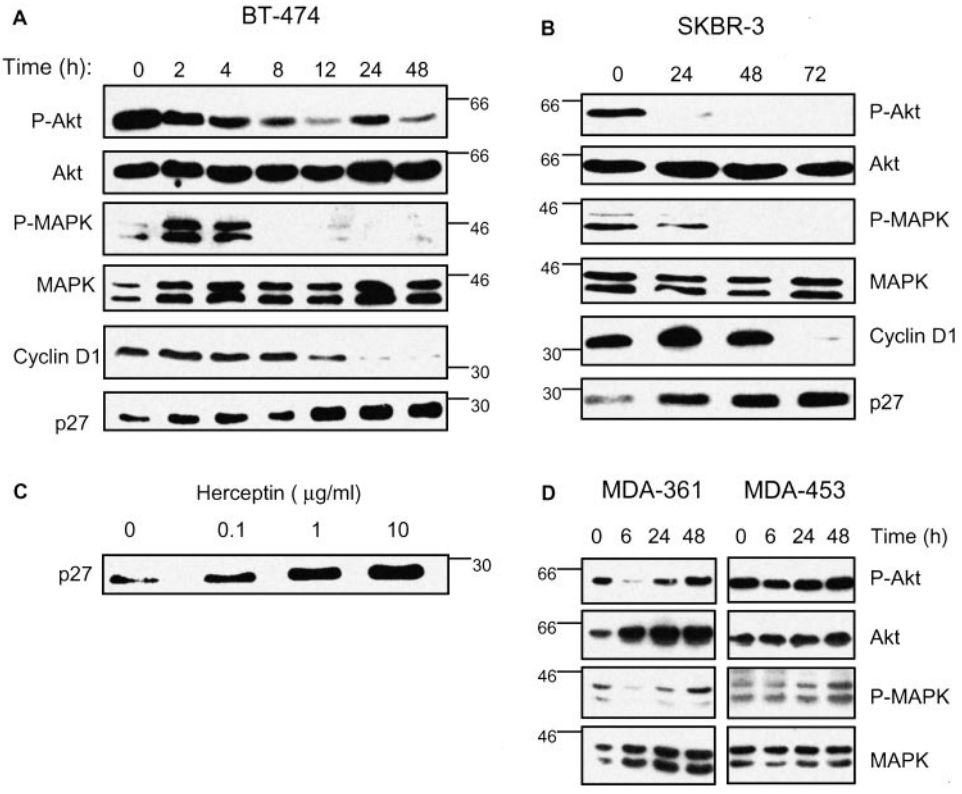

Fig. 2. Herceptin inhibits active Akt and MAPK and modulates cyclin D1 and p27 protein levels. In *A* and *B*, BT-474 and SKBR-3 breast cancer cells in IMEM/10% FCS were treated with 10 μg/ml Herceptin. At the indicated times, the cells were washed and harvested in lysis buffer. Seventy μg of protein from whole cell lysates were resolved by SDS-PAGE and subjected to the indicated immunoblot procedures. *To the side of each panel*, M_r in thousands. In *C*, dose-dependent effect of Herceptin on p27 levels. BT-474 cells were treated with 0.1–10 μg/ml Herceptin for 24 h. At this time, cells were washed, lysed, and tested for p27 protein content by immunoblot analysis. In *D*, exponentially growing MDA-361 and MDA-453 cells in IMEM/10% FCS were incubated with 10 μg/ml Herceptin. After 6–48 h, the cells were lysed and tested for the content of P-Akt, total Akt, P-MAPK, and total MAPK by immunoblot analysis as described in "Materials and Methods." *Each lane*, 70 μg of protein from whole cell lysates.

mined *HER2* gene copy number by FISH analysis. The method used determines HER2 copy number corrected to the number of copies of chromosome 17 (53). BT-474 and SKBR-3 exhibited 5-fold *HER2* gene amplification, whereas MDA-361 and MDA-453 cells contained a single copy of the HER2/*neu* gene (Fig. 1*B*). Compared with BT-474 cells, HER2 protein levels were approximately similar in MDA-361 but much lower in MDA-453 cells, as measured by immunoblot (Fig. 1*C*).

We next examined whether the signaling-pathways downstream HER2 that regulate cell cycle progression and/or cell death are modified by Herceptin. In BT-474 and SKBR-3 cells, Herceptin inhibited active MAPK and active Akt as measured by antibodies specific to P-MAPK and phospho-Ser473 Akt, respectively, without changes in total MAPK and total Akt (Fig. 2, *A* and *B*). In BT-474 cells, these changes were noticed as early as 8–12 h after treatment with the HER2 antibody. In addition, cyclin D1 was down-regulated, and p27 protein levels were increased (Fig. 2, *A* and *C*). In SKBR-3 cells, the down-regulation of cyclin D1 was observed after 72 h of treatment with Herceptin (Fig. 2*B*). Consistent with the lack of a growth-inhibitory effect, Herceptin treatment was unable to reduce P-MAPK or P-Akt in MDA-453 cells, whereas in MDA-361 cells, it resulted in a transient decrease of these phosphoproteins at 6 h, with recovery at 24 h (Fig. 2*D*).

