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A senescence-like growth arrest is induced in mouse primary embryo fibroblasts by inhibitors of phosphoinositide 3-kinase (PI3K). We observed that senescence-like growth arrest is correlated with an increase in p27Kip1 but that down-regulation of other cyclin-dependent kinase (CDK) inhibitors, including p15INK4b, p16INK4a, and p21Cip1, as well as other negative cell cycle regulators such as p53 and p19ARF, implies that this senescence-related growth arrest is independent of the activity of p53, p19ARF, p16INK4a, and p21Cip1, which are associated with replicative senescence. The p27Kip1 binds to the cyclin/CDK2 complexes and causes a decrease in CDK2 kinase activity. We demonstrated that ectopic expression of p27Kip1 can induce permanent cell cycle arrest and a senescence-like phenotype in wild-type mouse embryo fibroblasts. We also obtained results suggesting that the kinase inhibitors LY294002 and Wortmannin arrest cell growth and induce a senescence-like phenotype, at least partially, through inhibition of PI3K and protein kinase B/Akt, activation of the forkhead protein AFX, and up-regulation of p27Kip1. In summary, these observations taken together suggest that p27Kip1 is an important mediator of the permanent cell cycle arrest induced by PI3K inhibitors. Our data suggest that repression of CDK2 activity by p27Kip1 is required for the PI3K-induced senescence.

Normal somatic cells undergo a limited number of divisions when cultured in vitro before entering an irreversible state of cell cycle arrest known as replicative senescence (1). This process has been demonstrated to occur also in vivo (2) and is believed to play a major role in safeguarding against tumor formation by suppressing the emergence of immortal cells (3). The biological significance of replicative senescence has been highlighted by observations showing that the in vitro life span of cells is related to the age of the donor as well as the general life expectancy of the species (4). The molecular basis underlying this physiological process and how it is overcome in tumor cells is at present not very well understood. Nevertheless, replicative senescence is associated with specific physiological and morphological changes (3), including a reduction in proliferative capacity that is refractory to mitogenic stimulation, telomere shortening, adoption of a flat and enlarged cell shape, and the appearance of senescence-related \( beta \)-galactosidase activity in human cells (2). The molecular mechanism that regulates the replicative senescence is not well understood, but the accompanied growth arrest is associated specifically with the up-regulation of negative regulators of cell cycle progression, including the tumor suppressor p53 and the cyclin-dependent kinase inhibitors (CKIs), p21Cip1 and p16INK4a (5–8). Overexpression of p53, p16INK4a, or p21Cip1 has been shown to cause premature senescence-related cell cycle arrest in low passage fibroblasts (9, 10). The CKIs p21Cip1 and p16INK4a can arrest cell cycle progression through inhibiting the activity of cyclin-dependent kinases (CDKs) directly (11–13), whereas p53 presumably may act indirectly by inducing the transcription of p21Cip1 (14). It has also been shown that pRB is present in its hypophosphorylated forms in senescent cells (15). Thus, it is conceivable that the increased expression of p21Cip1 and...
p16\textsuperscript{INK4a} detected in senescent cells can arrest cell cycle progression through inhibition of the G1 cyclin-dependent CDK activity and, thereby, can prevent the phosphorylation of pRB. The hypophosphorylated pRB will in turn repress transcription factors, including E2F, which regulate the expression of genes essential for cell cycle progression (16-18). Indeed, E2F-regulated genes including cyclin A and E, CDK2, CDC2, dihydrofolate reductase (DHFR), and E2F1 have been demonstrated to be down-regulated in a variety of senescent cells (19-21). These genes that have been implicated in the senescence program are somatically mutated in a variety of cancers, and such mutations to these genes may contribute to development of malignant clones (22).

The \textit{INK4a} /\textit{ARF} locus encodes two potent tumor suppressor proteins, p16\textsuperscript{INK4a} and p19\textsuperscript{ARF}, that regulate the antiproliferative and tumor suppressor functions of pRB and p53 proteins (23, 24), respectively. Recent evidence has shown that expression of p19\textsuperscript{ARF} alone is sufficient to induce cell cycle arrest (25), and the ability of p19\textsuperscript{ARF} to induce cell cycle arrest depends on the presence of functional p53 and is achieved through stabilization of p53; p19\textsuperscript{ARF} sequesters the oncogene MDM2, thus preventing the MDM2-induced degradation of p53 (26-29).

