Efficient Down-Regulation of Cyclin A-Associated Activity and Expression in Suspended Primary Keratinocytes Requires $p21^{Cip1}$

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Abstract

When suspended in methylcellulose, primary mouse keratinocytes cease proliferation and differentiate. Suspension also reduces the activity of the cyclin-dependent kinase cdk2, an important cell cycle regulatory enzyme. To determine how suspension modulates these events, we examined its effects on wild-type keratinocytes and keratinocytes nullizygous for the cdk2 inhibitor $p21^{Cip1}$. After suspension of cycling cells, amounts of cyclin A (a cdk2 partner), cyclin A mRNA, and cyclin A-associated activity decreased much more rapidly in the presence than in the absence of $p21^{Cip1}$. Neither suspension nor $p21^{Cip1}$ status affected the stability of cyclin A mRNA. Loss of $p21^{Cip1}$ reduced the capacity of suspended cells to growth arrest, differentiate, and accumulate $p27^{Kip1}$ (a second cdk2 inhibitor) and affected the composition of E2F DNA binding complexes. Cyclin A-cdk2 complexes in suspended $p21^{+/+}$ cells contained $p21^{Cip1}$ or $p27^{Kip1}$, whereas most of the cyclin A-cdk2 complexes in $p21^{-/-}$ cells lacked $p27^{Kip1}$. Ectopic expression of $p21^{Cip1}$ allowed $p21^{-/-}$ keratinocytes to efficiently down-regulate cyclin A and differentiate when placed in suspension. These findings show that $p21^{Cip1}$ mediates the effects of suspension on numerous processes in primary keratinocytes including cdk2 activity, cyclin A expression, cell cycle progression, and differentiation.

Introduction

Most mammalian cell types grow in an adhesion-dependent manner both *in vivo* and *in vitro*. Oncogenic transformation overrides the need for attachment for cell proliferation, and anchorage-independent growth correlates strongly with tumorigenicity. When deprived of a substratum, nontransformed cells typically arrest in G_1, and studies addressing this phenomenon have focused on the cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins (1). Complexes containing the D cyclins and cdk4, cyclin E and cdk2, and cyclin A and cdk2 become active in mid-G_1, in late G_1, and at G_1-S, respectively (2). All complexes phosphorylate the retinoblastoma (Rb) protein, an event required for S phase entry (3). When hyperphosphorylated by cdk4 and cdk2, Rb no longer functions as a transcriptional repressor, and numerous gene products (including many involved in DNA replication) are expressed (4, 5). Events that regulate the activity of cdk4 and cdk2 include the periodic synthesis and destruction of the cyclins. In addition, proteins such as $p21^{Cip1}$ and $p27^{Kip1}$ interact with and inhibit the activity of complexes containing cdk4 or cdk2 and their cyclin partners (6, 7). The overall abundance of $p21^{Cip1}$ and $p27^{Kip1}$ varies during the cell cycle, and sequestration of $p21^{Cip1}$ and $p27^{Kip1}$ by D cyclin-cdk4 complexes facilitates cdk2 activation.

Most notably, nontransformed cells do not express cyclin A, and consequently do not form cyclin A-cdk2 complexes, when cultured in suspension (8–18). Conversely, cyclin A is readily detectable in cells that grow in suspension as a result of enforced expression of E7, c-Myc, activated Ras, cyclin D1, or integrin-linked kinase (11, 13, 15–17, 19, 20). When ectopically expressed, cyclin A allows or contributes to the anchorage-independent proliferation of NRK, Rat 1a, and ER-1-2 fibroblasts (8, 11, 13). Collectively, these studies identify cyclin A as a key target of suspension-induced growth inhibitory pathways.

Transcriptional repression of the cyclin A promoter accounts (at least in part) for the absence of cyclin A from suspended cells (10, 14, 21, 22). Repression occurs when Rb sequesters a CCAAT-binding protein required for transactivation and/or when Rb or the Rb-related protein p107 interacts with a variant E2F site in the cyclin A promoter. In both instances, Rb and p107 lose their capacity to repress *cyclin A* gene transcription when hyperphosphorylated by cdk4 and cdk2. Suspension inhibits cyclin E-cdk2 activity in most cell types and cdk4 activity in some cell types; as a result, Rb and p107 are typically hypophosphorylated in suspended cells (9–12, 15–17, 19, 23, 24). Suspension does not alter the abundance of cyclin E or cdk2 or the assembly of cyclin E-cdk2 complexes; however, more cyclin E-cdk2 complexes contain $p21^{Cip1}$ or $p27^{Kip1}$ in suspended cells than in attached cycling cells (9, 19, 23, 24). $p21^{Cip1}$ and $p27^{Kip1}$ often accumulate in suspended cells, and in some instances, the absence of the D cyclins precludes the sequestration of $p21^{Cip1}$ and $p27^{Kip1}$ by D cyclin-cdk4 complexes (9, 10, 12, 16, 19, 20, 23, 24). Thus, suspension increases the availability of $p21^{Cip1}$ and $p27^{Kip1}$, which results

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in cyclin E-cdk2 inactivation, Rb hypophosphorylation, and ultimately, the repression of *cyclin A* gene transcription. Suspension may also inhibit cyclin A expression by processes other than those described above (9, 11).