Herceptin Inhibits PI3 and Akt Kinases in *HER2* Gene-amplified Cells. Because of (*a*) the effect of Herceptin on the levels of phosphorylated (active) Akt, a target of PI3K, (*b*) the potent ability of HER3, present in BT-474 and SKBR-3 cells, to couple to PI3K directly (29, 30), and (*c*) the constitutive association of HER2 with HER3 reported in HER2-overexpressing tumor cells (20, 39), we examined the effect of Herceptin on the coupling of HER2 with HER3 and on the catalytic activity of PI3K and Akt. Treatment of BT-474 cells with Herceptin eliminated PI3K activity as measured by the ability of P-Tyr immunoprecipitates from BT-474 cell lysates to induce the formation of PIP_3 from PIP_2 *in vitro* (Fig. 3*A*). Similarly, the Akt kinase activity, as measured by the ability of Akt precipitates from Herceptin-treated BT-474 cells to phosphorylate a GSK-3β substrate *in vitro*, was severely reduced compared with that present in untreated cells (Fig. 3*B*). The inhibitory effect of Herceptin against PI3 and Akt kinases was similar to that induced in intact BT-474 by the PI3K inhibitor LY294002 (Fig. 3, *A* and *B*). At the same concentrations in which PI3K and Akt were inhibited, both Herceptin and LY294002 inhibited the growth of BT-474 cells (Fig. 3*C*), which suggested a link between the inhibition of PI3K-Akt and the antiproliferative effect of Herceptin.

In lysates from exponentially growing cells, HER3 antibodies were able to coprecipitate p85 and HER2 (Fig. 4*A*, *middle* and *right panels*), and p85 antibodies coprecipitated both HER3 and HER2 (Fig. 4*A*, *left* and *right panels*). In addition, a M_r ≈180,000 P-Tyr immunoreactive band was detectable in the HER3 precipitates (Fig. 4*B*, *Lane 1*), which indicated that, in BT-474 cells, HER3 is constitutively phosphorylated on Tyr and is associated with both HER2 and p85. Treatment with Herceptin induced a transient increase in HER3 phosphorylation at 1 h without disrupting the association with p85 (Fig. 4*B*, *Lane 2*). However, consistent with the inhibition of PI3K and Akt shown in Fig. 3, treatment with Herceptin for 24 h eliminated the basal phosphorylation of HER3 and the constitutive association of HER3 with p85 (Fig. 4*B*).

Inhibition of PI3K and Modulation of p27. Blockade of HER2 with Herceptin or HER kinase inhibitors has been reported to redirect the Cdk inhibitor to p27 to Cdk2, thus leading to the growth arrest of HER-overexpressing cells (39, 42). Therefore, we examined whether the inhibition of PI3K with either Herceptin or LY294002 induced similar modulation of p27 in BT-474 cells. By immunofluorescence microscopy, we observed that most of p27 was present in the cytosol of proliferating BT-474 cells. Treatment with growth-inhibitory concentrations of Herceptin or LY294002 for 20 h resulted in an almost complete translocation of immunofluorescent p27 from cytosol to cell nuclei (Fig. 5*A*). This effect was evident as early as 8 h after a

