Constitutive MEK/MAPK Activation Leads to p27Kip1 Deregulation and Antiestrogen Resistance in Human Breast Cancer Cells*

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Antiestrogens, such as the drug tamoxifen, inhibit breast cancer growth by inducing cell cycle arrest. Antiestrogens require action of the cell cycle inhibitor p27Kip1 to mediate G1 arrest in estrogen receptor-positive breast cancer cells. We report that constitutive activation of the mitogen-activated protein kinase (MAPK) pathway alters p27 phosphorylation, reduces p27 protein levels, reduces the cdk2 inhibitory activity of the remaining p27, and contributes to antiestrogen resistance. In two antiestrogen-resistant cell lines that showed increased MAPK activation, inhibition of the MAPK kinase (MEK) by addition of U0126 changed p27 phosphorylation and restored p27 inhibitory function and sensitivity to antiestrogens. Using antisense p27 oligonucleotides, we demonstrated that this restoration of antiestrogen-mediated cell cycle arrest required p27 function. These data suggest that oncogene-mediated MAPK activation, frequently observed in human breast cancers, contributes to antiestrogen resistance through p27 deregulation.

p27Kip1 is a member of the KIP1 (kinase inhibitory protein) family of cdk inhibitors that regulate the cyclin-cdk complexes governing cell cycle transitions (1). The importance of p27 as a G1-to-S phase regulator is highlighted by the finding that antisense-mediated inhibition of p27 expression is sufficient to induce cell cycle entry in quiescent fibroblasts (2) and in steroid-depleted breast cancer cells (3). p27 protein levels are high in G0 and early G1 during which time p27 binds tightly and inhibits cyclin E1-cdk2. p27 translation rates decrease, and its proteolysis increases during G1-to-S phase progression, leading to p27 protein loss as cells enter S phase (4–6). p27 proteolysis is regulated by phosphorylation of p27 on threonine 187 (Thr-187) by cyclin E1-cdk2 (7, 8). While mutations or deletions in the p27 gene are uncommon (9, 10), p27 degradation is increased in many cancers, including breast cancer (11, 12).

An increasing body of data suggests that p27 is regulated by mitogenic signal transduction pathways, including Ras-dependent activation of the mitogen-activated protein kinase (MAPK) pathway (13–17). Many mitogens increase the cellular levels of GTP-bound Ras, leading to activation of the downstream target, Raf-1. The Raf-1 kinase can phosphorylate and activate the dual specificity kinases MEK1 and MEK2, which in turn activate MAPK (also known as p42ERK2 and p44ERK1). Once activated, MAPK can phosphorylate several nuclear transcription factors including Myc, Elk, and Rsk (for review, see Ref. 18). p27 itself has several MAPK consensus sites, and MAPK can phosphorylate p27 in vitro (16) and reduce the ability of recombinant p27 to bind and inhibit cdk2 in vitro (15). While constitutive activation of Ras-MAPK can reduce p27 inhibitory function in immortal and cancer-derived lines, it is not clear whether MAPK directly regulates p27 during cell cycle progression in normal cell types.

Studies of p27 regulation by the Ras-MAPK pathway were initially carried out in fibroblasts (15, 19, 20). In NIH3T3 fibroblasts, Ras signaling is required for the down-regulation of p27 as cells approach the G1-to-S phase transition (13, 20). Introduction of a dominant negative ras mutant prevented the loss of p27 in response to serum and inhibited S phase entry. Others have reported that Ras-MAPK activation reduces the ability of p27 to inhibit cdk2 through sequestration of p27 into cyclin D1-cdk4 complexes, rather than by promoting p27 protein loss (14).

Constitutive activation of the MAPK cascade may contribute to malignant progression of many human cancers (21). Although the causes of MAPK activation differ among tumors, in many cancers constitutive signaling from cell surface tyrosine kinase receptors contributes to activation of the Ras-Raf-1-MEK-MAPK pathway. For example, the epidermal growth factor receptor and HER2/c-ErbB-2, both of which activate the Ras-MAPK pathway, are overexpressed in up to 20 and 30% of breast cancers, respectively. Overexpression of these receptors has been associated with antiestrogen resistance and poor prognosis in primary breast cancers (22–27). Tissue culture models suggest that elevated MAPK activity may contribute to estrogen-independent growth of breast cancer cells (28–30).