Most previous studies examined the effects of suspension on cells stimulated from quiescence (8–16, 22, 23). In contrast, our studies (18) examined the effects of suspension on growing cells, which express active cyclin A-cdk2 complexes at the time of suspension. Our studies were performed on primary mouse keratinocytes, which cease proliferation and differentiate when placed in a semisolid medium (25). This system allows us to determine how suspension affects cyclin-CDK activity and how changes in cyclin-CDK activity affect both cell cycle progression and differentiation. We found that $p27^{Kip1}$ accumulated in suspended keratinocytes and interacted with cyclin A-cdk2 complexes and that amounts of cyclin A and cyclin A-associated activity declined (18). Suspension of cells in the presence of $p27^{Kip1}$ antisense oligonucleotides, and thus in the absence of $p27^{Kip1}$ accumulation, did not prevent the loss of cyclin A or cyclin A-associated activity or cell cycle arrest; however, the differentiation-specific marker keratin 1 (K1) was not induced.

On the basis of the results of our previous report (18), we suggest that $p27^{Kip1}$ promotes keratinocyte differentiation by actions unrelated to the inhibition of cdk2 activity. We also suggest that $p27^{Kip1}$ (or $p27^{Kip1}$ accumulation) contributes to but is not required for cdk2 inactivation and growth arrest in suspended cells. To identify additional mediators of these events, we examined the effects of suspension on keratinocytes lacking $p21^{Cip1}$. We show that efficient down-regulation of cyclin A and cyclin A-associated activity in suspended keratinocytes requires $p21^{Cip1}$ as do growth arrest, differentiation, and maximal accumulation of $p27^{Kip1}$. Determination of how suspension abrogates cell cycle controls in normal keratinocytes may be relevant to studies of cancer cells, which typically grow in suspension.

Results

Requirement for $p21^{Cip1}$ for Efficient Down-Regulation of Cyclin A, Cyclin A Messenger RNA, and Cyclin A-Associated Activity in Suspended Keratinocytes

To assess the need for $p21^{Cip1}$ for suspension-induced decreases in cyclin A expression and cyclin A-associated activity, primary keratinocytes derived from wild-type and $p21^{Cip1}$-null mice were suspended in methylcellulose and harvested at various times thereafter. At the time of suspension, keratinocytes in both populations were actively cycling (data not shown). Amounts of cyclin A were determined by Western blotting of cell extracts, and kinase activity was assayed in cyclin A immune complexes using histone H1 as substrate. Although cyclin A associates with both cdk2 and the mitotic CDK cdc2 (26), $p21^{Cip1}$ selectively inhibits the activity of cdk2-containing complexes (27). It is noted that $p21^{Cip1}$ preferentially interacts with cyclin A-cdk2 complexes rather than cyclin A or cdk2 alone (27).

Cyclin A was expressed and cyclin A-associated kinases were active in attached $p21^{+/+}$ keratinocytes and, to a greater extent, in attached $p21^{+/+}$ keratinocytes (Fig. 1, A and B, *lanes 1* and *6*). Cyclin A-associated activity decreased within 3 h of suspension of $p21^{+/+}$ keratinocytes and was barely detectable at 6 h and times thereafter (Fig. 1B, *lanes 1–5*), even when the blots were overexposed (*lanes 11–15*). Amounts of cyclin A declined more slowly; cyclin A abundance was modestly reduced 6 h after suspension and substantially reduced 12 h after suspension of $p21^{+/+}$ cells (Fig. 1A, *lanes 1–5*). Importantly, cyclin A was detectable in cells suspended for 6–12 h, whereas cyclin A-associated activity was not. This indicates that factors other than (or in addition to) loss of protein account for loss of activity during this time period.

In contrast to $p21^{+/+}$ keratinocytes, $p21^{-/-}$ keratinocytes expressed comparable amounts of cyclin A or cyclin A-associated activity whether attached to plates or suspended for 12 h (Fig. 1A, *lanes 6–10*, and Fig. 1B, *lanes 6–9*). Amounts of both, however, eventually declined (Fig 1B, *lane 10*, and data not shown). The data in Fig. 1, A and B, indicate that loss of $p21^{Cip1}$ greatly delays the suspension-induced down-regulation of cyclin A and cyclin A-associated activity in primary keratinocytes.