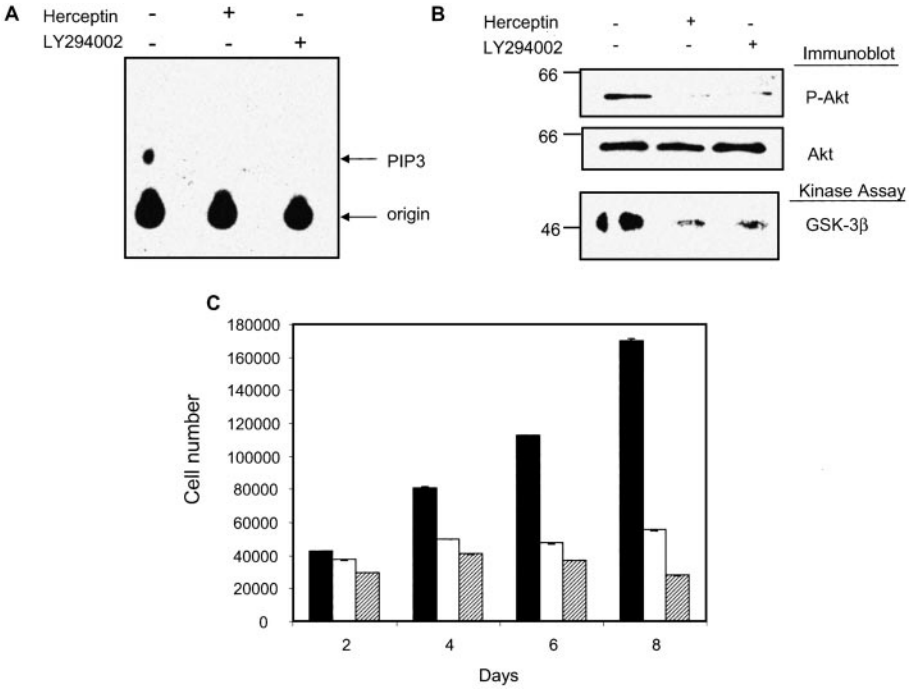

Fig. 3. Herceptin inhibits PI3K and Akt kinase activities *in vitro*. In *A*, BT-474 cells were treated with 10 μg/ml Herceptin or 40 μM LY294002 for 24 h. Whole cell lysates were precipitated with P-Tyr antibodies. Immune complexes were next assayed for PIP_3-forming activity using PIP_2 (origin) as a substrate. The reaction products were resolved by TLC and detected by autoradiography as described in "Materials and Methods." In *B*, identically treated cells were washed and lysed in NP40 lysis buffer; 100 μg per treatment condition were precipitated with immobilized Akt-Sepharose. Identical volumes of Sepharose slurry containing adsorbed immune complexes were then tested in immunoblot procedures for phospho-Ser473 Akt and total Akt (*top panels*) as well as in a kinase reaction against GSK-3β as substrate (*bottom panel*). In *C*, 3×10^4 cells/well were seeded in 6-well plates in IMEM/10% FCS in the absence (■) or presence of either 10 μg/ml Herceptin (□) or 40 μM LY294002 (▨). At the indicated times, cells were trypsinized and cell numbers determined in a Coulter counter. Fresh IMEM/10% FCS, either alone or supplemented with Herceptin or LY294002, was added on days 3, 5, and 7. *Each data point*, the mean ± SD of triplicate wells.

treatment with LY294002 but required >16 h with the saturating dose of Herceptin. By immunoblot of cell fractions, we observed that most of p27 was in the cytosol of the proliferating cells. Treatment with Herceptin or LY294002 increased the p27 protein levels in both nucleus and cytosol but more so in the nucleus (Fig. 5*B*). In proliferating untreated cells, a low level of nuclear p27 precipitated with Cdk2 antibodies. However, in cells that were treated for 20 h with Herceptin or LY294002, higher levels of p27 associated with nuclear Cdk2, which resulted in the suppression of the ability of nuclear Cdk2 precipitates to phosphorylate HH1 *in vitro* (Fig. 5*C*). Similar results were obtained when using GST-Rb as a Cdk2 kinase substrate (data not shown).

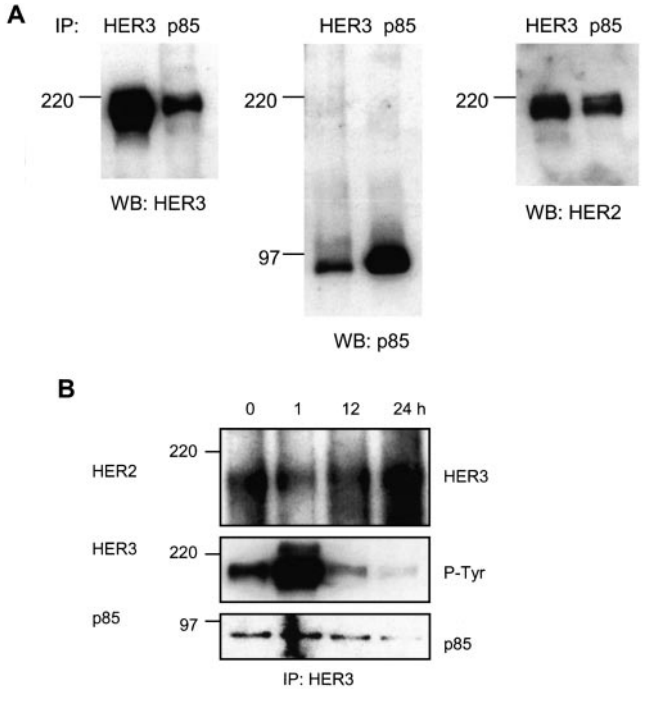

Fig. 4. Herceptin inhibits HER3 phosphorylation and its association with p85. In *A*, exponentially growing BT-474 cells in IMEM/10% FCS were lysed as described in "Materials and Methods." Total protein (0.5 mg) was immunoprecipitated overnight with either a HER3 or a p85 antibody. Immune complexes were divided into equal parts, resolved by SDS-PAGE, and finally subjected to Western blot (*WB*) analysis for HER3 (*left panel*), p85 (*middle panel*), and HER2 (*right panel*). In *B*, BT-474 cells were treated or not with 10 μg/ml Herceptin for 1–24 h, washed with ice-cold PBS, and solubilized in lysis buffer. Total protein from whole cell lysates (0.5 mg) was precipitated with a HER3 antibody. Immune complexes were divided into equal parts and analyzed for HER3, P-Tyr, and p85 by immunoblot analysis as described in "Materials and Methods." *On the left of each panel*, M_r in thousands.

