Blockade of the Extracellular Signal-regulated Kinase Pathway Induces Marked G1 Cell Cycle Arrest and Apoptosis in Tumor Cells in Which the Pathway Is Constitutively Activated

UP-REGULATION OF p27Kip1*

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Constitutive activation of the ERK pathway is associated with the neoplastic phenotype of a relatively large number of human tumor cells. Blockade of the ERK pathway by treatment with PD98059, a specific inhibitor of mitogen-activated protein (MAP) kinase/ERK kinase (MEK), completely suppressed the growth of tumor cells in which the pathway is constitutively activated (RPMI-SE and HT1080 cells). Consistent with its prominent anti-proliferative effect, PD98059 induced a remarkable G1 cell cycle arrest, followed by a modest apoptotic response, in these tumor cells. Selective up-regulation of p27Kip1 was observed after PD98059 treatment of RPMI-SE and HT1080 cells. Overexpression in RPMI-SE cells of either a kinase-negative form of MEK1 or wild-type MAP kinase HT1080 cells. Overexpression in RPMI-SE cells of either a kinase-negative form of MEK1 or wild-type MAP kinase phosphatase-3 also induced up-regulation of p27Kip1. The up-regulation of p27Kip1 correlated with increased association of p27Kip1 with cyclin E-cyclin-dependent kinase (CDK) 2 complexes, a concomitant inhibition of cyclin E-CDK2 kinase activity, and a consequent decrease in the phosphorylation state of retinoblastoma protein, which would culminate in the marked G1 cell cycle arrest observed in these tumor cells. These results suggest that the complete growth suppression that follows specific blockade of the ERK pathway in tumor cells in which the pathway is constitutively activated is mediated by up-regulation of p27Kip1.

The 41-43-kDa mitogen-activated protein (MAP)1 kinase pathway, also called the extracellular signal-regulated kinase (ERK) pathway, is activated in a variety of cell types by diverse extracellular stimuli and is among the most thoroughly studied of signaling pathways that connect different membrane receptors to the nucleus (1, 2). Activation of the ERK pathway involves the activation of Ras at the plasma membrane, and the sequential activation of a series of protein kinases. Initially, Ras interacts with and activates Raf-1, which in turn activates MAP kinase/ERK kinase (MEK)-1 and -2 by serine phosphorylation. MEK-1/2 then catalyze the phosphorylation of 41- and 43-kDa MAP kinases (ERK2 and ERK1, respectively) on tyrosine and threonine residues, and these activated MAP kinases can phosphorylate cytoplasmic and nuclear targets. The ERK pathway participates in a wide range of cellular programs including proliferation, differentiation, and movement (1, 2).

Ablative activation of signal transducing proteins has been linked with cancer. For example, constitutively active mutants of Ras (3) and Raf-1 (4) have been observed in several human tumors, and constitutively active mutants of MEK-1 have been shown to transform mammalian cells (5, 6). We recently examined whether constitutive activation of the ERK pathway is associated with the neoplastic phenotype of human tumor cells. Constitutive activation of ERKs and MEK was observed in a relatively large number of tumors; tumor cells derived from pancreas, colon, lung, ovary, and kidney tissues showed especially high frequencies (30–50%) and a high degree of kinase activation (7, 8). Activation of the ERKs is also associated with prostate cancer progression (9). The precise cause of constitutive activation of the ERK pathway in many of these tumor cells remains unclear. However, such high frequencies of ERK/MEK activation in human tumors indicate that specific inhibitors might be developed against these protein kinases for cancer therapy, especially for treatment of tumors showing constitutive activation of the ERK pathway.