The effects of suspension on cyclin A mRNA abundance in $p21^{+/+}$ and $p21^{-/-}$ keratinocytes were similar to its effects on cyclin A protein abundance. After suspension, amounts of cyclin A mRNA decreased rapidly and substantially in $p21^{+/+}$ keratinocytes (Fig. 1C, *lanes 1–5*), but remained elevated in $p21^{-/-}$ keratinocytes for 12 h (*lanes 6–10*). When $p21^{+/+}$ and $p21^{-/-}$ keratinocytes were suspended in the presence of the mRNA synthesis inhibitor DRB, the cyclin A transcript decayed with similar kinetics in both populations (*lanes 11–18*). Thus, differences in cyclin A mRNA stability do not account for the differences in cyclin A mRNA abundance in suspended $p21^{+/+}$ *versus* $p21^{-/-}$ keratinocytes. Amounts of cyclin A mRNA also decreased with similar kinetics in $p21^{+/+}$ keratinocytes suspended in the presence (*lanes 11–14*) and absence (*lanes 2–5*) of DRB. By default, these data suggest that loss of $p21^{Cip1}$ prevents suspension-induced decreases in the activity of the cyclin A promoter. This premise is consistent with previous findings showing transcriptional repression of the *cyclin A* gene in nonadherent fibroblasts (10, 14, 21, 22).

To ensure that $p21^{Cip1}$ status accounts for the different expression patterns of cyclin A in wild-type *versus* $p21^{Cip1}$-null keratinocytes, we ectopically expressed $p21^{Cip1}$ in $p21^{-/-}$ keratinocytes and amounts of cyclin A were determined. In these experiments, $p21^{-/-}$ keratinocytes were infected with adenovirus without insert or adenovirus encoding human $p21^{Cip1}$ (Ad-$p21^{Cip1}$). Cells were suspended in methylcellulose 19 h after infection and harvested 19 h later. For comparative purposes, $p21^{+/+}$ keratinocytes were also examined (Fig. 2, *lanes 1* and *2*). The expression of human $p21^{Cip1}$ in $p21^{-/-}$ keratinocytes was verified by Western blotting (*lanes 4* and *7*). After suspension, amounts of cyclin A declined but were readily detectable in uninfected $p21^{-/-}$ keratinocytes and $p21^{-/-}$ keratinocytes infected with virus alone (compare *lanes 3*, *5*, and *6*). In contrast, cyclin A was not detected in $p21^{-/-}$ keratinocytes infected with Ad-$p21^{Cip1}$ and suspended for 19 h (*lane 7*). These findings show that nonadherent $p21^{-/-}$ keratinocytes gain the capacity to efficiently down-regulate cyclin A when supplied with $p21^{Cip1}$. It is unclear why Ad-$p21^{Cip1}$ more completely reduces cyclin A abundance in suspended keratinocytes (*lane 7*) than in attached keratinocytes (*lane 4*).

