Reversible G\textsubscript{1} Arrest Induced by Inhibition of the Epidermal Growth Factor Receptor Tyrosine Kinase Requires Up-regulation of p27\textsuperscript{KIP1} Independent of MAPK Activity*  

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\textsuperscript{b}To whom correspondence should be addressed: Div. of Medical Oncology, Vanderbilt University School of Medicine, 22nd Ave. South, 1956 TVC, Nashville, TN 37232-5536. Tel.: 615-936-3524; Fax: 615-936-1790; E-mail: carlos.arteaga@mcmail.vanderbilt.edu.\textsuperscript{c}The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; IMEM, improved minimal essential medium; FCS, fetal calf serum; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; TGF, transforming growth factor.}

We have used quinazoline inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase to study the link between EGFR signaling and G\textsubscript{1} to S traverse. Treatment of A431 and MDA-468 human tumor cells with 0.1–10 \textmu M AG-1478 inhibited basal and ligand-stimulated EGFR phosphorylation without a decrease in receptor content, EGF-binding sites, or binding affinity. Incubation of A431 cells with 0.1–1 \textmu M AG-1517 abrogated 125I-EGF internalization. Both AG-1478 and AG-1517 markedly inhibited A431 and MDA-468 colony formation in soft agarose at concentrations between 0.01 and 1 \textmu M. Daily injections of AG-1478 at 50 mg/kg delayed A431 tumor formation in athymic nude mice. A transient exposure of A431 cells to AG-1478 resulted in a dose-dependent up-regulation of the cyclin-dependent kinase inhibitor p27, down-regulation of cyclin D1 and of active MAPK, and hypophosphorylation of the retinoblastoma protein (Rb). These changes were temporally associated with recruitment of tumor cells in G\textsubscript{1} phase and a marked reduction of the proportion of cells in S phase. Upon removal of the kinase inhibitor, EGFR and Rb phosphorylation and the levels of cyclin D1 protein were quickly restored, but the cells did not reenter S phase until p27 protein levels were decreased. Phosphorothioate p27 oligonucleotides decreased p27 protein in A431 cells and abrogated the quinazoline-mediated G\textsubscript{1} arrest. Treatment of A431 cells with PD 098509, a synthetic inhibitor of MEK1, inhibited MAPK activity without inducing G\textsubscript{1} arrest or increasing the levels of p27. However, treatment with LY 294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K), inhibited basal Akt activity, up-regulated p27, and recruited cells in G\textsubscript{1}. These data suggest that p27 is required for the growth arrest that follows interruption of the EGFR kinase in receptor-overexpressing cells. In addition, the G\textsubscript{1} arrest and up-regulation of p27 resulting from EGFR blockade are not due to the interruption of MAPK, but to the interruption of constitutively active PI3K function.

The epidermal growth factor receptor (EGFR; ErbB-1 or HER1) is a 170-kDa member of the ErbB family of transmembrane receptor tyrosine kinases that plays a central role in proliferation, development, differentiation, migration, and oncogenesis of several cell lineages (1–3). Critical for EGFR function are its tyrosine kinase, which becomes activated upon binding of ligand to the receptor’s extracellular domain, and subsequent receptor dimerization (4). The activated tyrosine kinase then autophosphorylates tyrosine residues in the receptor’s C terminus, which recruit and phosphorylate several intracellular substrates, leading to mitogenic signaling and other cellular activities (1–3). The requirement for the EGFR tyrosine kinase activity in cellular signaling is based upon the observations that receptors with mutations in the ATP-binding site lack kinase function and do not display a full range of biochemical responses following ligand binding (1). Several epithelial tumors display EGFR overexpression often associated with increased production of EGFR ligands, which, in turn, activate endogenous receptors via an autocrine mechanism (5). In support of the operative nature of these autocrine pathways in receptor-overexpressing tumor cells, perturbation of the EGFR with bivalent antibodies or with small molecule inhibitors of the EGFR tyrosine kinase results in inhibition of tumor cell proliferation (6). The observation that some human tumor cells depend on EGFR function for proliferation and/or viability, the ability to identify EGFR-overexpressing tumors, the association of EGFR overexpression with poor patient outcome, and the lack of an obvious role for this receptor in normal adult physiology have all suggested the EGFR as a rational molecular target for antitumor strategies.