Though oncogenic Ras can transform immortal rodent cells to a tumorigenic state, introduction of oncogenic Ras into primary fibroblasts can trigger premature senescence through the activation of the tumor suppressors p16\textsuperscript{INK4a}, p19\textsuperscript{ARF}, and p53 (30, 31). The ability of the oncogenic Ras to transform immortal rodent cell lines involves its capacity to interact and activate a range of downstream effectors, including Raf-1, phosphoinositide 3-kinases (PI3Ks), and Ral.GDS (32-34). These signaling molecules, in turn, activate their respective downstream targets and signaling pathways. Although the involvement of these signaling pathways in mediating the senescence process is unclear, constitutive activation of molecules along the Raf-1/mitogen-activated protein kinase-signaling cascade, including Raf-1, mitogen-activated protein kinase kinases, and mitogen-activated protein kinases, have been demonstrated to induce premature senescence through activating p16\textsuperscript{INK4a} and p53 (35, 36).

PI3Ks are a group of lipid kinases that catalyze the specific phosphorylation of the inositol ring of phosphoinositides at position 3 (37) and are involved in a variety of cellular responses, including cell growth, survival, metabolism, differentiation, cytoskeletal organization, and membrane trafficking (38). Several nematode genes, including \textit{age-1}, \textit{daf-2}, \textit{akt-1}, \textit{akt-2}, and \textit{daf-16}, shown to affect the life span of \textit{Caenorhabditis elegans}, have been identified to encode homologues of molecules making up the PI3K signal transduction pathway. For instance, \textit{age-1} is a nematode homologue of the p110 subunit of PI3K; DAF-2 encodes a member of the insulin/insulin-like growth factor 1 receptor family, which generally signals through PI3K; AKT-1 and AKT-2 are nematode homologues of mammalian Akt/protein kinase B, which commonly acts downstream of PI3K; DAF-18 encodes the phosphatase and tensin homologue deleted from chromosome 10, which is an antagonist of PI3K activity, by removing the 3-phosphate from 3-phosphoinositides; and DAF-16 is a member of the forkhead/winged-helix family of transcriptional regulators (39). These findings from \textit{C. elegans} strongly suggested that the PI3K signal transduction pathway has a role in mediating senescence signals. Apart from an isolated study showing that inhibitors of PI3Ks can shorten the life span of the human diploid fibroblast cell line WI-38 (40), the role of PI3Ks in mediating senescence in mammalian cells has not been investigated. In the present study, we explore the role of PI3Ks in regulating cell proliferation and senescence as well as the mechanisms involved.

**EXPERIMENTAL PROCEDURES**

**MEFs Isolation and Cell Culture**—Wild-type mice and mice with deletion of \textit{Ink4a} /\textit{ARF} (41), p27\textsuperscript{kip1} (42), or p53 (43) genotypes were maintained at the animal facilities of the Imperial College (London) and Centro Nacional de Biotecnologia (Madrid). Primary mouse embryo fibroblasts (MEFs) were isolated from day 13.5 embryos derived from the corresponding colonies of wild-type or gene "knock-out" mice as described previously (45). Briefly, each embryo was dispersed and trypsinized for 20 min at 37 °C, and the resultant cells were grown for 1 day in a 10-cm diameter tissue culture plate. The cells were then replated onto a 15-cm dish and allowed to grow for 2 days. These cells, designated passage number 0 cells, were stored in liquid nitrogen for later use. MEFs derived from \textit{ARF}−/− mice were kindly provided by Dr. Charles Sherr. The MEFs were cultured and passaged as described previously (46). Briefly, 10°6 cells were replated every 3 days on 10-cm plates. MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and 100 μg/ml penicillin/streptomycin. For kinase inhibitor treatment, fibroblasts were grown to 60% confluence and the tissue culture medium changed before addition of inhibitors. Cells were treated for 24-48 h with PD98059 (50 μM; Calbiochem) or LY294002 (25 μM; Calbiochem) or Wortmannin (10 nM; Calbiochem) unless specified otherwise.