In the present study, we have examined the effect of blockade of the ERK pathway on the proliferation of human tumor cells. We utilized small molecule inhibitors of this pathway, PD98059 (10) and U0126 (11), which specifically inhibit MEK activity. Our results demonstrate that these MEK inhibitors induce a remarkable G1 cell cycle arrest, followed by a modest apoptotic response, in tumor cells in which the ERK pathway is constitutively activated. Up-regulation of the CDK inhibitor p27Kip1 was observed in these G1-arrested tumor cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human cell lines A-172 (glioblastoma), TGW (neuroblastoma), GOTO (neuroblastoma), HT1080 (fibrosarcoma), RPMI-SE (renal cell carcinoma), Colo320 (colon adenocarcinoma), and TIG-3 (diploid fibroblasts) (7) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Antibodies and Reagents—The polyclonal anti-ERK antibody has been described previously (12, 13). Antibodies against p16INK4a (SC-1661), p19INK4d (SC-1063), p21Cip1 (SC-6246), p27Kip1 (SC-528), p57Kip2 (SC-1040), cyclin A (SC-239), cyclin D1 (SC-6281), cyclin E (SC-247), and pRb (SC-102) were obtained from Santa Cruz Biotechnology. Anti-
cyclin B1 antibody (CC-03) was from Calbiochem. 2-(2-Amino-3-methoxyphenyl) chromone, which is identical to the published compound PD98059 (10), was synthesized as described previously (14). U0126 (11) was purchased from Promega. Other chemicals and reagents were of the highest grade available.

**Cell Growth Analysis**—For monolayer growth, cells were plated at a density of 1 × 10^4 cells per 35-mm dish and incubated for 24 h at 37 °C. Cells were then mock-treated or treated with 50 μM PD98059 or 20 μM U0126 for up to 5 days. Cells were harvested by trypsinization, and viable cells which excluded trypan blue were counted using a hemocytometer. For anchorage-independent growth, 1 × 10^4 viable cells which excluded trypan blue were counted using a hemocytometer. For monolayer growth, cells were plated at a density of 1 × 10^4 cells per 35-mm dish and incubated for 24 h at 37 °C. Cells were then mock-treated or treated with 50 μM PD98059 or 20 μM U0126 for up to 5 days. Cells were harvested by trypsinization, and viable cells which excluded trypan blue were counted using a hemocytometer. For anchorage-independent growth, 1 × 10^4 viable cells which excluded trypan blue were counted using a hemocytometer.

**Immunoblotting**—Cells were scraped off plates in IB cell lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 mM sodium orthovanadate, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and lysed by sonication for 60 s. Lysates were cleared by centrifugation at 15,000 × g for 30 min, and protein concentrations were determined using the BCA protein assay reagent (Pierce). Cell lysates (50 μg of protein) were separated by SDS-PAGE, electrophoretically transferred to an Immobilon-P membrane (Millipore Corp), and probed with the appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized with the enhanced chemiluminescence system (Amersham Pharmacia Biotech) (7, 15).

**ERK Assay**—ERK activity was measured in an immune complex kinase assay as described previously (7, 12, 13). Briefly, cell lysates prepared as described above (10 μg of protein) were immunoprecipitated by incubating for 3 h at 4°C with polyclonal anti-ERK antibody preabsorbed to protein-A Sepharose (Amersham Pharmacia Biotech). After washing twice with kinase buffer A (50 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, and 10% glycerol), each immunoprecipitate was incubated for 30 min at 30°C with 20 μM ATP, 1 μCi of [γ-^32P]ATP (Amersham Pharmacia Biotech), and 7.5 μg of myelin basic protein (Sigma) in 30 μl of kinase buffer A. Radioactivity incorporated into myelin basic protein was determined by liquid scintillation spectrometry.

**Flow Cytometry**—Cells were fixed in 70% ethanol, treated with 100 μg/ml RNase A (DNase-free; Sigma), and stained with 20 μg/ml propidium iodide (16). At least 1 × 10⁶ cells from each sample were analyzed for DNA content using a Coulter EPICS XL flow cytometer (Coulter Electronics). Percentages of cells in G₁, S, and G₂/M phases were determined using Multicycle AV software (Phoenix Flow Systems). Nuclear Staining—Cells grown on glass coverslips were treated with 50 μM PD98059 for 96 h. After fixing with 3.7% paraformaldehyde, apoptotic cells with condensed or fragmented nuclei were visualized by DAPI staining (0.4 mg/ml, 30 min).