To determine whether p27 played a role in the cell cycle arrest on blockade of HER2 with Herceptin, we used p27 AS oligonucleotides. In cells treated with cytofectin alone (control) or with MM oligonucleotides, but not in cells treated with AS p27 phosphorothioates, Herceptin up-regulated p27 levels (Fig. 6*B*). In cells treated with cytofectin alone or with MM oligonucleotides, treatment with Herceptin reduced the proportion of cells in S phase [11 *versus* 5% ($P = 0.01$) and 5.2% ($P = 0.03$), respectively] and increased the G_1 cell fraction [70 *versus* 85% ($P = 0.001$) and 83% ($P = 0.002$), respectively]. In cells in which p27 was down-regulated by AS p27 oligonucleotides, Herceptin failed to statistically reduce the proportion of cells in S phase compared with controls [11 *versus* 8% ($P = 0.28$)]. Although the AS oligonucleotides did not eliminate p27 content completely (Fig. 6*B*), it is likely that they prevented a threshold level of p27 required for redirection to and inhibition of Cdk2. In cells preincubated with AS p27 oligonucleotides, Herceptin still induced an increase in the G_1 cell fraction. However, this increase was less significant than that induced in cells treated with MM oligonucleotides [70 *versus* 76% ($P = 0.03$); Fig. 6A), which, all together, suggested that the modulation of p27 levels and/or its localization play a partial role in the growth arrest that follows HER2 blockade with Herceptin.

Constitutively Active Akt Abrogates the Antitumor Effect of Herceptin. We next examined whether forced expression of active Akt prevented Herceptin action. For this purpose, we used Myr-Akt, which consists of Akt1 ligated to a myristoylation sequence, resulting in an enzyme ~10-fold more active than the wild-type enzyme (48). In cells infected with a control β-gal adenovirus, both Herceptin and LY294002 inhibited PI3K as indicated by a marked reduction of

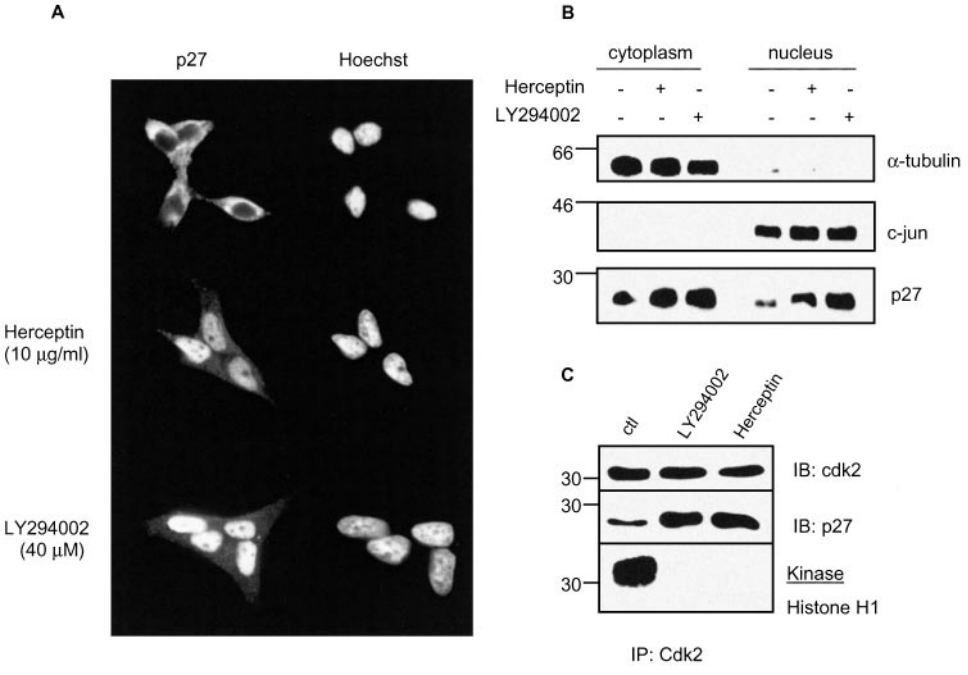

Fig. 5. Blockade of HER2 or PI3K results in nuclear accumulation of p27 and inhibition of nuclear Cdk2 activity. In *A*, BT-474 cells were treated with 10 μg/ml Herceptin or 40 μM LY294002 for 24 h and fixed. p27 was detected by immunohistochemistry with a p27 mAb. Localization of p27 was detected by indirect immunofluorescence with Cy3-conjugated antimouse IgG (*left panels*). The cells were counterstained with Hoechst to confirm the localization of the nuclei (*right panels*). In B, BT-474 cells were treated as in *A* and partitioned into cytoplasmic and nuclear fractions as described in "Materials and Methods," resolved by SDS-PAGE, and analyzed by p27 immunoblot. The fractions were also probed with antibodies representative of cytoplasmic (*α-tubulin*) and nuclear (*c-jun*) proteins, respectively. *C*, Cdk2 activity. Simultaneously treated BT-474 cells were harvested and nuclear fractions prepared as described in "Materials and Methods." Cdk2 was precipitated with Cdk2 antibodies. Immune complexes were divided into equal parts and subjected to Cdk2 and p27 immunoblot procedures (*top panels*) and tested for Cdk2 activity against HH1 (*bottom panel*).