Antiestrogenic drugs, such as tamoxifen, are effective in the treatment and prevention of breast cancer (31–33). However, only two-thirds of estrogen receptor (ER)-positive breast cancers respond initially to antiestrogen therapy, and even sensitive tumors invariably acquire antiestrogen resistance (34). In most cases, acquired resistance is not due to a loss or mutation of the ER (35, 36). Numerous mechanisms have been proposed to explain the phenomenon of tamoxifen-resistant ER-positive breast cancer, including altered drug metabolism (37), altered binding of co-activator and co-repressor molecules to the antiestrogen-ER complex (38), and altered signal transduction

* This work was supported in part by grants from the United States Army Department of Defense Breast Cancer Research Program (Ideas and Career Development to J. M. S. and Pre-doctoral Award to A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: KIP, kinase inhibitory protein; cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ER, estrogen receptor; BrdUrd, bromodeoxyuridine; 2D-IEF, two-dimensional isoelectric focusing; AS, antisense.
pathways that modulate ER activity (39) or regulate the cell cycle machinery (3).

The cell cycle inhibitor, p27Kip1, is an essential mediator of cell cycle arrest by tamoxifen and other antiestrogenic drugs. We recently demonstrated that antisense-mediated down-regulation of p27Kip1 abrogated antiestrogen-induced cell cycle arrest in the ER-positive MCF-7 breast cancer line (3). p27 protein levels are frequently reduced in primary breast cancers compared with the normal breast epithelium, and low p27 protein levels are associated with poor prognosis and hormone independence (11, 40, 41). These observations stimulated the present study to investigate the relationships between Ras-MAPK pathway activation, antiestrogen resistance, and p27 function. Our data indicate that constitutive MEK activation alters p27 phosphorylation, reduces p27 inhibitory activity, and contributes to antiestrogen resistance in breast cancer.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 cells (42) and LY-2 cells (43) were obtained from the laboratory of M. Lippman. The LCC2 line was a gift from G. Clarke (44). MCF-7 cells, stably transfected with full-length HER2 cDNA (MCF-7/HER2–18), were kindly provided by C. Arteaga. All lines were grown in improved-modified essential medium (option Zn2+ supplemented with insulin and 10% fetal calf serum.

Flow Cytometric Analysis—Cells were lysate-labeled with 10 μM bromodeoxyuridine (BrdUrd) for 2 h and then fixed, stained with anti-BrdUrd-conjugated fluorescein isothiocyanate (Becton Dickinson) and counterstained with propidium iodide as described (45). Cell cycle analysis was carried out on a Becton Dickinson FACScan and Cell Quest Software. Values given for flow cytometric analysis represent the mean of at least three assays.

Cell Cycle Effects of Antiestrogens and MEK Inhibition—For comparison of the effects of antiestrogens in MCF-7, MCF-7 transfectants, or LY-2 cell lines, cultures were treated with addition of 1 μM 4-hydroxy-tamoxifen (4-OHT) (Sigma) or 10 nM ICI 182780 (7α-9-(4,4,5,5,5-pentafluoropentyl sulfonyl) nonyl-estrastra-1,3,5, (10)-trien-3,17β-diol, from Zeneica Pharmaceuticals) to complete medium, and samples were collected at 48 h thereafter for protein and flow cytometric analysis. The effects of MEK inhibition on the cell cycle were assayed following addition of 0.1 μM U0126 (Promega) for 2 or 24 h prior to recovery for immunoblotting or flow cytometric analysis. The effects of MEK inhibition on antiestrogen sensitivity in the antiestrogen-resistant lines, LY-2 or MCF-7/HER2–18, were assayed by treating cells with 0.1 μM U0126 for 2 h followed by an additional 48 h with either 1 μM 4-OHT or 10 nM ICI 182780 prior to recovery of cells for protein or flow cytometric analysis.

Antisense Oligonucleotide Transfections—Antisense experiments were carried out as described (3). Phosphorothioate oligonucleotide sequences were as follows: GS5422 antisense p27 (ASp27) 5′-TG-GCTGCTCTGCGCC-3′; GS5585 mismatch p27 (MSPm27) 5′-TG-GCTGCTCTGCGCC-3′; X indicates the G-clamp modification of these oligonucleotides. The specificity of these oligonucleotides for p27 has been documented (2, 3). LY-2 cells were rendered quiescent by the addition of 0.1 μM U0126 (Promega) for 2 h followed by antiestrogen treatment (10 nM ICI 182780, or 1 μM 4-OHT) for a further 48 h. Quiescent cells were then transfected with 120 nM oligonucleotides using 2.5 μg/ml cefotax G3815 (Gilead Scientific, Foster City, California) for 6 h, followed by replacement with complete medium supplemented by U0126 and antiestrogen. Flow cytometry and proteins were analyzed prior to transfection and at 21 h thereafter.