**DNA Fragmentation Analysis**—Cells were lysed in TET lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 0.5% Triton X-100) on ice for 10 min. After centrifugation, supernatants were treated with 100 μg/ml of RNase A for 60 min. DNA was extracted and resolved by electrophoresis on 2% agarose gels as described (17). Pulsed-field gel electrophoresis was performed using a CHEF system (Bio-Rad). DNA was visualized with ethidium bromide and detected using a Molecular Dynamics Storm 840 Cromemco 880 Chemiluminescence Imaging System (Molecular Dynamics). Cell lysates (10 μg of protein) were immunoprecipitated with 2 μg of anti-Cdk2 antibody (SC-163, Santa Cruz Biotechnology) or 3 μg of anti-cyclin E antibody (14761A, Pharmingen) for 3 h at 4°C. Immunocomplexes were collected on protein-A-Sepharose beads, washed twice with kinase buffer B (50 mM Hepes, pH 7.3, 10 mM MgCl₂, and 1 mM dithiothreitol), and resuspended in 30 μl of kinase buffer B supplemented with 2 μg of histone H1 (Roche Molecular Biochemicals), 25 μM ATP, and 10 μCi of [γ-^32P]ATP. Reaction mixtures were incubated for 30 min at 30°C, and the extent of histone H1 phosphorylation was determined by SDS-PAGE and autoradiography.

**Plasmid and Transient Transfection**—The expression vector for MKP-3 (pMT-SM-Myk-MKP-3) (19) was kindly provided by Dr. Steve Arkin (Seronova Pharmaceutical Research Institute), and the plasmid expressing a kinase-negative form of MEK1 (pcDNAinco-Myc-MEK1/AA) (19) was purchased from Promega. Other chemicals and reagents were of the highest grade available.

**RESULTS**

**MEK Inhibitor Completely Suppresses the Growth of Human Tumor Cells in Which the ERK Pathway Is Constitutively Activated**—We have recently proposed that human tumor cells can be classified into four groups with regard to the activation of the ERK pathway (7). Tumor cells in which constitutive activation of the ERK pathway is detected are classified as type III or type II; type III tumor cells are those in which the degree of activation of the ERK pathway is especially high. Tumor cells in which constitutive activation of the ERK pathway is not detected are classified as type I or type 0. Type II tumor cells are those in which the ERK pathway is markedly activated when serum-starved cells are growth-stimulated with 10% serum; this response is identical to that observed in normal diploid fibroblasts. Type 0 tumor cells are abnormal with respect to activation of the ERK pathway, i.e. significant activation of the pathway is undetectable even when serum-starved cells are growth-stimulated with 10% serum.

We first examined the inhibitory effect of two MEK inhibitors, PD98059 and U0126, on the activation of ERKs in exponentially growing tumor cells. Activation of ERKs was determined by two different assay procedures as follows: by performing a direct in vitro kinase assay of the immunoprecipitates using myelin basic protein as the substrate, and by measuring the appearance of the phosphorylated/activated forms of ERKs, which show reduced mobility in SDS-PAGE. As described previously (7), type III (RPMI-SE and HT1080) and type II (A-172) tumor cells exhibited a significantly high degree of ERK activation under exponentially growing conditions. Treatment of these tumor cells with U0126 or PD98059 suppressed the activation of ERKs in a dose-dependent manner; U0126 inhibited ERK activation more efficiently than PD98059, with virtually complete suppression being observed at 20 or 50 μM, respectively. Although only a limited degree of ERK activation was detected in exponentially growing type I (TGW) and type 0 (Colo320 and GOTO) tumor cells, treatment with PD98059 or U0126 also reduced the degree of ERK activation in these cells. Fig. 1C shows the PD98059/U0126-mediated inhibition of ERK activation in RPMI-SE and Colo320 cells.