P-Ser473 Akt, P-GSK-3β, and cyclin D1 protein levels (Fig. 7*A*). However, in cells infected with Myr-Akt, neither inhibitor reduced the P-Akt levels. This unchanged P-Akt band likely represents the membrane-associated Myr-Akt protein phosphorylated in Ser-473. Similarly, P-GSK-3β and cyclin D1 levels were not reduced by Herceptin or LY294002 in cells expressing Myr-Akt (Fig. 7*A*), which supports the conclusion that Myr-Akt was indeed functional. In fractions from BT-474 cells that were infected with β-gal virus, and consistent with results with uninfected cells (Figs. 5, *A* and *B*), the majority of p27 was present in the cytosol. Myr-Akt eliminated the low basal nuclear levels of p27 but did not alter the basal cytosolic content of p27 (Fig. 7*B*). In cells infected with β-gal adenovirus, both inhibitors up-regulated p27 in the cytosol and in the nucleus. However, this accumulation of p27 in both compartments was markedly reduced in cells infected with a similar MOI of Myr-Akt adenovirus (Fig. 7*B*), which suggested that the accumulation of p27 that follows the blockade of HER2 and PI3K results from the inhibition of Akt.

Finally, we examined whether active Akt was able to counteract the antiproliferative effect of Herceptin in BT-474 cells, which in culture are highly sensitive to Herceptin-mediated growth arrest (39) but not to apoptosis (54). Treatment with Herceptin or LY294002 over a period of 6 days reduced cell proliferation >50% compared with untreated controls. However, infection with Myr-Akt almost completely prevented the antiproliferative effect of each inhibitor (Fig. 8*A*). The ability of active Akt to counteract the pro-apoptotic effect of Herceptin was studied in cultured SKBR-3 cells. Herceptin does not induce SKBR-3 cell cycle arrest as markedly as in BT-474 cells (39, 55) but has recently been shown to induce apoptosis of these cells (54). Treatment with either Herceptin or LY294002 for 72 h induced a 3-fold increase in the basal level of SKBR-3 cell apoptosis that was completely prevented by infection with Myr-Akt (Fig. 8*B*).

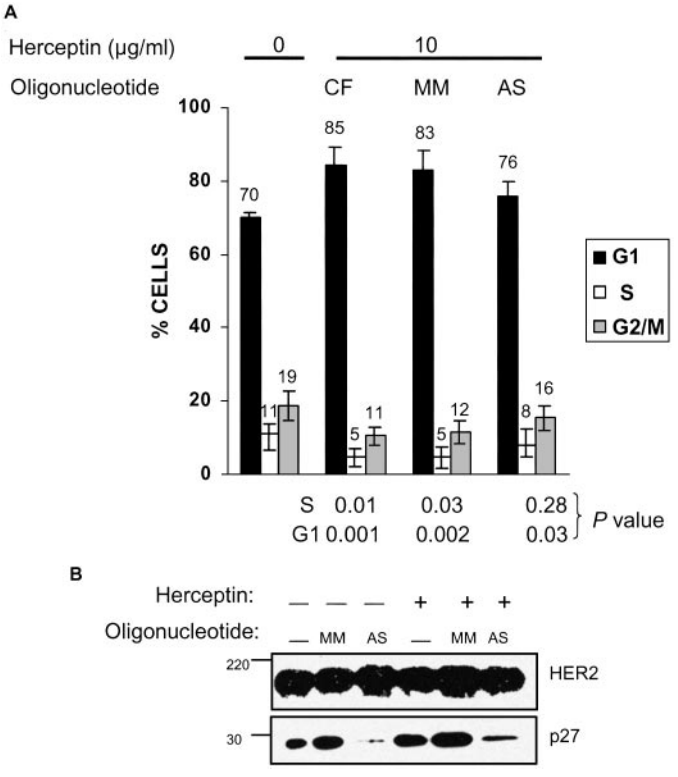

Fig. 6. AS-mediated down-regulation of p27 prevents Herceptin-induced reduction in S phase. In *A*, BT-474 cells (2×10^5/60-mm dish) were transfected with 30 nM MM or AS p27 oligonucleotides in the presence of 2 μg/ml cytofectin (*CF*) as described in "Materials and Methods." To control for nonspecific effects of cytofectin, cells treated with neither oligonucleotide were still treated with cytofectin alone (*B, oligonucleotides lanes*). The cells were then exposed to 10 μg/ml Herceptin. After 48 h in the presence of the anti-HER2 IgG1, cells were trypsinized, their nuclei were labeled with propidium iodide, and DNA histograms were generated by flow cytometry of 10,000 labeled nuclei. *Each bar*, the mean ± SD of four independent experiments. Statistics were calculated by Student's unpaired *t* test. In *B*, identically treated dishes were harvested simultaneously in NP40 lysis buffer and tested for HER2 and p27 content by immunoblot. AS p27 but not MM oligonucleotides nor cytofectin alone prevented the up-regulation of p27 induced by Herceptin.