Immunoblotting—Cells lysis and immunoblotting were as described (45). Equal protein loading was verified by blotting for β-actin. To assay cyclin E1 complexes, cyclin E1 was immunoprecipitated from 200 μg of protein lysate. Immunoprecipitates were resolved, transferred, and blotted with cyclin E1, cdK2, and p27 antibodies. Antibody alone controls were run along side immunoprecipitates.

Antibodies—Monoclonal antibodies to p27 were obtained from Neomarker (Transduction Laboratories). p27 rabbit polyclonal serum (pAB5588) was provided by H. Toyoshima and T. Hunter (Salk Institute). Antibodies to p27 were from Santa Cruz, to cdK2 (PSTAIRE) from S. Reed (The Scripps Research Institute), to MEK, MAPK, and phospho-MApK from New England Biolabs, to β-actin from Sigma, and to cyclin E1 (mAbs E12 and E172) from E. Harlow (Massachusetts General). These cyclin E1 antibodies are specific for cyclin E1(46). The ER antibody H222 was provided by D. G. Greene.

Cyclin-dependent Kinase Assays—Cyclin E1 or p27 complexes were immunoprecipitated from 100 μg of protein lysate and reacted with [γ-32P]ATP and histone H1 as described (45). Radioactivity incorporated in histone substrate was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software. To determine background IgG-associated activity, nonspecific mouse IgG (for cyclin E1-cdk2 assays) or polyclonal rabbit IgG (for p27-associated kinase) immunoprecipitates from test lysates were collected on protein A, washed, and reacted with the kinase mixture as for cyclin E1 and p27 immunoprecipitates. Radioactivity incorporated in control nonspecific IgG immunoprecipitates was subtracted from test kinase values prior to graphing as in Refs. 45 and 47.

Production of Cyclin E1-cdk2 by Baculovirus Infection of SF-9 Cells—SF-9 cells and TNM-FH media were obtained from Invitrogen. Adherent SF-9 cells were co-infected with baculoviruses encoding human cyclin E1 or human cdK2 genes, and cyclin E1 and cdK2 were prepared as described (46). SF-9 cell lysates containing cyclin E1 and cdK2 were used directly in p27 inhibitor assays.

Assays of p27 Inhibitory Function—Cell lysates (250 μg) from asynchronously proliferating MCF-7, MCF-7/MEK (line M2), or LY-2 cells were immunoprecipitated with pAb5588 anti-p27 serum or control polyclonal rabbit IgG, and precipitates were collected on protein A-Sepharose beads. For testing heat-stable p27 inhibitor activity, p27 immunoprecipitates were washed, then boiled for 5 min in 200 μl of reaction buffer, placed on ice, and then lysates were loaded. Uninhibited control was recovered, and recombinant cyclin E1-cdk2 and dithiothreitol (1 μM final) were added and incubated at 30 °C for 30 min, followed by immunoprecipitation with either anti-cyclin E1 (mAbE172) or control polyclonal mouse IgG (Sigma). The un-inhibited control recombinant cyclin E1-cdk2 was treated with reaction buffer and incubated at 30 °C for 30 min without any added p27. Complexes were subsequently assayed for H1 kinase activity, and resulting activities were graphed as a % maximum un-inhibited cyclin E1-cdk2 activity.

Two-dimensional Isoelectric Focusing (2D-IEF) and Phosphatase Treatment—Cells were lysed in ice-cold 0.1% Tween 20 lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 2.5 mM EGTA, pH 8.0, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM Na3VO4, 1 mM sodium orthovanadate, 2 mM sodium fluoride, and 0.02 mM each of aprotinin, leupeptin, and pepstatin). For 2D-IEF, p27 immunoprecipitates were denatured in 8 μl urea, loaded on immobilized non-linear pH gradient (pH 3–10) IEF strips and focused at 50,000 volt-hour using the IPGphor IEF system (Amersham Pharmacia Biotech). These assays yield highly reproducible electrophoretic resolution of isoforms because of the covalent linkage of the electrophoresis medium to the gel matrix. The IEF strip was equilibrated in 50 mM Tris, pH 8.8, 6% urea, 30% glycerol, and 2% SDS for 30 min before loading for SDS-polyacrylamide gel electrophoresis. Gels were transferred to polyvinylidene difluoride membrane, p27 isoforms were detected by immunoblotting using p27 antibody from Transduction Labs, and proteins were detected using enhanced chemiluminescence (ECL). For 2D-IEF, p27 cyclin E1-bound p27, 3 mg of protein lysate was immunoprecipitated with the monoclonal MAb E172 antibody. Densitometric analysis of multiple ECL film exposures from repeat assays was used to determine the ratios of the various p27 isoforms. For phosphate treatment, p27 immunoprecipitates were washed twice with phosphatase buffer (50 mM Tris, pH 8.0, 10% glycerol) and then incubated at 37 °C overnight with 66 units per μl of reaction of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals). To confirm that the most positively charged p27 isoforms represented unphosphorylated p27, cells were labeled with [32P]orthophosphate (1 mCi/100 dish) for 3 h, and p27 immunoprecipitates were isolated and subjected to 2D-IEF. p27 immunoprecipitated from unlabeled cells was resolved by 2D-IEF in parallel with the labeled p27, and the resolution pattern of the cold p27 was compared with the phosphate-labeled p27.