Treatment with PD98059 or U0126 inhibited the growth of all of the tumor cells examined (Fig. 1A). These results seemed reasonable, because the ERK pathway is the major cytoplasmic kinase pathway and is activated commonly by numerous mitogenic stimuli that interact with a diversity of structurally distinct receptors. In addition, activation of the ERK pathway has been shown to be necessary for fibroblast proliferation (5, 6, 21). However, susceptibility to these MEK inhibitors showed distinct differences among cell types. The growth of tumor cells in which the ERK pathway was constitutively activated was totally abolished (type III tumor cells) or almost completely inhibited (type II tumor cells) by 50 μM PD98059 or 20 μM U0126. PD98059/U0126 inhibited the growth of TGW type I
tumor cells and TIG-3 diploid fibroblasts (data not shown) to a similar extent; the inhibition was considerable but not complete.

Although proliferation of type 0 tumor cells appeared not to depend on the activation of the ERK pathway, PD98059/U0126 slightly inhibited the growth of these cells. Basal ERK activity (Fig. 1C) may be partially responsible for the proliferation of type 0 tumor cells; inhibition of the basal ERK activity by PD98059/U0126 treatment might have resulted in the slight growth inhibition observed in these cells.

Consistent with its antiproliferative effect on monolayer growth, PD98059 completely inhibited the anchorage-independent growth of RPMI-SE type III tumor cells but did not significantly affect Colo320 type 0 tumor cells (Fig. 1B).

**MEK Inhibitor Induces Marked G1 Cell Cycle Arrest in Tumors in Which the ERK Pathway Is Constitutively Activated**—To investigate the mechanism underlying the antiproliferative effect of MEK inhibitors, cells were treated with 50 μM PD98059 for 0–96 h, stained with propidium iodide, and subjected to flow cytometric analysis of cell cycle distribution. Consistent with the marked inhibitory effect on cell proliferation, PD98059 induced remarkable G1 cell cycle arrest in type III tumor cells (Fig. 2). The onset of G1 phase-arrest was apparent as early as 12 h after PD98059 treatment of RPMI-SE and HT1080 cells, and almost complete G1 cell cycle arrest was observed by 24 h, at which time the proportion of cells in G1 phase had increased from 57.5 to 93.2% or 45.1 to 77.4%, and the proportion of cells in S phase had declined from 27.8 to 2.4% or 34.8 to 2.9% in RPMI-SE or HT1080 cells, respectively. Furthermore, PD98059 treatment induced in these tumor cells a significant increase in the proportion of dead cells with fractional DNA content, which is a characteristic feature of apoptosis (22). The proportion of such dead cells 96 h after PD98059 treatment was 10.7% in RPMI-SE cells and 26.1% in HT1080 cells; the proportions in mock-treated control cells were 2.3 and 3.5%, respectively. PD98059 also induced prominent G1 cell cycle arrest in A-172 type II tumor cells. However, the accumulation of PD98059-treated A-172 cells in G1 phase was slower than that observed in type III tumor cells; the proportion of A-172 cells in G1 phase had increased from 38.4 to 74.7% by 96 h, at which time the proportion of cells in S phase had decreased from 40.3 to 7.6%.

In contrast, MEK inhibition affected the cell cycle distribution of TIG-3 type I tumor cells and TIG-3 diploid fibroblasts (data not shown) to only a small extent. PD98059 induced a slight increase (at most 5–7%) in the proportion of these cells in G1 phase and a slight decrease in the proportion of cells in S phase. This modest effect of PD98059 treatment on the cell cycle distribution of these cells appeared to result in the considerable growth suppression described above (Fig. 1A). PD98059 did not significantly affect the cell cycle distribution of Colo320 or GOTO type 0 tumor cells.