Several data have linked EGFR signaling with one aspect of epithelial transformation, \textit{i.e.} alterations in cell cycle progression. The Ras/MAPK pathway has been proposed as the major mitogenic signaling pathway initiated by the EGFR tyrosine kinase (7, 8). A number of recent reports have connected the Ras/MAPK pathway with alterations in normal cell cycle progression. In mouse fibroblasts, conditional expression of oncogenic Ras induces shortening of the G\textsubscript{1}/S transition and degradation of the cyclin-dependent kinase inhibitor p27 (8–10). p27 is a member of the KIP family, which also includes p21\textsuperscript{WAF1/CIP1} and p57\textsuperscript{KIP1} (11). KIP molecules interact with the catalytic subunits of all three cyclins that mediate the G\textsubscript{1}/S transition
(Cdk2, Cdk4, and Cdk6) (12) by binding to cyclin-Cdk complexes to either prevent their activation by Cdk-activating kinase or to inhibit directly their kinase activity (11, 12). p27 was originally discovered as a Cdk inhibitory activity induced by extracellular antimitotic signals (13, 14). It accumulates in serum-starved and density-arrested cells, and its overexpression causes cell cycle arrest in G1 (13–15). Furthermore, depletion of p27 by antisense oligonucleotides prevents cell cycle arrest in serum-deprived cells (15). Notably, some reports indicate that perturbation of EGFR signaling with tyrosine kinase inhibitors or bivalent antibodies against the receptor's ectodomain results in stabilization of p27 and G1 arrest (16–19). Low levels of p27 and increased proteasome-dependent degradation of p27 have both been reported in several epithelial neoplasias, suggesting an association between loss of p27 and oncogenesis or tumor progression (reviewed in Ref. 20).

Other reports indicate that activation of Ras/MAPK results in induction of expression of cyclin D1 and abrogation of the adhesion dependence of kinases associated with cyclins E and A, thus accelerating G1 progression and anchorage-independent growth (8). In addition, constitutive activation of Ras/MAPK per se can markedly increase cyclin D1 transcription and expression in the absence of added growth factors. Therefore, p27 and cyclin D1 may be essential elements in pathways that connect EGFR-mediated mitogenic signals to the cell cycle at the G1/S boundary.

Using small molecule quinazoline inhibitors of the EGFR tyrosine kinase (21, 22) as experimental tools, we have studied the cell cycle effects that follow interruption of EGFR function in receptor-overexpressing tumor cells focusing on molecules involved in the G1/S transition. Both of the quinazolines used inhibit the EGFR kinase activity at submicromolar concentrations by reversibly binding to the receptor's ATP site and inducing the formation of inactive EGFR homodimers (23), thereby preventing the phosphorylation of downstream cellular substrates. In turn, these reversible biochemical responses result in G1 arrest of tumor cells expressing high levels of autoinhibited EGFR, thus allowing examination of the temporal correlation between EGFR signal transduction and cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Kinase Inhibitors—**The A431 human epidermoid carcinoma and MDA-468 human breast cancer cell lines, both exhibiting EGFR gene amplification and >1.5 × 10^6 EGFR sites/cell (24, 25), were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were grown in improved minimal essential medium (IMEM; Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS) at 37 °C in a humidified incubator with 5% CO2. The quinazolines AG-1478 and AG-1517 were purchased from the American Type Culture Collection (Manassas, VA). Bedform, MA) was then added for 1–6 min at 37 °C. In some cases, 0.1–10 μM AG-1517 was added to the medium containing radiolabeled ligand. After the indicated times, the cells were placed on ice and washed twice with ice-cold phosphate-buffered saline (PBS) and 0.2% bovine serum albumin, pH 7.4. This was followed by a 60-min incubation with 0.2 μg acetic acid washed with 0.5 ml of PBS. Data were expressed as the ratio of internalized versus surface radioactivity over time. For determination of the number of EGF-binding sites and receptor binding affinity, A431 cells were seeded on 24-well plates and labeled with different concentration of ^125I-EGF ranging from 0.002 to 20 nM in binding buffer (IMEM, 0.1% bovine serum albumin, and 20 μM Hepes, pH 7.6) for 4 h at 4 °C. In some cases, cells were pretreated with 10 μM AG-1517 for 1 h at 37 °C and then incubated with radiolabeled ligand in the presence of the kinase inhibitor for 4 h at 4 °C. The labeled monolayers were washed three times with ice-cold PBS and 0.2% bovine serum albumin and solubilized in 1 ml of 1 N NaOH, and cpm were determined in a γ-counter. Bound cpm/dish were determined in triplicate and subjected to Scatchard-type analysis (28) using the program L programs (29).