**Western Blot Analysis and Antibodies**—Western blot cell extracts were prepared by lysing cells with a cell volume of lysis buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM MgCl\textsubscript{2}, 5 mM EDTA (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 5 mM sodium orthovanadate) packed three times on ice for 20 min. The protein yield was quantified using the Bio-Rad DC protein assay kit. Samples corresponding to 50 μg of lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and recognized by appropriate antibodies. The antibodies against p15 (M-20), p16\textsuperscript{INK4a} (M-156), p18\textsuperscript{INK4c} (M-168), p19\textsuperscript{INK4a} (M-167), p21\textsuperscript{Cip1} (M-19), p53\textsuperscript{ARF} (M-20), p107 (C-18), DAF-2 (M-2), p27\textsuperscript{kip1} (R25020), p21\textsuperscript{Cip1} (M-25), DAF-18 (M-21), and cyclin E (M-20) were purchased from Santa Cruz Biotechnology. Anti-p19\textsuperscript{ARF} (R562) antibody was purchased from ABCam. Anti-p130 (anti-PR2) and anti-p27\textsuperscript{kip1} (R25020) monoclonal antibodies were acquired from Transduction Laboratories. The antibodies were detected using horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Dako) and visualized by the enhanced chemiluminescent (ECL) detection system (Amersham Pharmacia Biotech).

**Immunoprecipitation and AKT Kinase Assays and Immunodepletion**—

For immunoprecipitation and CDK2 kinase assays, cell collected were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1 mM MgCl\textsubscript{2}, 5 mM EDTA (pH 8.0), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride. Protein lysates (100 μg) were then incubated with 5 μg of indicated antibodies conjugated to agarose beads for 2 h at 4 °C. At 4 °C, 50% protein A-Sepharose beads (20 μl; Amersham Pharmacia Biotech) in lysis buffer were added and the mixture was incubated for an additional 2 h. The anti-CDK2 immunoprecipitates were then washed substantially and resuspended in 20 μl of kinase buffer (20 mM Tris-HCl (pH 8.0), 10 mM MgCl\textsubscript{2}, 1 mM EDTA, 1 mM dithiothreitol), supplemented with 2.5 μg of histone H1 (Sigma) and 10 μCi of [γ-32P]ATP (3000 Ci/mmol; Amersham Pharmacia Biotech). Reaction mixtures were incubated for 15 min at room temperature, and the phosphorylated histone H1 was resolved on 10% SDS-polyacrylamide gel electrophoresis. The gels were then dried and exposed to x-ray films. For immunodepletion experiments, two extra immunoprecipitations were performed with the anti-p27\textsuperscript{kip1} antibody (R25020).

**Retroviral Infections**—Phoenix cells (5 × 10°6) were plated in a 10-cm dish, incubated for 24 h, and then transfected by calcium phosphate precipitation (47) with 20 μg of the p27\textsuperscript{kip1}-expressing retroviral plasmid, pLPC-h p27\textsuperscript{kip1}, or the empty control vector, pLPC (16 h at 37 °C) (30). After 48 h, the virus-containing medium was filtered (0.45-μm filter; Millipore) and supplemented with 4 μg/ml Polybrene. Recipient cells were plated the night before the infection at 8 × 10°6 cells per 10-cm dish. Cells were maintained by calcium phosphate transfection for 24 h, washed with fresh medium, and then incubated for 24 h. The next day, cells were treated with 5 μg/ml sodium metaperiodate. Cells were then washed substantially and resuspended to the target cell density, and plates were centrifuged at 1 h at 1500 rpm and incubated at 37 °C overnight. Infected cells were selected 16 h later by incubating the cell population with medium containing 2 μg/ml puromycin. For AFX and p27\textsuperscript{kip1} transduction, MEFs were infected with 20 μg of retroviral plasmid, pBabe-p27\textsuperscript{kip1}, or pBabe-AFX (48) or the empty control vector pBabe-puro for 16 h at 37 °C.

**Cell Cycle Analysis**—Cell cycle analysis was performed by combined
LY294002 Arrests Early Passage Mouse Embryo Fibroblasts at G1 Phase of the Cell Cycle—A previous report has described that the specific PI3K inhibitor LY294002 induces a senescence-like growth arrest in human diploid fibroblasts (40). To further define the nature of this LY294002-induced cell cycle arrest and to explore the underlying molecular mechanism involved, we studied the effects of LY294002 on the proliferative activity of primary mouse fibroblasts. To this end, asynchronous exponentially growing early passage (<2) embryo fibroblasts plated at low cell density were treated with various doses of LY294002. The proliferative activity was monitored by cell count and by flow cytometric analyses of BrdUrd and propidium iodide incorporation. The growth curves (Fig. 1A) demonstrated that, while the untreated population continued to grow exponentially over the time course, there was a significant decline in proliferation rates in the LY294002-treated cells. At 25 μM or higher concentrations of LY294002, complete growth arrest was evident by 24 h. Similar results were observed in the flow cytometric analyses (Fig. 1B). Again, ≥25 μM LY294002 was sufficient to induce a noticeable diminution in BrdUrd uptake, suggesting these concentrations of LY294002 caused a significant decrease in cells entering S phase (Fig. 1B). Both the BrdUrd and propidium iodide staining indicated that the LY294002-treated cells were arrested predominantly at the G1 phase of the cell cycle. These dose-dependent proliferation results also showed that, under our experimental conditions, 25 μM LY294002 could induce cell cycle arrest by 24 h but did not trigger noticeable cell death. This concentration of LY294002 has also been demonstrated previously to selectively inhibit PI3K activity in vivo (49) and, therefore, is used in the rest of this study for treating mouse embryo fibroblasts.