RESULTS

p27 Deregulation in Antiestrogen-resistant LY-2—We compared p27 levels in antiestrogen-sensitive MCF-7 (42) and the resistant MCF-7 derivative, LY-2 (43). The loss of antiestrogen resistance in LY-2 was not due to a loss of p27 protein (Fig. 1A).

The association of p27 with cyclin E1-cdk2 was assayed in asynchronously growing and antiestrogen-treated MCF-7 and LY-2 cells (Fig. 1B). Asynchronously proliferating LY-2 and MCF-7 cells had similar cell cycle profiles (Fig. 1B). When equal amounts of cyclin E1 were immunoprecipitated, the...
asynchronously proliferating cells were analyzed for levels of total and kinase activity as described under p21, and cdk2 by immunoblotting or analyzed for associated histone H1 from asynchronously proliferating and ICI E1 immunoprecipitates (data not shown). Asynchronously growing MAPK-activated lines and empty vector controls showed similar cell cycle profiles (Fig. 2B). Lines with constitutive MAPK activation showed partial resistance to 4-OH-TAM or ICI compared with the parental or vector alone controls (Fig. 2B).

Levels of cyclin E1-associated p21 and p27 were assayed in asynchronously MEEK transfectants and in the empty vector controls (Fig. 2C). Although densitometric analysis showed that total p27 levels in asynchronously growing MEEK transfectants were reduced by up to 3-fold compared with controls, the amount of p27 detected in cyclin E1-cdk2 complexes was not reduced (Fig. 2, C and D). Despite the similar amounts of both p27 and p21 bound to cyclin E1 in proliferating MEEK and control lines, equal amounts of cyclin E1-cdk2 showed approximately 2-fold higher kinase activity in MEEK transfectants compared with empty vector controls (Fig. 2, C and D). There was no change in cdk2 binding to cyclin E1, and the subtle increase in the proportion of the faster mobility, CAK-activated cdk2 bound to cyclin E1 would not suffice to mediate the 2-fold increase in cyclin E1-cdk2 activity in the MEEK lines (Fig. 2C). MCF-7 lines with constitutive MEK1 activation showed no increase in p27 binding to cyclin E1 following antiestrogens compared with that in parental MCF-7 or in the empty vector controls (representative data in Fig. 2D). The modest increase of p21 binding to cyclin E1-cdk2 may mediate the partial cell cycle inhibition after antiestrogen treatment of the M2 clone. Earlier work has established that increased KIP binding to cyclin E1-cdk2 in MCF-7 is essential for G1 arrest by antiestrogens (3, 48). Thus, MAPK activation via MEEK or ERK2-MEK transfection may contribute to antiestrogen resistance, at least in part, by impairing the antiestrogen-mediated increase in p27 binding to cyclin E1-cdk2.