**In Vitro and in Vivo Growth Assays—**The growth effects of inhibition of the EGFR kinase were tested in vitro in a soft agarose colony survival assay. A431 and MDA-468 cells were plated at a density of 3 × 10^4 cells/35-mm dish in triplicate in IMEM, 10% FCS, 0.8% agarose, and 10 μM Hepes in the absence or presence of 0.01–10 μM either AG-1478 or AG-1517. Dishes were incubated in a humidified CO2 incubator at 37 °C, and colonies measuring >50 μm in diameter were counted after 7 days using an Omnicon FAS III image analyzer (Bausch & Lomb, Rochester, NY). The effects on tumor growth in vivo were tested in a xenograft model in athymic nude mice. A431 cells (7.5 × 10^6) were injected subcutaneously in 5–6-week-old female Balb/c nu/nu mice (Harlan Sprague Dawley, Madison, WI) just caudal to the right forelimb. Beginning 1 day post-implant, eight mice per group were randomly allocated to treatment with either 50 mg/kg AG-1478 in Me2SO or Me2SO alone (control) in a 50-μl volume, both given intraperitoneally daily via a 26-gauge needle for 21 days. At different intervals, tumor diameters were measured with calipers, and tumor volumes in mm^3 were calculated by the following formula: volume = (width × length)^2/2. Statistical differences in tumor volumes between AG-1478-treated and control mice were evaluated by Student's t test. p values <0.05 were considered to be statistically significant.

**Flow Cytometric Analysis of Cell Cycle Distribution—**Cells were harvested by trypsinization, washed twice with ice-cold PBS, resuspended in cold PBS, and fixed by adding absolute ethanol while vortexing to a final concentration of 67%. After overnight refrigeration at −20 °C and subsequent rehydration in PBS for 30 min at 4 °C, the cell nuclei were washed and rehydrated in 50 μg/ml propidium iodide (PI) containing 125 units/ml protease-free RNase (Calbiochem), both diluted in PBS. Cells were filtered through 95-μM pore size nylon mesh (Small Parts, Inc., Miami Lakes, FL), and a total of 15,000 stained nuclei were analyzed in a FACS caliber flow cytometer (Becton Dickinson, Mansfield, MA). DNA histograms were modeled off-line using ModFit-LT software (Verity, Topsham, ME).

**Immunoprecipitation and Immunoblot Analyses—**Cells were washed twice with ice-cold PBS and then lysed with EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 2 μg/ml each aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and pepstatin) for 20 min at 4 °C. Cell lysates were clarified by centrifugation at 14,000 × g for 10 min at 4 °C, and the protein content of the supernatants was determined by the BCA method (Pierce). Equal amounts of total protein were resolved by 7% (for EGFR, Tyr(P), and p27) SDS-polyacrylamide gel electrophoresis and then immobilized to nitrocellulose membranes. After blocking the nonspecific binding sites by incubation for 1 h with Tris-buffered saline (25 mM Tris and 150 mM NaCl, pH 7.5) containing 0.05% Tween 20 and 5% nonfat dry milk, the membranes were incubated with mouse monoclonal antibodies against p27 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cyclin D1 (Pharmingen, San Diego, CA), Rab (Pharmingen), phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY), and EGFR (Transduction Laboratories, Inc., Lexington, KY) and rabbit polyclonal antibodies against active MAPK (Promega, Madison, WI), Ser^{416}-phosphorylated Akt (New England Biolabs Inc.), and total Akt (New England Biolabs Inc., Beverly, MA). DNA histograms were modeled off-line using ModFit-LT software (Verity, Topsham, ME).
Inc.) Bound antibodies were detected with horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Amersham Pharmacia Biotech), followed by enhanced chemiluminescence and exposure to x-ray film. In some cases, cell lysates were immunoprecipitated at 4 °C using the 986 polyclonal EGFR antisera (kindly provided by Dr. Graham Carpenter) or human protein A-Sepharose CL-4B (Sigma). After four washes, the EGFR immune complexes were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for subsequent immunoblot analysis as described above.