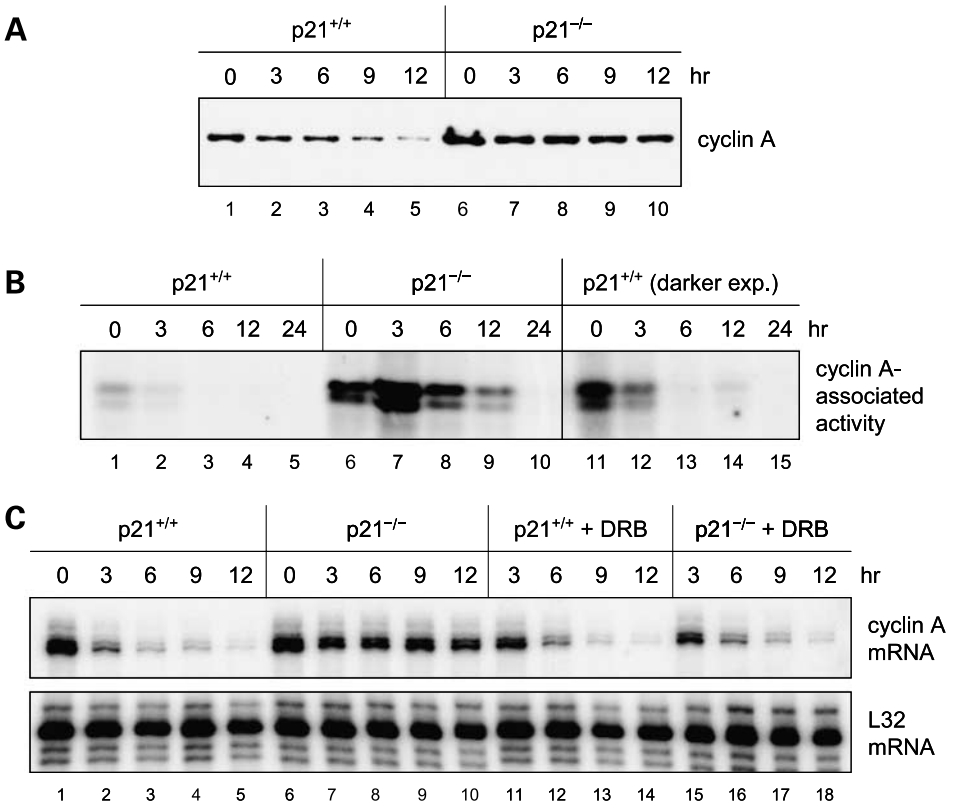

FIGURE 1. Amounts of cyclin A protein, mRNA, and associated activity in p21$^{+/+}$ and p21$^{-/-}$ keratinocytes suspended in methylcellulose. **A** and **B.** Proliferating p21$^{+/+}$ and p21$^{-/-}$ keratinocytes were detached from tissue cultures plates and suspended in methylcellulose as described in "Materials and Methods." Cells were harvested at the times indicated. **A.** Amounts of cyclin A were determined by Western blotting. **B.** Cell extracts were immunoprecipitated with antibody to cyclin A, and cyclin A immune complexes were assayed for kinase activity using histone H1 as substrate. *Lanes 1–10,* results of a single exposure of the autoradiogram. *Lanes 11–15,* darker exposure of *lanes 1–5*, respectively. The darker exposure allows normalization of the activities at time 0 for p21$^{+/+}$ (*lane 11*) and p21$^{-/-}$ keratinocytes (*lane 6*). **C.** Asynchronously cycling p21$^{+/+}$ and p21$^{-/-}$ keratinocytes were suspended in methylcellulose in the presence or absence of 100 μM 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) and harvested at the times indicated. Amounts of cyclin A mRNA and L32 mRNA (loading control) were determined by RNase protection assay.

Submaximal Accumulation of p27^{Kip1} in Suspended p21^{Cip1}-Null Keratinocytes

We have shown that amounts of p27^{Kip1} increase in suspended keratinocytes, as do amounts of p27^{Kip1}-associated cyclin A-cdk2 complexes (18). As shown here, loss of p21^{Cip1} prevents the rapid decline in cyclin A-associated activity in suspended cells. Together, these findings indicate that p27^{Kip1} contributes to cdk2 inactivation in suspended cells but does not compensate for the absence of p21^{Cip1}. As a possible explanation of this phenomenon, we examined p27^{Kip1} expression in p21$^{+/+}$ and p21$^{-/-}$ keratinocytes in methylcellulose.

Amounts of p27^{Kip1} increased substantially when p21$^{+/+}$ keratinocytes were placed in suspension (Fig. 3A); (18). Increases occurred within 6 h of suspension and amounts of p27^{Kip1} remained elevated for at least 24 h. p27^{Kip1} was less abundant in p21$^{-/-}$ keratinocytes than in p21$^{+/+}$ keratinocytes at 0 h (*i.e.*, before detachment) and did not accumulate appreciably in p21$^{-/-}$ keratinocytes suspended for 12 h; submaximal increases were observed at 24 h. Unlike protein, amounts of p27^{Kip1} mRNA were similar in attached and suspended p21$^{+/+}$ and p21$^{-/-}$ keratinocytes (Fig. 3B). These findings show that p21^{Cip1} elicits events that increase the

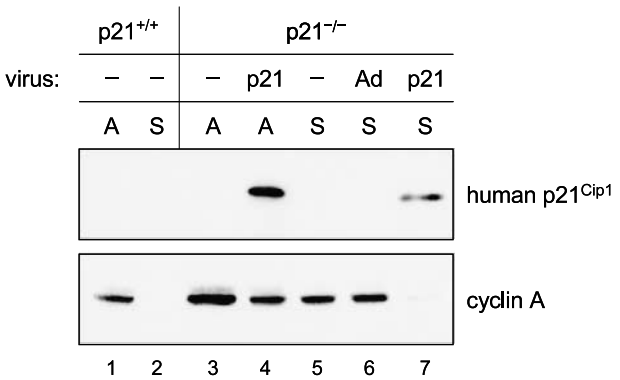

FIGURE 2. Amounts of cyclin A in suspended p21$^{-/-}$ keratinocytes ectopically expressing p21^{Cip1}. Proliferating p21$^{-/-}$ keratinocytes were infected with adenovirus without insert (*Ad*) or Ad-p21^{Cip1} (*p21*) at a multiplicity of infection (MOI) of 250. Cells were suspended in methylcellulose 19 h after infection and harvested 19 h later for determination of p21^{Cip1} and cyclin A abundance by Western blotting. Uninfected p21$^{+/+}$ and p21$^{-/-}$ keratinocytes were also suspended in methylcellulose for 19 h. The p21^{Cip1} antibody used for Western blotting recognizes ectopic (human) p21^{Cip1} but not endogenous (mouse) p21^{Cip1}. *A,* attached cells; *S,* suspended cells.