DISCUSSION

We have examined whether the blockade of HER2 with the humanized IgG1 Herceptin inhibits tumor cell growth by inhibiting PI3K function. Herceptin inhibited colony formation, P-MAPK, and

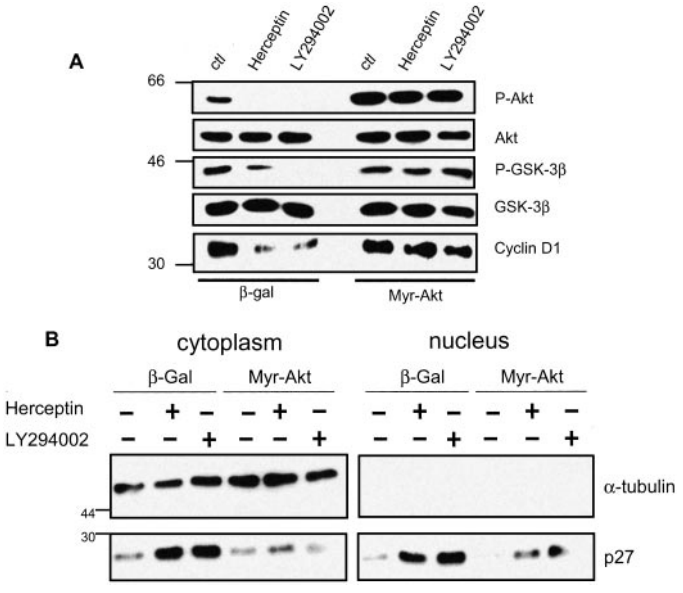

Fig. 7. Dominant active Akt rescues BT-474 cells from Herceptin action. BT-474 cells (3×10^5 cells/60-mm dish) were infected with either β-gal or Myr-Akt adenoviruses for 48 h as indicated in "Materials and Methods" followed by treatment with either 10 μg/ml Herceptin or 40 μM LY294002 for 24 h. Whole cell lysates were prepared and 70 μg of protein resolved by SDS-PAGE followed by the indicated immunoblot procedures. *On the left of each panel*, M_r in thousands. In *B*, nuclear and cytoplasmic fractions were prepared as described in "Materials and Methods" from identically treated cells. Fractions were next analyzed for p27 or α-tubulin levels by immunoblot; α-tubulin was detected only in the cytosolic fractions.

P-Akt in BT-474 and SKBR-3 cells, which exhibit *HER2* gene amplification, but not in MDA-361 and MDA-453 cells, both with a single copy of the HER2/*neu* gene. This result is consistent with a previous report in which the latter two cell lines exhibited a modest reduction in [^{3}H]thymidine incorporation but no reduction in the S-phase fraction when treated with 10 μg/ml Herceptin (56). Although MDA-361 and MDA-453 cells exhibit robust levels of constitutively phosphorylated HER2 (20, 54), quantitation of HER2 gene copy number as measured by DNA dot-blot analysis was only 2-fold, relative to normal human DNA (50). It is likely that this last method overestimated the gene copy number as suggested by the results with the more controlled FISH analysis (Fig. 1).

The differential sensitivity among the four cell lines that we used in this study is in agreement with preliminary data showing that the clinical responses to Herceptin were limited to breast cancers with *HER2* gene amplification and/or with the highest level of receptor protein expression (57). Because Herceptin is specific to HER2, and considering that only *HER2* gene-amplified tumors are inhibited by the antibody, we speculate that only *HER2* gene-amplified breast cancers are dependent on activated HER2. Along those lines, a recent study reported that breast tumors that stained positively with a mAb specific for Tyr-1248 phospho-HER2 were those with the highest level of total HER2 expression (21). This is also consistent with the ability of p185$^{c\text{-}neu}$, the rat homologue of HER2, to spontaneously oligomerize and activate its kinase when present at high density, whereas, at low concentrations, the presence of a ligand was required for receptor multimerization and activation (22). The latter result also implies that at levels of HER2 below those seen in *HER2* gene-amplified cells, activation of the receptor may depend on ligand-activated coreceptors. Several studies suggest that blocking the HER2 ectodomain with Herceptin may not be effective in interrupting lateral signaling from HER coreceptors to HER2. In OVCA420 ovarian cancer cells, EGF induced phosphorylation of HER2 that was not blocked by mAb 4D5, the corresponding mouse hybridoma of Herceptin (58). In a recent report, tumor growth factor-α-induced proliferation of BT-474 cells was inhibited by ZD1839, a low-molecular-weight EGFR Tyr kinase inhibitor, but not by Herceptin (54). Finally, Herceptin did not inhibit the growth of MKN gastric cancer cells, which exhibit *HER2* gene amplification (59) but which also overexpress highly phosphorylated EGFR (39). These data imply that Herceptin may not be effective: (*a*) in HER2-overexpressing cells with a single copy of the *HER2* gene, like MDA-361 and MDA-453, in which HER2 may rely on lateral signals from HER coreceptors for activation; and (*b*) in *HER2* gene-amplified cells in which Herceptin-bound HER2 can potentially still receive signaling input from co-overexpressed HER family members.