Altered p27 Phosphorylation in Antiestrogen-resistant Lines—The MAPK-activated transfectants and LY-2 cells show a number of similarities. Both showed more abundant p27 binding to cyclin E1-cdk2 in asynchronously proliferating cells than would have been predicted from the respective total cellular abundance of p27, and antiestrogens failed to cause an accumulation of p27 in cyclin E1-cdk2 complexes. We postulated that differences in p27 phosphorylation may be associated with these differences in p27 function. Under most one-dimensional SDS-polyacrylamide gel electrophoresis conditions, p27 does not show reproducible differences in gel mobility. 2D-IEF allowed resolution of different p27 phospho-isofoms that are not apparent on single dimension gel electrophoresis. 2D-IEF showed a reproducible difference between the phosphorylation profile of p27 in the antiestrogen-sensitive and -resistant lines. 2D-IEF of p27, using an amphoteric carrier with a non-linear pH range of 3–10, showed five p27 isoforms present in all three lines (labeled 1–5 in Fig. 2E). Form 1 migrates at the predicted isoelectric focusing point for p27 (pH = 6.54). Phosphatase treatment of the p27 immunoprecipitates confirmed that most of these different isoforms represent different phosphoforms of p27 (Fig. 2E). The minor amount of form 2 remaining after phosphatase treatment may reflect incomplete dephosphorylation. Alternatively, this may represent a hypophosphorylated form of p27 in which post-translational modification (e.g. myristylation) confers a more negative charge. When cells were [32P]orthophosphate-labeled prior to p27 immunoprecipita-
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The Arrest of LY-2 by MEK Inhibition and Antiestrogens Is p27-dependent—The increase in p27 association with cyclin E1-cdk2 in LY-2 cells treated by the combination of 0.1 μM U0126 and 10 nM ICI was similar to that seen following antiestrogen treatment in the sensitive MCF-7 line (see Figs. 1B and 3C). We postulated that MAPK inhibition in LY-2 enhanced the cdk2 inhibitory function of p27 to facilitate cell cycle arrest by antiestrogens. If this were the case, then antisense-mediated inhibition of p27 expression in the U0126/ICI treated cells should abrogate this drug-mediated arrest. U0126/ICI-arrested LY-2 cells were transfected with either antisense p27 (Asp27) oligonucleotides or mismatch control oligonucleotides (Msp27) or mock transfected with lipid only (control, C), and cells were recovered for flow cytometry and protein analysis at 21 h following completion of Asp27 transfection. The inhibition of p27 expression in Asp27-treated cells lead to cell cycle re-entry with ~23% cells in S phase at 21 h, in contrast to 8 and 9% of cells in S phase following lipid only (control, C) or mismatch (Msp27) transfection (Fig. 3D). The Asp27-mediated cell cycle re-entry was associated with loss of cyclin E1-bound p27 and cyclin E1-associated kinase activation (Fig. 3D). Control (lipid alone) and Msp27-transfected groups showed no cyclin E1-cdk2 activation. We also observed a similar result using the combination of 0.1 μM U0126 and 1 μM 4-OH-TAM (data not shown). Thus, in the LY-2 line, p27 became an essential mediator of G₁ arrest by antiestrogens following partial MEK/MAPK inhibition.

p27-immunoprecipitable Kinase Activity in Antiestrogen-treated LY-2 Cells—Proliferating LY-2 cells, with and without antiestrogen treatment, showed more abundant p27 association with active cyclin E1-cdk2 than was detected in inhibited cyclin E1-cdk2 complexes from antiestrogen-arrested MCF-7 cells (Fig. 1B). These data suggested impaired inhibitory function of cyclin E1-bound p27 in LY-2 cells. p27 immunoprecipitates were tested for associated histone H1 kinase activity in

FIG. 2. MAPK activation contributes to p27 deregulation and antiestrogen resistance. A, the levels of active phospho-MAPK, p27, and p21 were analyzed in two control cell lines transfected with empty vector (C1, C2) and in two MEK overexpressing MCF-7 clones (M1, M2). B, the cell cycle profiles of asynchronously proliferating and antiestrogen-treated MEK transfectants were compared with empty vector controls. C, cyclin E1-bound p27 and cdk2 and cyclin E1-associated kinase activities were assayed as in Fig. 1B. D, cyclin E1-bound p21 and p27 were assayed before (−) and after (+) ICI treatment. E, p27 immunoprecipitates from asynchronously proliferating MCF-7, LY-2, and MEKEE-transfectant and (M2) were analyzed by 2D-IEF. The 2D-IEF of phosphatase-treated p27 from MCF-7 cells is shown in the upper panel. The different p27 isoforms were quantitated by densitometry.
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FIG. 3. Inhibition of MAPK restores sensitivity to antiestrogens in LY-2. A, asynchronously growing LY-2 or LY-2 cells treated with 0.1 μM U0126 were assayed for levels of total and active MAPK by immunoblotting. B, the cell cycle profile of LY-2 and MCF-7/HER2 cells were measured in asynchronously proliferating, ICI-treated and 0.1 μM U0126 + ICI (U+ICI)-treated cells. C, LY-2 cells treated for 48 h with ICI, U0126 (U) or the combination of U0126 + ICI (U+ICI) and cell cycle profiles assayed by flow cytometry. Cyclin E1 immunoprecipitates were resolved and analyzed by immunoblotting with the indicated antibodies. D, LY-2 cells treated with the combination of U0126 + ICI were lysed before (0 h) or 21 h after transfection with lipid only (control group, C), ASp27 oligonucleotides (ASp27), or MSp27 oligonucleotides (MSp27). Cyclin E1 immunoprecipitates were resolved and associated proteins detected by immunoblotting. The % S phase activities in each treatment group are shown.