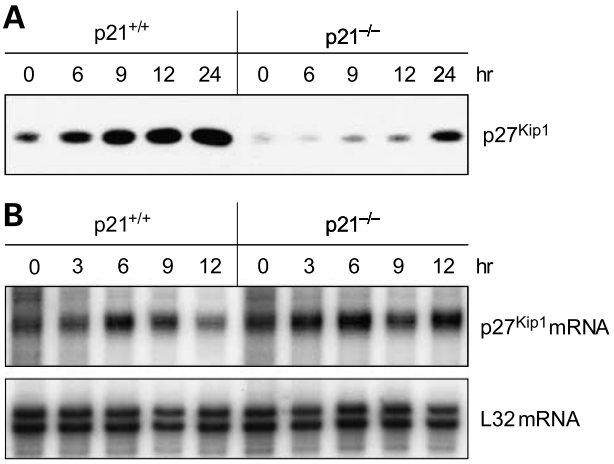

FIGURE 3. Amounts of $p27^{Kip1}$ in suspended wild-type keratinocytes. **A** and **B.** Exponentially growing $p21^{+/+}$ and $p21^{-/-}$ keratinocytes were suspended in methylcellulose and harvested at the times indicated. **A.** Amounts of $p27^{Kip1}$ protein were determined by Western blotting. **B.** Amounts of $p27^{Kip1}$ mRNA and L32 mRNA (loading control) were determined by RNase protection assay.

translation and/or stability of $p27^{Kip1}$ in both adherent and nonadherent keratinocytes. In $p21^{-/-}$ keratinocytes, amounts of $p27^{Kip1}$ are subnormal, and as a result, the potential capacity of $p27^{Kip1}$ to compensate for the absence of $p21^{Cip1}$ is attenuated. Similar to our findings, loss of $p21^{Cip1}$ reduced the accumulation of $p27^{Kip1}$ protein (but not of $p27^{Kip1}$ mRNA) in interleukin-treated astrocytoma cells (28).

Increased Association of CDK Inhibitors With Cyclin A Complexes in Suspended Wild-Type Keratinocytes

In contrast to $p27^{Kip1}$, $p21^{Cip1}$ did not accumulate in suspended $p21^{+/+}$ keratinocytes (Fig. 4A). In fact, $p21^{Cip1}$ decreased in abundance 6–9 h after suspension and was not detected at 24 h. Although total amounts of $p21^{Cip1}$ were constant for at least 6 h after suspension, amounts of $p21^{Cip1}$ not sequestered by D cyclin-cdk4 complexes (and thus available for interaction with cdk2 complexes) may increase. Consistent with this hypothesis, we have shown that amounts of cyclin D1 decrease after suspension of wild-type keratinocytes (18). To test this hypothesis, we determined the amounts of $p21^{Cip1}$-associated cyclin A and $p21^{Cip1}$-free cyclin A in $p21^{+/+}$ keratinocytes at various times after suspension. Cell extracts were immunoprecipitated with antibody to $p21^{Cip1}$, and $p21^{Cip1}$ immune complexes were Western blotted with antibody to cyclin A ($p21^{Cip1}$-associated cyclin A). $p21^{Cip1}$-immunodepleted extracts were immunoprecipitated and Western blotted with antibody to cyclin A ($p21^{Cip1}$-free cyclin A). Western blots using $p21^{Cip1}$ antibody confirmed that immunodepleted extracts contained little if any $p21^{Cip1}$ (data not shown).

Suspension of $p21^{+/+}$ keratinocytes for 3 or 6 h resulted in an increase in the amounts of $p21^{Cip1}$-associated cyclin A and a decrease in the amounts of $p21^{Cip1}$-free cyclin A (Fig. 4B, compare *lanes 2* and *3* with *lanes 7* and *8*). Thus, a greater percentage of cyclin A complexes contain $p21^{Cip1}$ in suspended keratinocytes than in attached keratinocytes. After 6 h, amounts of $p21^{Cip1}$-associated cyclin A declined (*lanes 4* and *5*), most likely because of reductions in $p21^{Cip1}$ abundance (see Fig. 4A). Although suspension reduced amounts of $p21^{Cip1}$-free cyclin A, it did not eliminate $p21^{Cip1}$-free cyclin A, at least for times up to 12 h (*lane 10*). On the other hand, little if any cyclin A remained in 12-h cell extracts immunodepleted of both $p21^{Cip1}$ and $p27^{Kip1}$ (Fig. 4C, *top panel*, *lane 8*). Comparison of the relative amounts of CDK inhibitor (CKI)-associated and CKI-free cyclin A at all time points shows that the ratio of associated to free increases progressively after suspension (Fig. 4C, *top panel*).

The data in Fig. 4 support our hypothesis that suspension-induced decreases in cyclin D1 result in the redistribution of $p21^{Cip1}$ from cyclin D1 complexes to cyclin A complexes and thus in the inhibition of cyclin A-cdk2 activity in wild-type keratinocytes. Redistribution of $p27^{Kip1}$ and/or increases in $p27^{Kip1}$ abundance also contribute to cyclin A-cdk2 inactivation in suspended $p21^{+/+}$ cells. Our studies also explain why cyclin A-cdk2 complexes are active in suspended $p21^{-/-}$ cells: most of these complexes lack $p27^{Kip1}$, as do most of the cyclin A-cdk2 complexes in attached $p21^{-/-}$ cells. This finding is consistent with our data showing subnormal amounts of $p27^{Kip1}$ in $p21^{-/-}$ keratinocytes (Fig. 3A).