Treatment with Herceptin inhibited the PI3K target Akt in antibody-sensitive but not in antibody-resistant breast tumor cell lines. Both HER3-associated PI3K activity and HER2/HER3 heterodimers have been previously shown in MDA-361 and MDA-453 cells (20). The inability of Herceptin to block PI3K activity suggests that, in these cells, HER3-associated PI3K depends on signals other than HER2. The inhibition of PI3K in BT-474 cells was associated with the uncoupling of phosphorylated HER3 from HER2 and from p85. The kinetics of these effects were slow and probably reflect the reported ability of mAb 4D5 to remove HER2 from the plasma membrane (60,

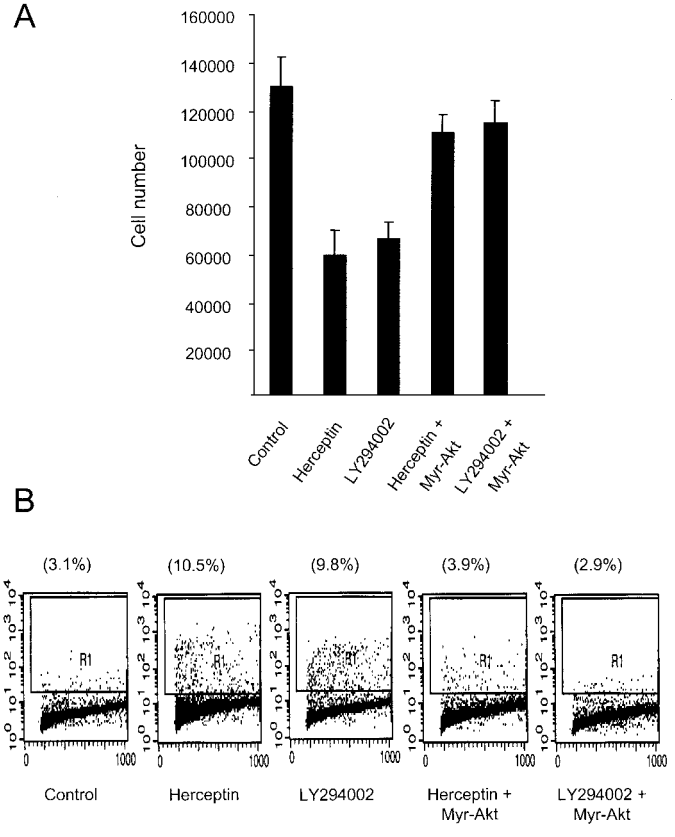

Fig. 8. Dominant-active Akt rescues from the antiproliferative and apoptotic effect of Herceptin. In *A*, BT-474 cells (3×10^4/well in 6-well plates) were infected with Myr-Akt or β-gal adenoviruses for 48 h followed by treatment with Herceptin (10 μg/ml) or LY294002 (40 μM). Fresh IMEM/10% FCS $\pm$ inhibitors was added on days 3 and 5. Cells were trypsinized and counted in a Coulter counter on day 6. *Each data point*, the mean $\pm$ SD of triplicate wells. Results were confirmed in two independent experiments. In *B*, 10^6 SKBR-3 cells were infected with Myr-Akt or β-gal (*Control*) adenoviruses treated with the same concentrations of Herceptin or LY294002 for 72 h. Adherent and floating cells were harvested and assayed for evidence of apoptosis by Apo-BrdU analysis in the presence of Tdt. *In parentheses on top of each panel*, the percentage of FITC-positive apoptotic cells in the *R1 gated area*, quantitated by flow cytometry.

61) and/or induce its homodimerization.[4] Down-modulation of receptor-ligand complexes is a major attenuation mechanism of receptor-induced signaling. Therefore, antibody-induced HER2 down-modulation from the cell surface and/or HER2 dimerization with itself should result in less receptor available for heterodimerization with other HER family members and thus impair growth signals in HER2-dependent tumor cells. Interestingly, in MKN gastric cancer cells, Herceptin did not down-regulate PI3K signaling nor inhibit growth (39). The low-to-undetectable levels of HER3 in these cells led to the suggestion that the collaboration of HER2 with HER3 and its disruption by Herceptin are markers of HER2 dependence and Herceptin sensitivity, respectively (39). This logical speculation requires further investigation in human tumors but is also supported by the data presented above.