Up-regulation of p27Kip1 Is Associated with LY294002-induced Senescence-like Arrest—It has been shown previously that replicative senescent cells display “flat cell” morphology, irreversibly arrest during the G1 phase of the cell cycle, and express increased levels of p16INK4a, p19ARF, and p53 and augmented activity of p53 (5–8, 50). To further establish that LY294002 could induce cellular senescence in mouse embryo fibroblasts, we investigated the cell morphology and expression of these senescence markers in LY294002-arrested cells. The ability of these LY294002-arrested fibroblasts to re-enter DNA synthesis after mitogenic stimulation was also assayed. To also confirm that the senescence induced by LY294002 was a result of selective blockade of PI3K activity, the effects of another specific inhibitor of PI3K, Wortmannin (51) was also investigated. As a control, an inhibitor (PD98059) (52) of another cellular kinase, mitogen-activated protein kinase kinase 1, which is important for cell proliferation, was also included in this study. Examination of the fibroblasts 24 h after PI3K inhibitor treatment indicated that the proliferation was ar-
rested (Fig. 2A) and that they started to acquire enlarged and flat cell morphology after either LY294002 or Wortmannin treatment (data not shown). There was no notable cell cycle arrest detected in the PD98059-treated fibroblasts (Fig. 2A). 5 days after LY294002 or Wortmannin treatment, almost all cells adopted enlarged and flat cell morphology (Fig. 2B). Similar to the untreated cells, no prominent change in cell morphology was observed in the PD98059-treated cells (Fig. 2B). Moreover, the great majority of these LY294002- or Wortmannin-treated cells remained arrested (>5 days) and did not re-commence DNA synthesis in the presence of mitogens (Fig. 2A). These results suggested that down-regulation of PI3K by either LY294002 or Wortmannin could induce unscheduled senescence-associated cell cycle arrest. Moreover, the propidium iodide- and BrdUrd-labeling experiments also showed that the LY294002-induced growth arrest is irreversible, because these

**FIG. 2.** Inhibition of PI3Ks induces permanent and irreversible cell cycle arrest and morphological changes. A, effects of kinase inhibitors on cell cycle progression of MEFs. Low passage MEFs were untreated or incubated with either LY294002, Wortmannin, or PD98059 in the presence of 10% FCS. At the times indicated, the MEFs were BrdUrd-labeled and harvested for FACS analysis as described in Fig. 1. B, effects of kinase inhibitors on cell morphology of MEFs. Photographs of MEFs untreated or treated with either LY294002, Wortmannin, or PD98059 in the presence of 10% FCS after 5 days. The non-cell cycle-arrested MEFs were split to achieve comparable cell density with the drug-arrested cells. C, ability of LY294002 to induce irreversible cell cycle arrest. MEFs were rendered quiescent in 0.5% FCS for 48 h in the presence or absence of LY294002. The serum-arrested cells were then either retained in 0.5% FCS or stimulated with 10% FCS for 24 h before labeling with BrdUrd prior to FACS analysis.

**FIG. 3.** Effects of kinase inhibitors on the expression of cell cycle inhibitors. A, Western blot analysis of cell cycle inhibitors after either LY294002 (LY), Wortmannin (W), or PD98059 (PD) treatment. Cell extracts were prepared from low passage MEFs 24 h after incubated with either LY294002, Wortmannin, or PD98059, in the presence of 10% FCS. The cell extract from untreated cycling MEFs was included as a control. The expressions of p15INK4b, p16INK4a, p19INK4d, p21Cip1, p53, and p19ARF were examined by Western blotting (p18INKc and p57Kip2 expressions were undetectable). B, dose-dependent expression of p27Kip1 and p21Cip1 in LY294002-treated cells. MEF lysates harvested in parallel with the dose-dependent cell cycle experiment described in Fig. 1B were Western-blotted with anti-p27Kip1 and -p21Cip1 antibodies. C, expression of p27Kip1 in MEFs devoid of p16INK4a, p21Cip1, p53, or p19ARF before and after LY294002 treatment.
LY294002-arrested cells did not re-commence DNA synthesis even after mitogenic stimulation (Fig. 2C). In contrast, the control, serum-starved cells re-entered S phase 24 h after stimulation with serum (Fig. 2C).