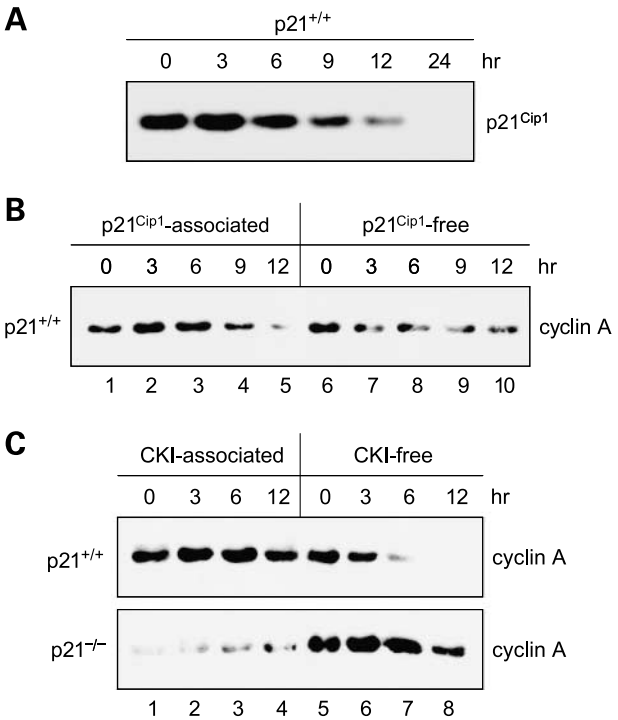

FIGURE 4. Association of cyclin A complexes with $p21^{Cip1}$ and $p27^{Kip1}$ in attached and suspended keratinocytes. **A.** Asynchronously cycling $p21^{+/+}$ keratinocytes were suspended in methylcellulose and harvested at the times indicated. Cell extracts were immunoblotted with antibody to mouse $p21^{Cip1}$. **B.** Cycling $p21^{+/+}$ keratinocytes were suspended in methylcellulose for the times indicated. *Lanes 1–4,* cell extracts were immunoprecipitated with antibody to $p21^{Cip1}$, and $p21^{Cip1}$ immune complexes were Western blotted with antibody to cyclin A. *Lanes 5–8,* after removal of $p21^{Cip1}$, cell extracts were immunoprecipitated with antibody to cyclin A, and cyclin A immune complexes were Western blotted with antibody to cyclin A. **C.** As in **B**, with the exception that cell extracts were immunoprecipitated with antibody to both $p21^{Cip1}$ and $p27^{Kip1}$ ($p21^{+/+}$) or with antibody to $p27^{Kip1}$ ($p21^{-/-}$).

Reduced Capacity of p21$^{-/-}$ Keratinocytes to Growth Arrest and Differentiate in Suspension

Although suspension typically arrests cells in G_1 (1), suspended primary mouse keratinocytes arrest in G_1, S, and G_2-M (29). To determine whether cell cycle arrest requires p21^{Cip1}, p21$^{+/+}$ and p21$^{-/-}$ keratinocytes attached to tissue culture plates received bromodeoxyuridine (BrdUrd) for 2 h to label S-phase cells. Cells were then suspended in methylcellulose in the absence of BrdUrd and harvested 12 h later, and the position of the BrdUrd-labeled cells in the cell cycle was determined by flow cytometry. The percentages of cells remaining in S phase 12 h after suspension were 52% and 31% for p21$^{+/+}$ and p21$^{-/-}$ populations, respectively (Fig. 5). Moreover, a greater percentage of p21$^{-/-}$ cells were in G_1 at this time than were p21$^{+/+}$ cells. These data suggest that p21$^{-/-}$ cells cycle for longer periods of time when placed in suspension than do p21$^{+/+}$ cells. In this respect, p21$^{-/-}$ keratinocytes mimic MK cells, which are immortalized mouse keratinocytes (30) and which express little if any p21^{Cip1} (data not shown).

To assess the need for p21^{Cip1} for differentiation, we placed p21$^{+/+}$ and p21$^{-/-}$ keratinocytes in suspension, and amounts of two differentiation markers, K1 (Fig. 6A) and loricrin (Fig. 6B), were determined. Both proteins increased in abundance after suspension of p21$^{+/+}$ keratinocytes. Increases in the abundance of loricrin were especially pronounced. Amounts of loricrin did not increase in suspended p21$^{-/-}$ keratinocytes, whereas amounts of K1 increased to a lesser extent and in a delayed manner. At 48 h, K1 was still much less abundant in p21$^{-/-}$ keratinocytes than in p21$^{+/+}$ keratinocytes (data not shown).

These findings show that efficient differentiation of primary keratinocytes requires p21^{Cip1}. To confirm that the differentiation-defective phenotype of p21$^{-/-}$ keratinocytes reflects the absence of p21^{Cip1}, p21$^{-/-}$ keratinocytes were infected with Ad-p21^{Cip1} and suspended in methylcellulose. Nineteen hours after suspension, Ad-p21^{Cip1}-infected p21$^{-/-}$ cells (Fig. 6C, *lane 4*) expressed more K1 than did uninfected p21$^{-/-}$ cells (*lane 2*) and p21$^{-/-}$ cells infected with adenovirus alone (*lane 3*).