**Results**

**Quinazolines Block Basal and TGF-α-induced EGFR Phosphorylation on Tyrosine**—We initially studied the inhibitory potency of AG-1517 on basal and ligand-induced EGFR phosphorylation in intact A431 and MDA-468 cells. These cells have a doubling time of approximately 24 h, exhibit EGFR gene amplification, and secrete TGF-α, thus expressing autoactivated EGFR in the absence of exogenous receptor ligands (31, 32). A 30-min preincubation with ≥0.1 μM AG-1517 inhibited the basal and TGF-α-stimulated tyrosine phosphorylation of the EGFR in both tumor cell lines without a detectable change in EGFR protein levels (Fig. 1, A and B). In contrast, treatment with SU-4231, a structurally related quinazoline that does not affect the EGFR kinase, but specifically blocks the HER2/ErBB-2 kinase in vitro, had no effect on basal and ligand-induced EGFR tyrosine phosphorylation in MDA-468 cells (Fig. 1B).

**Quinazoline-mediated Inhibition of the EGFR Kinase Does Not Decrease EGF-binding Sites and Receptor Binding Affinity, but Abrogates Receptor Internalization**—We next sought to determine the effect of the kinase inhibitor on the number of EGF-binding sites under conditions of binding equilibrium of 125I-EGF. Table 1 shows the results from Scatchard-type analysis of labeled EGF binding data from A431 cells incubated with or without 10 μM AG-1517. Treatment with the kinase inhibitor doubled the number of EGF sites with a negligible effect on the receptor’s equilibrium dissociation constant (K_D), indicating that the loss of receptor phosphorylation was not to be explained by a loss of EGFR binding capacity. On the other hand, EGFR internalization was markedly altered by inhibition of the EGFR kinase. To measure the rate of EGFR internalization, A431 cells were briefly exposed to 0.2 nM 125I-EGF at 37 °C in the absence or presence of 0.1–10 μM AG-1517. At all concentrations tested, AG-1517 prevented 125I-EGF internalization as measured by the rate of accumulation of intracellular radiolabeled EGF relative to the amount of surface-bound radiolabeled ligand (Fig. 2). These data are consistent with the requirement of EGFR autophosphorylation for ligand-depend-
The continuous presence of the kinase inhibitor and was totally reversible upon its removal, suggesting that, for adherent cells, the effect was predominantly cytostatic and not cytotoxic. Notably, a similar 4-day treatment of adherent MDA-468 cell monolayers with 1 µM AG-1478 or AG-1517, a concentration that almost completely blocked colony survival on soft agarose (Fig. 3), resulted at best in a modest (20%) inhibition of cell proliferation (data not shown).

The inhibitory effect against A431 cells was next confirmed in vivo against subcutaneous A431 xenografts implanted in athymic nude mice. Although all tumors still formed after subcutaneous inoculation of tumor cells, daily intraperitoneal injections of 50 mg/kg AG-1478 markedly delayed A431 tumor growth when compared with controls (p < 0.01) (Fig. 4) without any detectable host toxicity. Microscopic examination of both control and treated squamous carcinomas revealed no histological differences between them.

Quinazoline-mediated Inhibition of the EGFR Kinase Reversibly Arrests Cells in G1 Phase of the Cell Cycle, Simultaneous with a Decrease in Cyclin D1, Induction of p27, and Hypophosphorylation of Rb—To determine the cellular and biochemical mechanisms of growth arrest, we treated exponentially growing tumor cells with concentrations of AG-1478 that were known to inhibit the EGFR kinase activity (Fig. 1). We initially focused on the effects on the G1/S transition in the more sensitive A431 cells. These cells contain wild-type Rb, whereas the MDA-468 cells exhibit a homozygous deletion of most of the Rb gene (33). A 20-h exposure to 0.1–10 µM AG-1478 resulted in a dose-dependent inhibition of both EGFR phosphorylation and constitutive MAPK activity, hypophosphorylation of Rb, down-regulation of cyclin D1 protein, an increase in p27 protein levels, and recruitment of A431 cells into G1 phase of the cell cycle, but without any effect on EGFR protein levels (Fig. 5A).

When added in vitro, AG-1478 (10 µM) had no effect on the ability of Cdk2 precipitated from an A431 cell lysate to phosphorylate a glutathione S-transferase-Rb fusion protein (data not shown). In addition, the IC50 of AG-1478 against human Cdk2 in vitro using histone H1 as a substrate was >100 µM, suggesting further that the effect of AG-1478 on Rb phosphorylation in vivo was not due to a direct interaction of the quinazoline with Cdk2. In contrast to A431 cells and even though MDA-468 colony survival was markedly inhibited by inhibition of the EGFR kinase (Fig. 3), G1 arrest was not

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**Fig. 2.** Effect of the EGFR kinase inhibitor AG-1517 on the internalization of 125I-EGF. A431 cells were incubated in 24-well plates with 1 ng/ml 125I-EGF for 1–6 min at 37 °C in the presence or absence of 0.1–10 µM AG-1517. The amount of surface-bound and internalized radioactivity was determined at the end of each incubation as described under “Experimental Procedures.” The data were corrected for nonspecific binding in the presence of a 100-fold excess of unlabeled EGF, and the apparent rate of 125I-EGF internalization is expressed as the ratio of internalized versus surface radioactivity. Each data point represents the mean of triplicate wells.