In contrast to previous findings with replicative senescent cells, Western blot analysis of these kinase inhibitor-treated cells indicated that, surprisingly, neither p16INK4a, p21Kip1, p19ARF, nor p53 accumulated after either LY294002 or Wortmannin treatment (Fig. 3A). In marked contrast to replicative senescence, these proteins declined in their expression levels after LY294002 and Wortmannin treatment, implying that these cell cycle inhibitors were unlikely to be involved in this LY294002- or Wortmannin-induced cell cycle arrest. The expression levels of these proteins were not greatly affected by the inhibitor PD98059. These results also implied that the LY294002- or Wortmannin-induced senescence-like cell cycle arrest is not regulated by exactly the same negative cell cycle regulatory molecules that control replicative senescence. To understand in greater detail the molecular mechanisms involved in this senescence-like cell cycle arrest, we examined the expression levels of other CKIs (Fig. 3A). The subsequent results indicated that p27Kip1 increased in expression levels in response to either LY294002 or Wortmannin treatment while other CKIs, including p15INK4b and p19INK4d, decreased in levels after the administration of either LY294002 or Wortmannin. Consistent with a previous observation (53), we did not detect significant levels of p57Kip2 in primary mouse embryo fibroblasts. In the majority of cases, the expression levels of these cell cycle regulatory proteins studied are not influenced by PD98059 treatment (Fig. 3A). It is noteworthy that the dosage of PD98059 used could induce apoptosis in a B-cell line (WEHI 231) in a parallel experiment (data not shown).

To explore further the relationship between this LY294002-induced up-regulation in p27Kip1 expression and the G1 cell cycle arrest, we assessed the effect of different doses of LY294002 on the expression levels of p27Kip1 and on cell cycle arrest. Low passage cycling normal fibroblasts were treated with various doses of LY294002 in parallel with those used for earlier fluorescence-activated cell sorter (FACS) analysis illustrated in Fig. 1A, and the expression of p27Kip1 was analyzed by Western blotting (Fig. 3B). For comparison, the expression levels of p21Cip1, another member of the WAF/KIP family of CKIs, was also examined. The Western blotting results showed that 25 μM is the minimal dose level of LY294002 required to trigger the up-regulation of p27Kip1 expression. Significantly, this LY294002 dose corresponds with that required to trigger G1 cell cycle arrest (Fig. 1), suggesting that the LY294002-induced p27Kip1 expression is functionally related to this cell cycle arrest. It is notable that the level of p21Cip1 expression is again inversely correlated with the dose of LY294002. LY294002-induced Accumulation of p27Kip1 Is Independent of p16INK4a, p21Cip1, p53, and p19ARF—To better understand this cell cycle arrest-associated accumulation of p27Kip1 after LY294002 treatment, we treated mouse embryo fibroblasts isolated from mice devoid of negative cell cycle regulators previously shown to accumulate during replicative senescence and examined the expression of p27Kip1 using Western blotting after 24 h (Fig. 2C). As in wild-type cells, the p27Kip1 was found to accumulate after LY294002 treatment in the fibroblasts that are deficient of p16INK4a, p21Cip1, p53, and p19ARF, respectively. This observation confirmed our previous results and suggested further that the accumulation of p27Kip1 in response to LY294002 is independent of other tumor suppressors, including p16INK4a, p21Cip1, p53, and p19ARF, previously shown to be associated with replicative senescence.

The LY294002-induced Down-regulation of CDK2 Activity Is
levels of CDK2 kinase complexes are “free” of p27 Kip1 in the absence of LY294002. We next measured the level of CDK2-associated kinase activity using histone H1 as a substrate. The immunoprecipitation-kinase assays showed that the CDK2-containing kinase complexes were inactive in LY294002-treated fibroblasts, whereas the CDK2-dependent kinase activity from untreated cells remained high (Fig. 4A). When the immunoprecipitated proteins were subjected to Western blotting analysis, p27Kip1 in the CDK2 complexes was demonstrated to increase after LY294002 treatment, indicating increased p27Kip1 binding to CDK2 in response to LY294002.