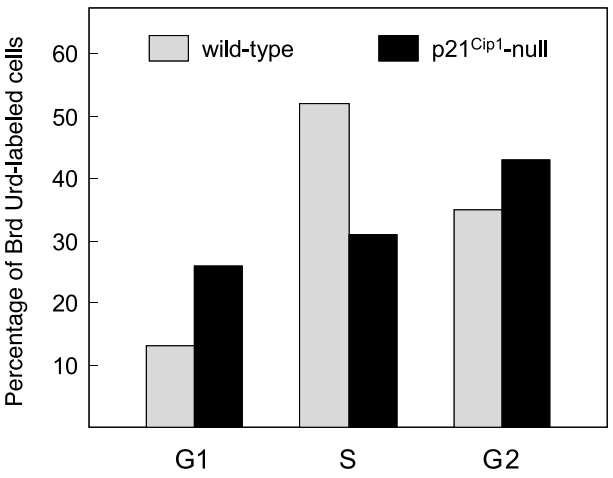

FIGURE 5. Proliferative capacity of suspended wild-type and p21^{Cip1}-null keratinocytes. Cycling p21$^{+/+}$ and p21$^{-/-}$ cells received 4 μM BrdUrd for 2 h. Cell pellets were processed as described in "Materials and Methods," and cell cycle distribution was determined by flow cytometry. Percentages of BrdUrd-labeled cells in G_1, S, and G_2 are shown.

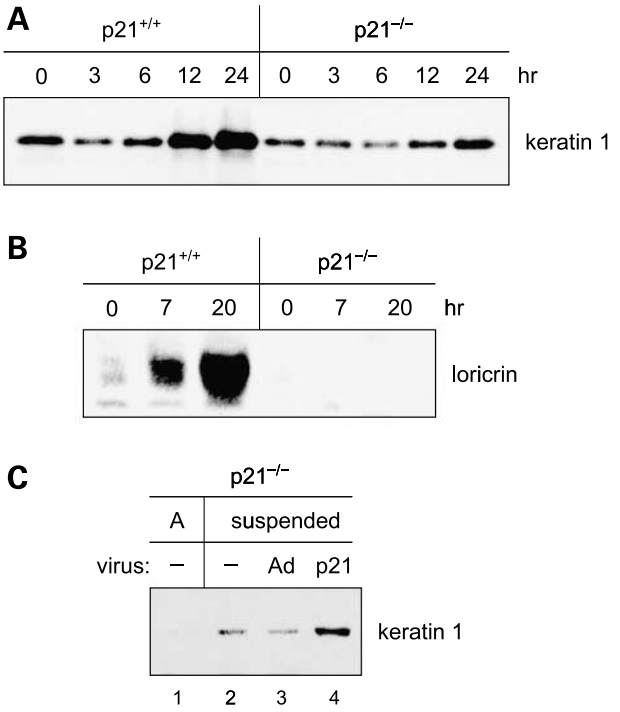

FIGURE 6. K1 and loricrin induction in p21$^{+/+}$ and p21$^{-/-}$ keratinocytes suspended in methylcellulose. **A** and **B.** p21$^{+/+}$ and p21$^{-/-}$ keratinocytes were suspended in methylcellulose for the indicated times, and cell extracts were immunoblotted with antibody to K1 (**A**) or loricrin (**B**). **C.** p21$^{-/-}$ keratinocytes were infected with control adenovirus (*Ad*) or Ad-p21^{Cip1} (*p21*) at an MOI of 250. Cells were suspended in methylcellulose 21 h after infection and harvested 19 h later for determination of K1 abundance by Western blotting. *Lane 1,* uninfected cells attached to plates.

E2F Complexes in Wild-Type and p21^{Cip1}-Null Keratinocytes in Suspension

Cyclin A is a member of a DNA binding complex that contains cdk2, the transcription factor E2F, the E2F binding partner DP1, and p107, an Rb family member (31–33). Previous studies have shown that p21^{Cip1} disrupts this complex both *in vivo* and *in vitro* (21, 34, 35). Thus, as another potential consequence of p21^{Cip1} loss, we monitored E2F DNA binding activity in p21$^{+/+}$ and p21$^{-/-}$ keratinocytes before and after suspension. As demonstrated by electrophoretic mobility shift assays, attached keratinocytes (wild-type and p21^{Cip1}-null) expressed an activity that interacted with an E2F binding site; this DNA/protein complex is designated complex I (Fig. 7A, *lanes 1* and *5*). Between 3 and 6 h after suspension of p21$^{+/+}$ keratinocytes, complex I disappeared and a more rapidly migrating complex (complex II) appeared (Fig. 7A, *lanes 3* and *4*). Complex II was not seen in suspended p21$^{-/-}$ keratinocytes, although amounts of complex I decreased somewhat (*lanes 6–8*). Infection of p21$^{-/-}$ keratinocytes with Ad-p21^{Cip1} resulted in the conversion of complex I to II (Fig. 7B). These findings show that suspension modulates the composition of E2F DNA binding complexes in a p21^{Cip1}-dependent manner.