**Discussion:**

LY-2 and MCF-7 cells. Cdk2 complexes, but not cdk4 and 6 complexes, can use histone H1 as substrate. Histone H1 kinase activity was detected in p27 immunoprecipitates from asynchronous and ICI-treated LY-2 cells, but was negligible in asynchronous and ICI-treated MCF-7 when background activity in nonspecific antibody control immunoprecipitates was subtracted (Fig. 4A). The combination of 0.1 μM U0126 and 10 nM ICI 182780 (U+ICI) inhibited the p27 immunoprecipitable kinase activity in LY-2 (Fig. 4A). Parallel p27 immunoprecipitates were resolved and blotted for associated cdk2, cyclin E1, and cyclin A. The amounts of cdk2 and cyclin A bound to p27 in ICI-treated LY-2 and MCF-7 were similar, and there was no loss of p27-bound cdk2 following ICI plus U0126 treatment LY-2 cells (data not shown).

**MEK Activation Modulates p27 Inhibitory Function**—The inhibitory activity of p27 toward recombinant cyclin E1-cdk2 was compared in the MCF-7 and LY-2 lines (Fig. 4B), in the MCF-7/MEK-EE transfectant, M2, and in the empty vector control line, C2 (Fig. 4C). Equal amounts of p27 protein were immunoprecipitated from the indicated cell lines and boiled to release associated proteins, and then heat-stable p27 was tested for its ability to inhibit a fixed amount of recombinant cyclin E1-cdk2. The cyclin E1-cdk2 complexes were then immunoprecipitated, and kinase activity was assayed. The activity of the p27-treated cyclin E1-cdk2 was expressed as a % of control, uninhibited cyclin E1-cdk2. p27 from the MCF-7 line had approximately four times the inhibitory potency as p27 from the LY-2 line (Fig. 4B). Similarly, MEK overexpression in the M2 line impaired the inhibitory function of p27 (Fig. 4C). The cyclin E1-cdk2 inhibitory activity of increasing amounts of p27 from the MEK2-K-transfected M2 line was compared with that of p27 from the vector alone control, C2. Even a 3-fold (3×) excess of p27 in the M2 line did not achieve the same level of cyclin E1-cdk2 inhibition shown by p27 (1×) from the control line.

**MEK Inhibition Modulates p27 Phosphorylation**—Our antisense experiments showed that p27 was essential for the antiestrogen arrest of LY-2 following partial MEK inhibition (Fig. 3D). Since MEK inhibition restored antiestrogen arrest, we postulated that MEK inhibition might alter p27 phosphorylation. As seen in asynchronously proliferating cells (Fig. 2F), the total IEP of p27 from antiestrogen-treated MCF-7 and LY-2 cells showed five distinct p27 isoforms (labeled 1–5 in Fig. 5A) with isoforms 1 and 3 again being the most abundant. p27 from antiestrogen-arrested MCF-7 showed a predominance of isoform 1, with the ratio of isoforms 1 to 3 being 2:1. In antiestrogen-treated LY-2 cells, form 3 was the predominant form, with the isoform 1:isoform 3 ratio at 1:2. Treatment with 0.1 μM U0126 together with either 4-OH-TAM or ICI changed the p27 phosphorylation profile in LY-2 cells to one that more closely resembled that in antiestrogen-arrested MCF-7 cells, with the p27 isoform 1 more abundant than isoform 3 at a ratio of 2:1 (data shown for ICI treatment in Fig. 5A). In all cell types, ICI treatment increased the relative abundance of isoforms 4 and 5 compared with that of untreated cells.

We tested whether the changes in total cellular p27 phosphorylation were reflected by changes in the phosphorylation of cyclin E1-bound p27 (Fig. 5B). Cyclin E1-bound p27 in the ICI-treated LY-2 line showed a predominance of isoform 3 (the ratio of isoform 1:isoform 3 was 1:6 by densitometry), whereas LY-2 cells arrested by the combination of MEK inhibition and antiestrogen showed a cyclin E1-associated p27 phosphorylation pattern more closely resembling that in antiestrogen-arrested MCF-7 (isoform 1:isoform 3 ratio nearly 1:1 in both). These data suggest that the combination of MEK inhibition and antiestrogen treatment may restore the cyclin E1-cdk2 inhibitory function of p27 in LY-2, at least in part, by altering p27 phosphorylation.
DISCUSSION