**Fig. 3.** Quinazoline-mediated inhibition of tumor cell colony formation. A431 (upper panel) and MDA-468 (lower panel) cells (3 x 104) were plated on 0.8% agarose, 10% FCS, and 10 mM Hepes in the absence or presence of the indicated concentrations (0.01–10 µM) of AG-1478 (●) and AG-1517 (■). After 7 days, colonies measuring ≥50 mm2 were counted as described under “Experimental Procedures.” Each data point represents the mean ± S.E. of triplicate dishes.

**Fig. 4.** Growth inhibition of A431 tumors in nude mice. A431 cells (7.5 x 106) were injected subcutaneously in Balb/c nu/nu mice on day 0. Beginning 1 day post-implant, the animals were treated with daily intraperitoneal injections of either 50 mg/kg AG-1478 or dimethyl sulfoxide (DMSO) alone (control) for the duration of the experiment (21 days). On the indicated days, tumor diameters were measured using calipers, and tumor volumes (in mm3) were calculated by the following formula: volume = (width2 x length2). Each data point represents the mean tumor volume ± S.E. obtained from eight mice. *, p < 0.01, AG-1478- versus Me2SO-treated mice.

Cdk2 in vitro using histone H1 as a substrate was >100 µM, suggesting further that the effect of AG-1478 on Rb phosphorylation in vivo was not due to a direct interaction of the quinazoline with Cdk2. In contrast to A431 cells and even though MDA-468 colony survival was markedly inhibited by inhibition of the EGFR kinase (Fig. 3), G1 arrest was not

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L. K. Shawver, unpublished data.
observed in adherent MDA-468 cells after a 4-day treatment with 1 μM AG-1478 or AG-1517, perhaps consistent with their lack of Rb (33).

The next study addressed the time course and reversibility of these biochemical events and cell cycle arrest in A431 cells. Despite the fact that the quinazoline-mediated inhibition of the EGFR kinase and the down-regulation of cyclin D1 levels were fairly rapid in A431 cells (i.e. <8 h), the recruitment into G1 phase of the cell cycle occurred 24 h after continuous exposure to 1 μM AG-1478, a time at which the levels of p27 were increased (Fig. 5B). These effects were reversible in that removal of the kinase inhibitor resulted in prompt phosphorylation of the EGFR and an increase in cyclin D1 levels. However, the cells remained in G1 and did not reenter S phase until 24 h after removal of AG-1478, a time at which p27 levels were down-regulated, suggesting that p27 was limiting for EGFR-mediated cell cycle progression (Fig. 5B). Similar results were obtained in three independent experiments. The content of cyclin A and p21 was not altered (data not shown).

**Up-regulation of p27 Partially Mediates Quinazoline-mediated G1 Arrest in A431 Cells**—We next examined whether p27 had a key role in mediating cell cycle arrest when the EGFR kinase was blocked by interfering with p27 with an antisense oligonucleotide approach. Cytofectin-mediated delivery of a 10 nM concentration of the G-clamp-modified 15-mer antisense p27 phosphorothioates to A431 cells blocked both the increase in p27 protein levels and the hypophosphorylation of Rb induced by 1–5 μM AG-1478, whereas mismatch controls or cytofectin alone did not (Fig. 6A). None of these conditions blocked the inhibitory effect of AG-1478 on basal EGFR phosphorylation in A431 cells. Overall, the levels of cyclin D1 were lower in all cells treated with AG-1478 compared with untreated controls. However, antisense p27 seemed to interfere with the decrease in cyclin D1 levels caused by AG-1478, whereas mismatch controls and cytofectin alone did not. Antisense p27 did not completely prevent quinazoline-mediated Rb hypophosphorylation. This could be due to remaining cyclin D/Cdk4 activity, not affected by the depletion of p27. Consistent with the effects observed on Rb phosphorylation, in A431 cells treated with cytofectin alone or with mismatch oligonucleotides, 1–5 μM AG-1478 was able to induce G1 arrest (from 57 to >80%) and to markedly decrease the proportion of cells in S phase (from 32 to 9–12%). This effect was minimal in cells preincubated with antisense p27 oligonucleotides (Fig. 6B) and in which levels of p27 protein had been down-regulated. These experiments were performed three times with similar results, thus suggesting that p27 is required for the growth arrest that follows interruption of EGFR function.