We next used anti-p27Kip1 antibodies to immunodeplete proteins binding to p27Kip1 from low passage cycling fibroblasts (Fig. 4B). The immunodepletion procedure removed almost all CDK2 proteins from the LY294002-treated cell lysate when compared with the input. In contrast, significant levels of CDK2 remained in the untreated control lysates after the immunodepletion procedure. These results indicated that the majority, if not all, of the CDK2 complexes are associated with p27Kip1, following LY294002 treatment, whereas significant levels of CDK2 kinase complexes are “free” of p27Kip1 in the absence of LY294002.

Ecotropic Expression of AFX Induces Up-regulation of p27Kip
Expression, Down-regulation of CDK2, Cyclin E-associated Kinase Activity, and a Senescence-like Cell Morphology in Primary Mouse Fibroblasts—Protein kinase B/Akt, an important downstream target of PI3K, directly phosphorylates and thereby inactivates the Forkhead family of transcription factors, including AFX, FKHR, and FKHR-L1 (54–58). A recent study demonstrated that AFX could induce G1 cell cycle arrest through inducing the expression of p27Kip1 at the transcriptional level (48). It is therefore likely that the PI3K/Akt pathway modulates the senescence-related cell cycle arrest through AFX and p27Kip1. To test this idea, we transduced low passage wild-type MEFs with empty retroviruses or retroviruses expressing either p27Kip1 or AFX (48). The results demonstrated that both the p27Kip1- and AFX-transduced MEFs irreversibly arrested growth (data not shown) and demonstrated “flat and enlarged” cell morphology, whereas the controls showed no sign of inhibited cell growth or abnormal morphology (Fig. 6A). Moreover, there was a drastic down-regulation of CDK2-associated kinase activity in the p27Kip1- and AFX-transduced MEFs but not in the control (Fig. 6B). Most importantly, the expression of p27Kip1 was induced significantly in both the p27Kip1- and AFX-transduced MEFs compared with the control (Fig. 6B). These results strongly suggested that inactivation of the PI3K/Akt pathway by ectopic expression of AFX induced up-regulation of p27Kip1 expression, down-regulation of CDK2-associated kinase activity, and senescence-like cell cycle arrest.

p27Kip1 Null Fibroblasts Arrested Growth after LY294002 Treatment—To further prove that p27Kip1 is important in mediating the cell cycle arrest induced by LY294002, we treated embryo fibroblasts derived from p27Kip1 null mice with various doses of LY294002 and monitored their growth rates. To our surprise, the p27Kip1−/− fibroblasts also underwent complete or partial growth arrest following LY294002 treatment (Fig. 7). Nevertheless, it is notable that at lower concentrations, e.g. 12.5 μM, LY294002 was more effective in arresting growth in wild-type than in p27Kip1−/− fibroblasts. Based on our earlier results that LY294002 induced the up-regulation of p27Kip1 and down-regulation of CDK2 activity, we therefore investigated whether the CDK2 activity is also inactivated following LY294002 treatment in p27Kip1−/− cells. The results indicated that immunoprecipitated CDK2 activity was almost completely repressed at higher LY294002 concentrations (e.g. 25 μM) in both wild-type and p27Kip1−/− cells. Interestingly, at lower concentrations (e.g. 12.5 μM), even though the CDK2 activity was completely inactivated in normal MEFs (Fig. 7), the inhibition in the p27 null counterparts was only partial. These findings indicated that p27Kip1 had a definitive role in mediating the senescence-related growth arrest induced by LY294002 and suggested that another CKI family member(s) or an unrelated protein functionally compensated for the loss of p27Kip1 in p27Kip1 null fibroblasts.

p130 Is Up-regulated upon LY294002 Treatment and Can Compensate for p27 Deficiency—To identify the mechanism for this LY294002-induced cell cycle arrest and repression of CDK2 activity and detect possible compensatory changes in expression of other CDK inhibitors, we examined the expres-
pression of both p107 and CDK2 declined in both wild-type and the p27 null fibroblasts. The Western blot results indicated that p130 expression was induced, whereas the expression of p130, p15INK4a, p16INK4a, p19ARF, and p53 were all down-regulated after LY294002 treatment, indicating that these proteins are unlikely to be involved in repressing the CDK2 activity (Fig. 8). Recent reports suggested that the pocket proteins p107 and p130 could bind to and repress CDK2 (53, 59, 60). We therefore also assayed for p107, p130, and CDK2 expression in LY294002-treated wild-type and the p27 null fibroblasts. The Western blot results indicated that p130 expression was induced, whereas the expression of both p107 and CDK2 declined in both wild-type and p27−/− fibroblasts after LY294002 treatment (Fig. 8). Nevertheless, the reduction in CDK2 expression in both wild-type and p27−/− fibroblasts is insufficient to account for the almost, if not complete, depletion of CDK2 activity in these LY294002-treated cells. The observation that p130 was up-regulated in response to LY294002 in the p27 null fibroblasts suggested that it could potentially substitute for p27 in binding to and thus repressing CDK2 activity in p27 null fibroblasts.