**MEK Inhibitor Induces a Modest Apoptotic Response in Tumors in Which the ERK Pathway Is Constitutively Activated**—To characterize the cell death caused by PD98059, we examined the nuclear morphology of dying RPMI-SE and HT1080 cells with a fluorescent DNA-binding agent, DAPI. After treatment with 50 μM PD98059 for 120 h, ~10% of the
RPMI-SE cell population and ~15% of the HT1080 cell population clearly exhibited condensed and fragmented nuclei, indicative of apoptotic cell death (Fig. 3). No such nuclear morphology was detected in mock-treated control cells. PD98059 treatment also induced internucleosomal DNA fragmentation in HT1080 cells; this was most evident after 120 h. PD98059 induced degradation of RPMI-SE DNA not into internucleosomal fragments but into fragments of high molecular weight. Pulsed-field gel electrophoresis revealed the presence of 10–40-kbp DNA fragments in PD98059-treated RPMI-SE cells after 120 h. It has recently been proposed, however, that the appearance of large DNA fragments (20–300 kbp) occurs prior to the appearance of internucleosomal DNA fragmentation and that such large DNA fragments serve as precursors for the smaller DNA fragments (18, 23). Thus, apoptosis appeared to be the major mechanism of PD98059-induced cell death in RPMI-SE and HT1080 tumor cells.

MEK Inhibitor Induces Selective Increase in the CDK Inhibitor p27Kip1 and Inhibition of CDK2 Kinase Activity—To investigate the molecular mechanism of the G1 cell cycle arrest observed in PD98059-treated RPMI-SE and HT1080 cells, we examined whether changes in G1-associated regulatory proteins had occurred in these cells, focusing on the retinoblastoma protein (Rb) (24). As shown in Fig. 4, PD98059 treatment decreased the phosphorylation state of Rb; the hypophosphorylated (pRb) species began to increase by 6 h, and virtually complete loss of the hyperphosphorylated (ppRb) species was observed 24–48 h after PD98059 treatment of RPMI-SE and HT1080 cells. In addition, PD98059 treatment markedly reduced the levels of cyclin A and B1. This is consistent with PD98059-induced inhibition of entry into S and G2/M phases, where peak expression of these cyclins is known to occur. Although the ERK pathway has been shown to regulate positively the expression of cyclin D1 (25), PD98059 inhibition of the pathway did not significantly affect the level of either cyclin D1 or cyclin E in these tumor cells.

We next explored possible reasons for Rb hypophosphorylation, focusing on the CDK inhibitors p21Cip1, p27Kip1, p57Kip2, p16INK4a, and p15INK4d (26). Exponentially growing RPMI-SE and HT1080 cells expressed only limited levels of these CDK inhibitors. PD98059 treatment induced a marked increase in p27Kip1 in these tumor cells, which began at 12–24 h and reached a maximum by 48 h. Immunostaining clearly revealed the up-regulation of p27Kip1 in the nuclei of PD98059-treated RPMI-SE cells (Fig. 6). In contrast, PD98059 did not induce any significant increase in p21Cip1, p57Kip2, p16INK4a, or p15INK4d in either of the tumor cell lines (data not shown).

Up-regulation of p27Kip1 has been shown to favor its association with G1-specific cyclin-CDK complexes such as cyclin E-CDK2, resulting in kinase inhibition and contributing to Rb hypophosphorylation (26). To investigate the functional significance of p27Kip1 up-regulation in PD98059-treated RPMI-SE cells, cell extracts were immunoprecipitated with antibodies against CDK2 or cyclin E, and CDK2 kinase activity and cyclin E-associated kinase activity were measured using histone H1 as a substrate. PD98059 treatment of the cells inhibited both CDK2 kinase activity and cyclin E-associated kinase activity, with complete inhibition being apparent by 48 h (Fig. 5). Immunoblot analysis of the immunoprecipitated proteins revealed an increase in p27Kip1 in both the CDK2 complexes and the
cyclin E complexes after PD98059 treatment, indicating increased binding of p27<sup>Kip1</sup> to cyclin E/CDK2 in response to PD98059. PD98059 treatment did not significantly affect the amount of either CDK2 or cyclin E in the immunoprecipitates.