**Inhibition of MAPK Activity in Cycling A431 Cells Does Not Induce G1 Arrest or Increase p27 Protein Levels**—Blockade of EGFR kinase activity in A431 cells simultaneously inhibits constitutively active MAPK, induces G1 arrest, and increases p27 protein levels. This temporal correlation between the effects on MAPK activity and cell cycle events as well as the observations linking active MAPK with G1/S progression suggested to us that the inhibition of MAPK may be mediating the G1 arrest that follows EGFR blockade in A431 cells. If so, interruption of MAPK per se should result in G1 arrest and up-regulation of p27 protein. To test this possibility, we used a synthetic inhibitor of the MAPK-activating enzyme (MEK) that lacks an inhibitory activity against MAPK itself (26). An overnight incubation of exponentially growing A431 cells with 50 μM PD 090859 inhibited MAPK activity without any effects on EGFR tyrosine phosphorylation and/or protein levels (Fig. 7). In cells in which MAPK activity was blocked, there was a modest decrease in cyclin D1 levels, but p27 protein and cell cycle distribution remained the same as those in untreated cells. This result was confirmed in at least two additional experiments and suggests that the G1 arrest that follows in-
terruption of the EGFR tyrosine kinase is not explained by inhibition of MAPK activity.

Blockade of Basal PI3K Activity in Cycling A431 Cells Results in Up-regulation of p27 and G1 Arrest—The inability of MAPK blockade to induce G1 arrest and to increase p27 levels prompted the study of alternative pathways downstream of the EGFR that may explain the cell cycle effects of AG-1478. Therefore, we focused on PI3K, an enzyme whose activity is also regulated by the EGFR tyrosine kinase (1–3). For this purpose, we used LY 294002, a well characterized inhibitor that binds to the ATP-binding sites of the PI3K p110 catalytic subunit (34). As a marker of PI3K activity, we measured the levels of Akt phosphorylated on Ser473. The Akt kinase, also called protein kinase B, is activated upon binding by PI3K lipid products by phosphorylation at Thr308 and Ser473 (35). By immunoblot analysis using anti-Ser473-phosphorylated Akt antibodies, activated Akt was detectable in exponentially growing A431 cells. Overnight treatment with either AG-1478 (1 μM) or LY 294002 (10–40 μM) eliminated Ser473-phosphorylated Akt protein levels, whereas a brief incubation with 100 ng/ml TGF-α increased them (Fig. 8, upper). Similar to AG-1478, treatment with LY 294002 resulted in an almost doubling of the proportion of cells in G1 as well as a marked reduction of the cells in S phase of the cell cycle (Fig. 8, lower). These effects were dose-dependent and reversible in that, upon removal of LY 294002, cell proliferation was restored with no evidence of toxicity (data not shown). By immunoblot analysis using anti-Ser473-phosphorylated Akt antibodies, activated Akt was detectable in exponentially growing A431 cells. Overnight treatment with either AG-1478 (1 μM) or LY 294002 (10–40 μM) eliminated Ser473-phosphorylated Akt protein levels, whereas a short incubation with 100 ng/ml TGF-α increased them (Fig. 8, upper). Similar to AG-1478, treatment with LY 294002 resulted in an almost doubling of the proportion of cells in G1 as well as a marked reduction of the cells in S phase of the cell cycle (Fig. 8, lower). These effects were dose-dependent and reversible in that, upon removal of LY 294002, cell proliferation was restored with no evidence of toxicity (data not shown). By immunoblot analyses of cell lysates, the cell cycle arrest induced by PI3K blockade with LY 294002 was not associated with changes in total Akt, cyclin D1, or active MAPK protein levels. However, p27 protein levels were markedly increased, whereas Rb became hypophosphorylated (Fig. 9), suggesting that the up-regulation of the Cdk inhibitor and cell cycle arrest that follow EGFR blockade might be due to the interruption of the downstream constitutive PI3K activity.