To test this possibility and to identify the LY294002-induced CDK2 inhibitory protein, we immunoprecipitated CDK2-containing complexes from LY294002-stimulated cells and identified the associated proteins by immunoblotting (Fig. 8). The results indicated that no detectable level of p21Cip1 is found associated with CDK2 in wild-type fibroblasts before and after LY294002 treatment. Although p21Cip1 was detected in CDK2 complexes in untreated cycling p27Kip1−/− fibroblasts, no p21Cip1 was detected in LY294002-treated p27Kip1−/− cells. Although only very low levels of p57Kip2 were detected in association with CDK2 in both normal and p27Kip1−/− cycling fibroblasts (only detectable after prolonged exposure), the level of p57Kip2 declined further after LY294002 treatments. These findings suggested that it is unlikely that either p21Cip1 or p57Kip2 play a predominant role in repressing CDK2 activity in both wild-type- and p27Kip1−/− deficient fibroblasts after LY294002 treatment. It is notable that the level of p57Kip2 was undetectable by direct immunoblotting but is probably significantly enriched through immunoprecipitation with CDK2.

We next assessed the binding of CDK2 to p130 before and after LY294002 treatment. The result illustrated that in wild-type fibroblasts there is an increase of p27Kip1 associating with CDK2 after LY294002 treatment. However, no p130 was detected binding to CDK2 before LY294002 treatment in both normal and p27Kip1 null fibroblasts. In p27Kip1 null fibroblast, there is a marked increase in p130 binding to CDK2 after LY294002 treatment, whereas in wild-type fibroblasts p130 binding to CDK2 was frequently undetectable or present at very low levels. Our results, taken together with previous findings, suggest that p130 can compensate for the loss of p27Kip1 through binding to and thereby repressing CDK2 activity in LY294002-treated cells.

DISCUSSION

In the present study, we examined the effects of inactivation of PI3Ks in primary mouse embryonic fibroblasts, a cell system commonly used for studying cellular senescence in vitro. LY294002 and Wortmannin are two structurally unrelated but selective inhibitors of PI3Ks and have been used extensively for studying the effects of PI3K. Using concentrations of these inhibitors that are specific for PI3K (49, 51), we showed that inhibition of PI3K could cause irreversible and permanent cell cycle arrest at the G1 phase of the cell cycle. This result is in agreement with previous studies showing that PI3K activity is important for G1 to S transition in NIH 3T3 fibroblasts, because microinjection of PI3K-neutralizing antibodies inhibited mitogen-induced DNA synthesis (61, 62). We further showed here that these LY294002-treated cells adopt a flat and enlarged morphology, which is characteristic of senescent cells. These findings confirmed and extended the results from a previous study demonstrating that LY294002 induced senescence-like cell cycle arrest and expression of neutral β-galactosidase activity in the human WT-38 cell line (40).

Although replicative senescence has previously been shown to be associated with an increase in expression of tumor suppressors p16INK4a, p21Cip1, p19ARF, and p53 (5–8, 26), we demonstrated here that the premature senescence induced by LY298004 is accompanied by the down-regulation of p16INK4a, p21Cip1, p19ARF, and p53 but the accumulation of p27Kip1. The present results are reminiscent of a previous report demon-
p27Kip1 Mediates Senescence-like Arrest Induced by PI3K Inhibitors

Recent studies have shown that other kinases, including PI4K (65–68), are also susceptible to inhibition by both LY294002 and Wortmannin at concentrations in excess of those required for inhibition of PI3K. To further confirm our results and to gain further insight into the mechanism by which the PI3K modulates p27Kip1 expression and senescence-related cell cycle arrest, we ectopically expressed AFX, a downstream negative regulator of the PI3K/Akt pathway to inhibit the PI3K/Akt pathway. The results demonstrated that, similar to LY294002 and Wortmannin treatment, AFX induced growth arrest and flat and enlarged cell morphology in low passage MEFs. Moreover, ectopic expression of AFX caused a drastic down-regulation of CDK2-associated kinase activity, accompanied by up-regulation in p27Kip1 expression. Taken together, these results suggested that the kinase inhibitors LY294002 and Wortmannin arrest cell growth and induce a senescence-like phenotype, at least partially, through inhibition of PI3K and protein kinase B/Akt, activation of the forkhead protein AFX, and up-regulation of p27Kip1 expression.