In Colo320 and GOTO type 0 tumor cells, PD98059 treatment did not induce a significant change in the phosphorylation state of Rb or in the levels of G<sub>1</sub>-associated regulatory proteins such as p27<sup>Kip1</sup>, cyclin A, cyclin B1, cyclin D1, or cyclin E; this was also the case in TGW type I tumor cells (Fig. 4 and data not shown). These results were in consistent with the slight inhibitory effect of PD98059 on the growth of these tumor cells (Fig. 1).

**DISCUSSION**

In the present study, we examined the effect of a specific blockade of the ERK pathway on the growth of human tumor cells in vitro, using the specific MEK inhibitors PD98059 and U0126 (10, 11). Both inhibitors efficiently suppressed ERK activation in all of the tumor cells examined (Fig. 1 C). This essentially complete inhibition of the ERK pathway suppressed the growth of all tumor cells examined. However, the susceptibility of cells to the blockade of the ERK pathway showed distinct differences among cell types and appeared to depend on the activation state of ERKs (7). The growth of tumor cells with constitutively high levels of ERK activation (type III tumor cells) was totally suppressed by PD98059/U0126, whereas the growth of tumor cells with barely detectable levels of ERK activation (type 0 tumor cells) was only slightly suppressed by the MEK inhibitors (Fig. 1A). The different effects of PD98059 on the anchorage-independent growth of type III and type 0 tumor cells are clearly shown in Fig. 1B. These results reinforce our previous observation that the requirement for the ERK pathway in proliferation differs markedly among human tumor cells; type III tumor cells depend absolutely on the activation of the ERK pathway for proliferation, whereas proliferation of type 0 tumor cells appears not to depend on the ERK pathway (7).

Consistent with its prominent antiproliferative effect, PD98059 induced striking G<sub>1</sub> cell cycle arrest in tumor cells in which the ERK pathway is constitutively activated (Fig. 2). These results are consistent with the idea that activation of the
ERK pathway is essential for cells to pass the G1 restriction point (21). It has recently been reported, however, that the ERK pathway functions not only in the G1/S transition but also in the transition from G2 to M phase in mammalian fibroblasts (27). In this respect, populations of HT1080 cells treated with 50 μM PD98059 for more than 24 h remained at G2/M; essentially complete depletion of the PD98059-treated cells in S phase was confirmed by pulsing the cells with BrdUrd and then double-staining with a fluorescein isothiocyanate-conjugated anti-BrdUrd antibody and propidium iodide for flow cytometric analysis (data not shown). Cells remaining at G2/M phase were not observed in PD98059-treated RPMI-SE or A-172 cell populations. Requirements for the ERK pathway in G2/M transition may differ significantly among tumor cells.

Orderly progression through the cell cycle is cooperatively regulated by several classes of CDKs, whose activities are in turn constrained by CDK inhibitors (26). We demonstrated that PD98059 induced selective up-regulation of p27Kip1 in RPMI-SE and HT1080 cells. PD98059 did not induce a significant increase in p27Kip1 in tumor cells in which constitutive activation of the ERK pathway is undetectable (Fig. 4). Furthermore, overexpression in RPMI-SE cells of either a kinase-negative form of MEK1 or wild-type MKP-3 clearly induced up-regulation of p27Kip1 (Fig. 6). These results suggest that specific blockade of the ERK pathway induces marked up-regulation of p27Kip1 only in tumor cells in which the pathway is constitutively activated.