**DISCUSSION**

We sought to examine the cellular and biochemical mechanisms of growth arrest that follow interruption of EGFR signaling in receptor-overexpressing A431 and MDA-468 human tumor cells. These cells are EGFR-dependent in that perturbation of their autoactivated receptors with bivalent antibodies against the ectodomain of the EGFR results in growth arrest and/or tumor cell death in vivo (36, 37). In these natural cell
By Scatchard analysis of 125I-EGF binding data, EGF-binding homodimers (21–23). Initially, we characterized the effects of inhibitors on the EGFR tyrosine kinase activity at sub-micromolar concentrations by reversibly binding to the receptor's ATP site and inducing the formation of inactive EGFR homodimers (21–23). At these concentrations, EGFRs are probably activated under basal conditions by multiple autophosphorylations in the receptor's carboxyl terminus (40). Previously, we have shown that genetic or biochemical inactivation of Rb results in failure to arrest in G1 following inactivation of the Ras/Raf pathway (27, 38). Consistent with this speculation, Peeper et al. (40) recently reported that genetic or biochemical inactivation of Rb results in failure to arrest in G1 following inactivation of the Ras/MAPK pathway, a major mitogenic pathway downstream of the EGFR, with an anti-Ras neutralizing antibody. Moreover, when an anti-Ras neutralizing antibody was microinjected, cells without Rb function were more resistant, although not completely, to the inhibitory effect of the antibody compared with cells with intact Rb (41). Confirmation of this hypothesis will require the inactivation of the Rb pathway by exogenous means in A431 cells as well as the reintroduction of Rb into MDA-468 cells followed in each case by the subsequent assessment of their sensitivity to EGFR kinase inhibitors.

Interruption of EGFR kinase function in A431 cells resulted in a dose-dependent inhibition of both Rb phosphorylation and MAPK activity, down-regulation of cyclin D1 protein levels, and an increase in p27. The content of cyclin A and p21 was not altered. Other studies have shown that perturbation of the EGFR with tyrosine kinase inhibitors or antibodies against the receptor's extracellular domain stabilizes p27 and arrests cells in G1 phase of the cell cycle (16–19), suggesting, together with our more direct data, that p27 is required for growth inhibition when the EGFR pathway is blocked. In two recent reports, the persistent overexpression of ectopic p27 resulted in apoptosis of several human tumor cell lines and lung fibroblasts (42, 43). However, we have been unable to detect any A431 cell DNA fragmentation on agarose gels following treatment with AG-1478. This result and the seemingly reversible nature of the growth inhibitory effect on adherent A431 cells suggest that AG-1478-mediated tumor cell apoptosis is not prominent under these experimental conditions. The hypophosphorylation of Rb is not due to a direct effect of AG-1478 in that its in vitro IC_{50} against recombinant Cdk2 exceeds 100 μM. Furthermore, addition (in vitro) of AG-1478 to Cdk2 immunoprecipitated from A431 cells did not inhibit its activity against glutathione-S-
transferrase-Rb. Therefore, we formally tested the role of p27 in quinazoline-mediated G1 arrest using an antisense oligonucleotide approach. Antisense p27 phosphorothioate oligonucleotides blocked the AG-1478-mediated increase in p27 levels, Rb hypophosphorylation, and G1 arrest, thus confirming for the first time the requirement of this Cdk inhibitor for the observed cell cycle arrest. This requirement of p27 is not unique for the cell cycle arrest that results from blocking the EGFR kinase. At 10-fold higher concentrations, these inhibitors also block the function of the homologous HER2 (ErbB-2) kinase in HER2-overexpressing breast tumor cells (23) and recruit cells into G1. This cell cycle arrest is also prevented by depletion of p27 with antisense phosphorothioates.3 Of note, the antisense phosphorothioates moderately dampened the down-regulation of cyclin D1 induced by AG-1478, but the interpretation of this finding remains unclear at this time.

The data obtained with antisense p27 support a causal association between the induced changes in the Cdk inhibitor and the G1 arrest that follows interruption of the EGFR pathway. Other antiproliferative signals can lead to accumulation of p27, including withdrawal of mitogens or cytokines, cell-cell contact inhibition, and agents such as cAMP and rapamycin (11). The role of p27 as critical regulator of cell proliferation is further illustrated by the p27 knockout mouse models, which exhibit gigantism, organomegaly, and enhanced spontaneous as well as induced tumorigenesis (44–47). In addition, several epidemiological surveys in a variety of human cancers using immunohistochemical analysis of p27 in tumor tissues support an association between low levels or the absence of p27 and more progressive stages of disease and poor clinical outcome (reviewed in Ref. 20). It has been suggested that the prognostic value of low or absent p27 correlates with high tumor cell proliferation, but a clear demonstration of such a correlation is missing. At a biological level, if p27-mediated G1 arrest is the predominant effect of perturbation of tumor cell EGFR in receptor-overexpressing human cancers, anti-EGFR interventions may not be effective against tumors with low or absent p27 compared with tumors that can mount a robust p27 response.