In a similar approach, we also demonstrated that expression of a constitutively active Akt could suppress the up-regulation of p27Kip1 expression. However, this constitutively active Akt did not abrogate the cell cycle arrest induced by LY294002 (data not shown). These results probably reflect the fact that either LY294002 or PI3K can target multiple cell cycle regulatory signaling cascades other than the PI3K/Akt/AFX pathway. Nevertheless, our results firmly implicate the PI3K/Akt/AFX pathway as one of the principal mediators of senescence-like cell cycle arrest and p27Kip1 expression in normal fibroblasts. The induction of p27Kip1 expression by AFX has been demonstrated to be mediated at transcriptional but not post-translational level (48). Interestingly, we have obtained data showing that LY294002 could also enhance the stability of the p27Kip1 protein in mouse embryo fibroblasts (data not shown). However, it is unclear whether this involves the AFX-dependent pathways and needs further investigation.

Although it is evident that the LY294002-induced senescence-related cell cycle arrest is mediated through up-regulation of p27Kip1, our results revealed that p27Kip1-deficient fibroblasts still underwent senescence-related cell cycle arrest following LY294002 treatment. Nevertheless, the findings that the p27Kip1 null counterparts are only partially sensitive to the low doses of LY294002, which completely arrested growth and CDK2 activity in normal MEFs, further indicate that p27Kip1 is functionally involved in the LY294002-mediated inactivation of CDK2 activity and cell cycle arrest. On the other hand, the observation that CDK2 kinase activity is wholly or partially inactivated in LY294002-treated p27Kip1 null fibroblasts also suggests that another CDK2 inhibitor compensates for the absence of p27Kip1. However, it is unlikely that other members of the CIP/KIP families of CDK inhibitors, including p21Cip1 and p57Kip2, are responsible for inactivating the CDK2 activity in p27Kip1 null fibroblasts after LY294002 treatment, because their expression levels are either down-regulated or undetectable. Moreover, the observation that the amounts of p21Cip1 and p57Kip2 binding to CDK2 decreased to almost undetectable levels after LY294002 treatment suggested further that the role of p27Kip1 in repressing CDK2 activity is not substituted by either p21Cip1 or p57Kip2 in p27Kip1 null fibroblasts. In our attempt to explore the mechanism for CDK2 repression and thus cell cycle arrest in p27Kip1 null cells, we detected binding of p130 to CDK2 after LY294002 treatment of p27Kip1−/− fibroblasts, which is associated with an increase in p130 expression and down-regulation of CDK2-dependent kinase activity. Intriguingly, the p130 binding to CDK2 is either undetecatable or present at low levels in either LY294002-treated or untreated
wild-type fibroblasts, although the expression of p130 is evident in these cells. The observation that the level of p130 associated with CDK2 is undetectable in untreated cells but accumulates in response to LY294002 treatment in both wild-type and p27Kip1 null fibroblasts shows that p130 has a physiological role in repressing CDK2 activity and, therefore, cell cycle progression in p27−/− cells.

In conjunction with previous results showing that direct binding of p130 to CDK2 complexes inhibits its catalytic activity both in vitro and in vivo (53, 60), our findings suggest that p130 functionally compensates for the loss of p27Kip1 after LY294002 treatment to induce cell cycle arrest in p27−/− deficient fibroblasts and lends further support to a recent report showing that p130 can functionally compensate for the loss of p27Kip1 to induce cell cycle arrest in p27−/− fibroblasts after serum deprivation (53). It is also notable that the amount of p130 binding to CDK2 in wild-type cells after LY294002 treatment is either undetectable or present at low levels compared with p27Kip1−/− cells, even though comparable levels of p130 are expressed in both LY294002-treated wild-type and p27Kip1−/− fibroblasts. This observation reinforces further a previous suggestion that p130 is subordinate to p27Kip1, in terms of CDK2 binding and inhibition (53). Consistent with this conclusion is our finding that almost all of the CDK2 complexes can be eliminated by p27Kip1 depletion in wild-type LY294002-treated fibroblasts. The ability of p130 to functionally compensate for the absence of p27Kip1 in mediating senescence reflects that multiple layers of compensatory mechanisms exist to enforce the senescence program, and if one pathway should become inactive, a safeguarding mechanism is in place to execute the senescence program.

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