The key roles of p21 and p27 in antiestrogen arrest have been demonstrated in earlier studies (3, 48). Antiestrogens increase cyclin E1-cdk2-KIP binding, and immunodepletion of p21 and p27 from steroid-depleted or tamoxifen-arrested cells removes essentially all cellular cyclin E1-cdk2 (49–51), suggesting that these cyclin complexes are fully saturated by p21 or p27 in arrested cells. Recent work with antisense (AS) p27 demonstrated that inhibition of expression of either KIP from antiestrogen-arrested cells leads to cell cycle re-entry (3, 48). In addition to increased KIP-cdk binding, other cell cycle effectors contribute to G1 arrest by antiestrogens. These include reductions in c-Myc and cyclin D1 and Cdc25A, increased p15, and potentially, the accumulation of cdk2 in a non-CAK-activated form (3, 50–53). However, while induction of cell cycle arrest by antiestrogens has multiple effectors, the antisense studies demonstrate that KIP function is required for maintenance of arrest. Moreover, the present work indicates that deregulation of p27 inhibits antiestrogen responsiveness.

Our data suggest that constitutive MEK/MAPK activation contributes to the development of antiestrogen resistance in ER-positive breast cancer cells, at least in part, by compromising the inhibitory function of p27. We show here that a non-cytostatic and non-cytotoxic dose of the MEK inhibitor, U0126, restored sensitivity to G1 arrest by antiestrogens in the widely used LY-2 model of antiestrogen resistance. Moreover, transfection of HER2 or MEKEE into MCF-7 impaired antiestrogen responses. In antiestrogen-treated LY-2 and MCF-7/MEK EE transfecants, p27 failed to accumulate in cyclin E1-cdk2 complexes and did not inhibit this kinase. MEK inhibition by
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FIG. 5. MEK inhibition modulates p27 phosphorylation. A and B, the 2D-IEF patterns of immunoprecipitated p27 (A) or cyclin E1-bound p27 (B) were assayed in MCF-7 and LY-2 cells after 48 h of treatment with ICI, and in LY-2 cells after 48 h treatment with both ICI and U0126.

U0126 in these antiestrogen-resistant lines altered p27 phosphorylation and restored the inhibitory binding of p27 to cdk2 following antiestrogen treatment. Thus, MEK/MAPK-dependent p27 phosphorylation events are associated with a reduced ability to inhibit cdk2.

Through the course of selection of the antiestrogen-resistant LY-2, p27 regulation has been altered such that its binding to cyclin E1-cdk2 is increased without a commensurate reduction in cyclin E1-cdk2 activity. In antiestrogen-mediated arrest of MCF-7, a 3-fold increase in p27 binding to cyclin E1-cdk2 is sufficient for cdk2 inactivation and cell cycle arrest (3). The approximately 4-fold increase in cyclin E1-bound p27 in asynchronously growing LY-2 cells relative to that in proliferating MCF-7 cells, and the failure of antiestrogens to increase p27 binding to cyclin E1-cdk2 in LY-2 prompted further investigation of p27 function in these lines. Indeed, both cyclin E1 and p27 immunoprecipitates contain detectable histone H1 kinase activity in LY-2 cells. While the p27-associated kinase activity could reflect dissociation of p27 from cyclin E1-cdk2 in vitro following immunoprecipitation of the complexes, the increased binding of p27 to cyclin E1 without loss of kinase activity in asynchronous LY-2 suggests that some of the cyclin E1-cdk2-p27 complexes may retain activity in vivo. Detection of p27-immunoprecipitable kinase activity has been reported by others (54, 55). The elevated level of p27 protein in the LY-2 line may reflect MAPK-independent events that have occurred throughout the course of selection of this line (44).

p27 levels were reduced in the MEK<sup>EE</sup> transfectants, consistent with the observation by others that Ras-MAPK contributes to p27 degradation (15, 20, 56). Despite the lower total p27 protein levels in these cells, cyclin E1-bound p27 levels were not reduced. Moreover, while cyclin E1-bound p21 and p27 levels were similar in MEK<sup>EE</sup> transfectants and control lines, cyclin E1-cdk2 activity was increased in asynchronously proliferating MEK<sup>EE</sup> transfecants compared with controls. Thus, MAPK activation at the levels achieved here, may favor the association of p27 with cdk2 in a poorly inhibitory form, such that some of the cyclin E1-cdk2-p27 complexes retain activity. This effect may be separable from the effect of MAPK on p27 stability. p27 forms that can bind cyclin E1-cdk2 but fail to inhibit this kinase have been modeled previously in vitro (8).