We next examined whether interruption of the MAPK pathway following blockade of the EGFR kinase explained the G1 arrest and up-regulation of p27 levels observed in AG-1478-treated A431 cells. Of note, several reports indicate that activation of the Ras pathway in mouse fibroblasts transfected with mutant Ras vectors results in degradation of p27 through MAPK signaling (8–10, 48), suggesting that both constitutively active MAPK may have the opposite effect and lead to stabilization of p27. However, blockade of constitutive MAPK activity in A431 cells did not result in an appreciable accumulation of cells in G1 or an increase in p27 protein levels, suggesting that interruption of other signaling pathways downstream of the EGFR is primarily responsible or is also required for cell cycle arrest and modulation of p27 content. It should be noted that transformation by activated MEK1, a MAPK-activating enzyme, is blocked by microinjected anti-Ras antibodies (49), strongly suggesting that other signals upstream of activated MAPK are involved in G1/S progression. It has also been shown that activation of the MEK1/MAPK pathway is not sufficient to trigger degradation of p27 unless cyclin D1 and Cdk4 subunits are co-overexpressed at levels achieved in cells stimulated by serum (50). These cyclin D1-Cdk4 complexes can then sequester p27, reduce its effective inhibitory threshold, and thus allow entry into S phase of the cell cycle.

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The inability of PD 098059 to induce G1 arrest and to increase p27 levels in A431 cells is entirely consistent with these reports.

Finally, we examined whether the interruption of the alternative PI3K pathway, also regulated by receptors of the EGF family (1–3), explained the cell cycle effects of AG-1478. PI3K is a lipid kinase that phosphorylates phosphoinositides at the 3′-position of the inositol ring. PI3K is composed of a p110 catalytic subunit and p85 regulatory subunit, which, via its SH2 domains, associate with the tyrosine-phosphorylated proteins (51–53). A number of signaling molecules have been implicated downstream of PI3K. One of these is Akt, a 480-amino acid protein-kinase that, via its pleckstrin homology domain, is recruited to the cell membrane by the membrane-bound lipids phosphorylated by PI3K, thus resulting in activation of its kinase activity upon phosphorylation at Thr308 and Ser473 (35). PI3K activation and its substrates have been causally associated with a number of cellular events, including cell cycle progression, protection from apoptosis, transformation, and cytoskeletal reorganization (54–60). Consistent with autocrine activation of the EGFR, A431 cells exhibited constitutively active Ser473-phosphorylated Akt, which was eliminated by blockade of the EGFR kinase with AG-1478 as well as by blocking the PI3K catalytic p110 subunit with LY 294002. Interruption of PI3K activity resulted in marked up-regulation of p27, Rb hypophosphorylation, and reversible cell cycle arrest. However, different from the EGFR kinase inhibitor, LY 294002 had no effect on active MAPK, further indicating that the cell cycle effects of AG-1478 were independent of the perturbation of constitutive MAPK function. We therefore infer that under basal conditions, PI3K signals, as a function of autocrine EGFR activity, are required for down-regulation of p27. Blocking constitutive PI3K function, in turn, results in stabilization of p27 and cell cycle arrest. Although this report focuses on cells with operative autocrine EGFR in the absence of added ligand(s), our results are entirely consistent with a recent paper showing interleukin-2-mediated induction of PI3K and Akt in lymphoid cells that results in enhanced E2F activity, hypophosphorylation of Rb, and down-regulation of p27, thus leading to cell cycle progression, all independent of MAPK function (56).

These data may have practical implications for current EGFR-targeted therapeutic strategies in human cancers. First, tumors with low or absent p27 may not respond as well compared with those with adequate p27 levels when treated with interventions aimed at blocking EGFR signals. Second, some tumors may also exhibit high levels of kinase activities downstream of the EGFR. For example, Akt2, a homologue of Akt, is overexpressed in ovarian cancer cell lines and primary tumors (61). In addition, several reports have now established a link between hyperactive PI3K/Akt via defects in PTEN, the phosphatase that negatively regulates PI3K signals, and human cancers (reviewed in Ref. 62). All these suggest the possibility that, in some tumors, the simultaneous hyperactivity of signaling pathways whose interruption is necessary for an antitumor effect by EGFR blockade may counteract the net antitumor effect of those interventions aimed only at the cell-surface receptor.

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