The pivotal role of p27Kip1 in controlling CDK function and thus cell cycle progression is well established (26). p27Kip1 mediates cell cycle arrest in response to various anti-mitogenic signals such as transforming growth factor-β, serum withdrawal, and cell-cell contact. p27Kip1 associates with and inhibits the catalytic activities of G1-specific cyclin-CDK complexes such as cyclin E-CDK2, whose activity is essential for entry of FIG. 5. Inactivation of cyclin E-CDK2 complexes by binding of p27Kip1 in PD98059-treated RPMI-SE cells. RPMI-SE cells were mock-treated (C) or treated with 50 μM PD98059 (PD) for the indicated times. After cell lysates (400 μg of protein) were immunoprecipitated (IP) with an antibody against CDK2 or cyclin E, CDK2 and cyclin E-associated kinase activities were measured using histone H1 (H1) as a substrate. The amounts of CDK2, cyclin E, or p27Kip1 in the CDK2/ cyclin E immunoprecipitates were analyzed by immunoblotting (IB). Data shown are representative of two separate experiments.

FIG. 6. Up-regulation of p27Kip1 induced by overexpression of MEK1(AA) or MKP-3 in RPMI-SE cells. RPMI-SE cells were mock-transfected or transfected with an expression vector encoding Myc-tagged MEK1(AA) or Myc-tagged MKP-3. Mock-transfected cells were then treated with 50 μM PD98059 for 48 h. For BrdUrd incorporation assays, the transfected cells were incubated with 20 μM BrdUrd for 24 h. Cells expressing either MEK1(AA) or exogenous MKP-3 were detected by immunofluorescence using the anti-Myc antibody. Localization of the phosphorylated (activated) ERKs was revealed by immunofluorescence using anti-phospho-ERKs antibody (pERKs), and localization of p27Kip1 was revealed with anti-p27Kip1 antibody. BrdUrd-positive cells were detected with fluorescein isothiocyanate-conjugated monoclonal anti-BrdUrd antibody (BrdU). The nuclei of all cells were visualized with DAPI. Arrowheads indicate cells expressing either MEK1(AA) or exogenous MKP-3. Experiments were repeated 3–5 times with similar results.
cells into S phase. One major target of cyclin E-CDK2 is Rb, which upon hyperphosphorylation dissociates from bound transcription factors such as E2F, enabling them to activate genes required for DNA replication. We demonstrated that the up-regulation of p27Kip1 observed in PD98059-treated RPMI-SE cells correlated with an increase in p27Kip1 associated with cyclin E-CDK2 complexes, a concomitant inhibition of cyclin E-CDK2 kinase activity, and a consequent decrease in the phosphorylation of Rb, which would culminate in the G1 cell cycle arrest of these cells. Taken together, our results suggest that the complete growth suppression that follows specific blockade of the ERK pathway in tumor cells in which the pathway is constitutively activated is mediated by up-regulation of p27Kip1.

Regulation of p27Kip1 is mediated by transcriptional, post-transcriptional, and post-translational mechanisms. We did not observe any significant increase in p27Kip1 mRNA levels in RPMI-SE cells treated with PD98059 for up to 48 h (data not shown), by which time p27Kip1 reaches a maximum level (Fig. 4). Given the recent demonstration that cyclin E-CDK2 directly phosphorylates p27Kip1 and promotes its destruction by the ubiquitin pathway (26, 28), it seems likely that the accumulation of p27Kip1 induced by inhibiting the ERK pathway is a result of stabilization of p27Kip1 at the protein level.

PD98059 treatment induced a significant increase in the proportion of cells with condensed and fragmented nuclei in tumor cells in which the ERK pathway is markedly activated (Fig. 3). PD98059 induced the degradation of chromosomal DNA into internucleosomal DNA fragments in HT1080 cells or into 10–40-kbp DNA fragments in RPMI-SE cells. This fragmentation was not observed if the cells were preincubated with the ERK pathway is constitutively activated. Importantly, blockade of the ERK pathway in tumor cells in which the pathway is constitutively activated is mediated by up-regulation of p27Kip1.

In conclusion, we have demonstrated in this report that specific blockade of the ERK pathway completely suppresses the growth of tumor cells in which the pathway is constitutively activated, and we presented evidence suggesting that this prominent growth inhibition is mediated by the up-regulation of p27Kip1.

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