Further evidence to support functional alteration of p27 in the LY-2 and MEK<sup>EE</sup> transfected MCF-7 lines is provided by analysis of p27 inhibitory function. p27 from both LY-2 and the MEK<sup>EE</sup>-transfected line, M2, both showed a reduced ability to inhibit recombinant cyclin E1-cdk2 in vitro. As an alternate model, MEK/MAPK activation may reduce the stability with which p27 binds to cyclin E1-cdk2 in vitro, allowing detection of this kinase activity in p27 immunoprecipitates through dissociation in vitro. A reduced ability to bind cyclin E1-cdk2 could also account for the reduced inhibitory activity of heat-stable p27 isolated from the resistant lines.

Signal transduction pathways have been shown to affect p27 inhibitory function (15, 57), raising the possibility that phosphorylation events may modulate p27 function. Overexpression of the integrin-linked kinase causes a reduction in inhibitory activity of p27 toward cyclin E1-cdk2 (57). Here we showed that the antiestrogen ICI182780 modulates p27 phosphorylation in MCF-7 cells, increasing the relative amounts of p27 isoforms 3, 5, and 6. We also showed an association between altered p27 inhibitory function and altered phosphorylation in LY-2 and MCF-7/MEK<sup>EE</sup> cells, suggesting that deregulated p27 phosphorylation may be causally linked to antiestrogen resistance. Although MAPK can phosphorylate p27 in vitro (15-17), it is not known at present whether direct phosphorylation of p27 by MAPK occurs in vivo. The effects of MAPK on the p27 phosphorylation profile may be indirect. p27 contains several potential MAPK consensus sites, including serine 10 (Ser-10), Ser-178, and Thr-187. Ser-10 has recently been shown to be a major p27 phosphorylation site in G<sub>0</sub>-arrested cells, although it may not be a physiological MAPK target site (16). Since a p27 mutation converting Ser-10 to alanine or aspartate did not affect the ability of p27 to inhibit cyclin E1-cdk2 in vitro (16), the MAPK-dependent pathway that modulates both p27 phosphorylation and its ability to inhibit cdk2 cannot uniquely affect Ser-10. Moreover, the phosphorylation of p27 at Thr-187 that regulates its recognition by the F box protein Skp-2, does not affect the cdk2 inhibitory function of p27 (8). Thus, phosphorylation at sites other than Ser-10 and Thr-187 may be required for the MEK/MAPK-dependent phosphorylation of p27 that modulates its cdk2 inhibitory function. The identity of the different 2D-IEF phospho-isoforms of p27 observed by 2D-IEF warrants further investigation.

The causes of MAPK activation in human cancers differ among different tumors. MAPK activation is increased in up to 50% of breast cancers compared with normal breast epithelium and is associated with poor patient prognosis (58-60). HER-2/ErbB-2 overexpression, seen in up to 30% of breast cancers is often associated with antiestrogen resistance (27). HER-2/ErbB-2 signaling has been shown to decrease p27 stability via MAPK activation (56). In the HER-2 overexpressing MCF-7/HER-2-18, MEK inhibition by U0126 restored sensitivity to antiestrogens. Taken together, the present study links HER-2/ErbB-2 activation and antiestrogen resistance through MAPK-dependent alterations in p27 function.

In addition to its mechanistic relevance to breast cancer, the observed link between p27 dysfunction and MAPK activation has implications for many types of cancers. The reduced levels of p27 observed in many cancers (colon, lung, prostate, gastric) may reflect oncogenic activation of the Ras/MAPK pathway (12). For example, the increased p27 proteolytic activity observed in colon cancer lysates may result from oncogenic activation of K-Ras in these cancers (61). There is a strong molecular rationale supporting the continued development of MEK/MAPK inhibitory drugs. A number of MEK inhibitors have shown good oral bioavailability and efficacy in preclinical trials (62). Tumor-specific MEK inhibitors may have the potential to restore p27 protein levels and inhibitory function and thereby restrain tumor growth.
Acknowledgments—We thank Drs. T. Hunter and H. Toyoshima for the pAB5588 p27 antibody, Dr. G. Greene for the ER (H222) antibody, Dr. D. Templeton and Dr. M. Cobb for the EE-CMV MEK2A and ERK2MEK1 plasmids, respectively, and Dr. M. Flanagan and Gilead Sciences for providing the p27 oligonucleotides. We also thank Drs. W. Hung, V. Subramanium and J-H. Lee for expertise in 2D-IEF and Dr. J. Liang for useful discussions regarding MEK inhibitors.

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Constitutive MEK/MAPK Activation Leads to p27Kip1 Deregulation and Antiestrogen Resistance in Human Breast Cancer Cells
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doi: 10.1074/jbc.M106448200 originally published online August 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106448200

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