We next studied whether or not the inhibition of the PI3K target Akt is required for the antitumor effect of Herceptin. The NH_2-terminal pleckstrin homology domain of Akt binds PI3K-induced PIP_3 in the plasma membrane, in which Akt is activated by 3-phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation. Once active, Akt phosphorylates an increasing number of substrates involved in apoptosis, cell cycle regulation, protein synthesis, and glycogen metabolism (Refs. 62–65 and Refs. therein). The substrates involved in regulation of cell death that are disabled by Akt include the Bcl-2 family member Bad, Forkhead transcription factors, IκB kinase, caspase-9, the cyclic AMP response-element binding protein (CREB; Refs. 62–65), and, more recently, p53 via MDM2-mediated phosphorylation and ubiquitination (66, 67). Other targets of Akt could potentially regulate cell cycle progression. Akt phosphorylates and inactivates GSK-3β, thus stabilizing nuclear β-catenin and increasing cyclin D1 transcription (68). By inactivating GSK-3β, Akt represses GSK-3β-mediated phosphorylation and the proteolytic turnover of cyclin D1, hence increasing cyclin D1 levels in the nucleus (69). Phosphorylation of the Cdk inhibitor $p21^{WAF1}$ by Akt causes its cytoplasmic retention, preventing it from exerting its antiproliferative action in the nucleus (41). Akt has been shown to induce E2F activity (70) and the transcription of c-Myc (71). In addition, Akt may contribute to the induction of cell cycle progression by regulating the Cdk inhibitor p27. By phosphorylating Forkhead transcription factors, it can inhibit AFX-mediated transcription of p27 (72). Akt can also inhibit p27 protein levels (73). Interestingly, ectopic expression of the 3-phosphoinositide-specific phosphatase PTEN (74) results in the inhibition of Akt, an increase in p27 levels, and growth arrest (75, 76). Conversely, loss of PTEN function, a frequent event in human cancers, leads to derepression of Akt activity, down-regulation of p27, and cellular transformation (77). Finally, recent work has revealed that Akt can phosphorylate p27 directly in Thr-157, which results in its cytoplasmic retention and the loss of its growth-inhibitory effects (personal communications[5] and Ref. 78).

Consistent with its ability to inhibit Akt function, Herceptin inhibited GSK-3β phosphorylation and increased p27 levels. As shown recently in BT-474 cells treated with a HER kinase inhibitor (42), the loss of phosphorylation in GSK-3β activates its catalytic activity against cyclin D1 *in vitro*, thus potentially explaining the reduction in cyclin D1 induced by Herceptin. All of these effects were abrogated by forced expression of active Akt (Fig. 7*A*). Treatment with Herceptin also resulted in higher nuclear and cytosolic levels of p27. The increase of p27 in the nucleus would increase the amount of p27 available for binding to and inhibiting Cdk2 and inducing cell cycle arrest (Fig. 5*C*). These responses were also prevented by Myr-Akt (Fig. 7*B*). Although the molecular determinants of a direct interaction between Akt and p27 require further investigation, these data suggest that the increase in the nuclear levels of p27 after treatment with Herceptin is attributable to the inactivation of Akt.

Finally, up-regulation of Akt activity by transfection of Myr-Akt prevented Herceptin-induced cytostasis of BT-474 cells and the apoptosis of SKBR-3 cells. LY294002 exhibited inhibitory effects that were similar to those induced by Herceptin, and these effects were also rescued by Myr-Akt (Fig. 8), supporting an important role for PI3K-Akt signaling in the HER2 dependence of these cells. BT-474 tumors in nude mice undergo complete regressions when treated with Herceptin (54, 79), which suggests that they are sensitive to Herceptin-induced apoptosis. However, Herceptin does not induce apoptosis of these cells in culture, impeding our ability to demonstrate a blocking effect of active Akt on Herceptin-induced BT-474 cell death. Nonetheless, the data presented suggest that the inhibition of PI3K-Akt may be an obligated step for the cell cycle arrest and/or apoptosis induced by the HER2 IgG1. These conclusions have important clinical implications for patients treated with Herceptin or other inhibitors of the HER network. Several studies suggest the presence of aberrant PI3K signaling in a cohort of breast carcinomas (reviewed in Ref. 80). Gershtein *et al.* (81) reported increased levels of p85 and PI3K catalytic activity in paired breast tumor over adjacent nontumor tissues in 79% of the specimens examined. Although mutations of PTEN occur in $<5\%$ of breast cancers, a recent report suggests that the complete lack of PTEN protein in breast cancers with hemizygous deletions of the *PTEN* gene is not uncommon (82). Akt1 kinase activity is often increased in breast cancers with a poor prognosis (83). Akt3 mRNA is up-regulated in estrogen receptor-negative breast tumors (84), linking Akt3 with a more rapidly progressive, hormone-independent breast cancer phenotype. The cytoplasmic protein tyrosine kinases Src and BRK are overexpressed in a high percentage of breast cancers, and both of these kinases up-regulate signaling via PI3K and Akt (85, 86). It is then likely that many HER2-overexpressing tumors will also harbor genetic alterations in the PI3K-Akt signaling pathway and exhibit very high levels of constitutive Akt activity. These tumors may not respond to therapy with HER2 inhibitors alone. This hypothesis can be now be tested prospectively in present clinical trials with inhibitors of the HER signaling network.

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