Antibody to cyclin A blocked the formation of complex I (or its entry into the gel; Fig. 7C, compare *lanes 1* and *4*, and *lanes 7–9* with *lanes 10–12*) but had no effect on complex

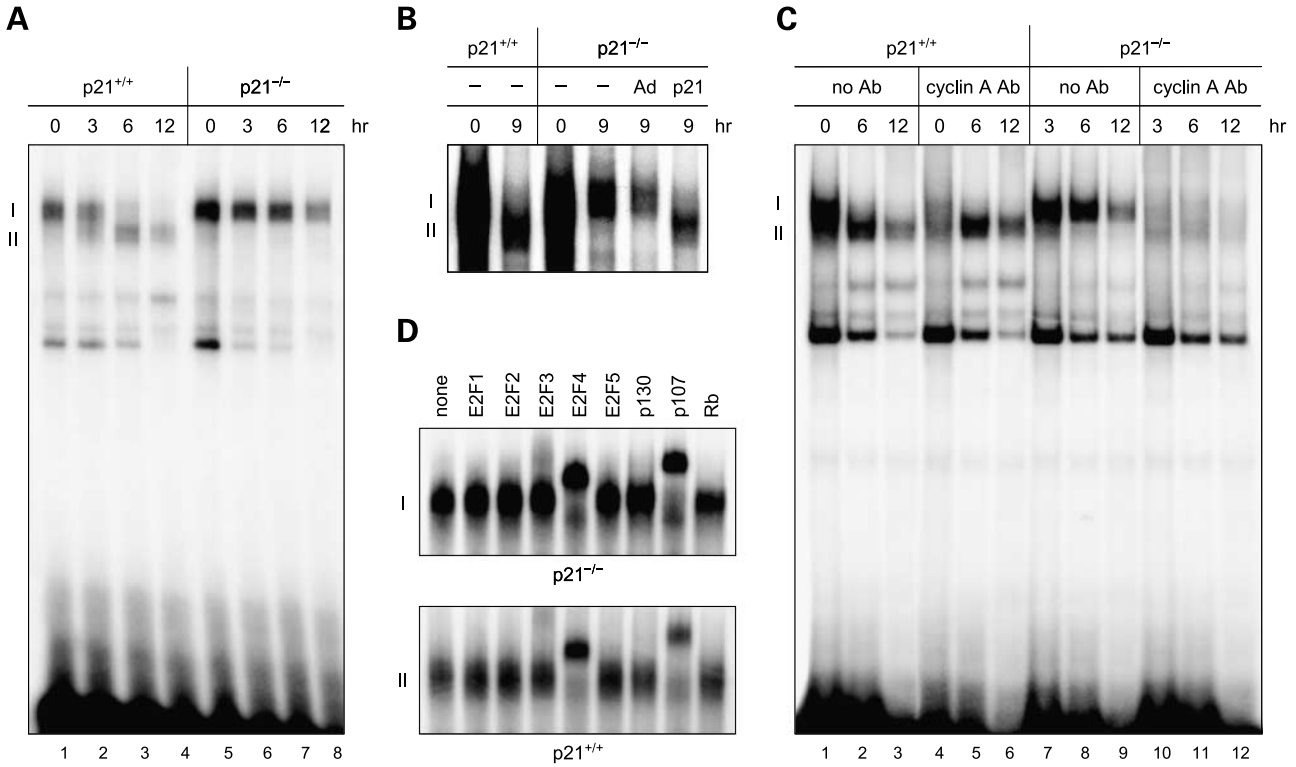

FIGURE 7. Composition of E2F DNA binding complexes in suspended $p21^{+/+}$ and $p21^{-/-}$ keratinocytes. **A–D.** Electrophoretic mobility shift assays were performed on cell extracts using a fragment of the dihydrofolate reductase promoter containing an E2F binding site. Antibodies to cyclin A (**C**), E2F1, E2F2, E2F3, E2F4, E2F5, p130, p107, or Rb (**D**) were added to binding reactions 30 min before addition of the radiolabeled DNA probe. **A.** $p21^{+/+}$ and $p21^{-/-}$ keratinocytes were suspended for the indicated times. **B.** $p21^{Cip1}$-null keratinocytes were infected with control adenovirus (*Ad*) or Ad-$p21^{Cip1}$ (*p21*) at an MOI of 250. Cells were suspended 19 h after infection and harvested 9 h later. Uninfected wild-type keratinocytes were also suspended for 9 h. **C.** $p21^{+/+}$ and $p21^{-/-}$ keratinocytes were suspended for the indicated times. **D.** $p21^{-/-}$ keratinocytes (*top panel*) and $p21^{+/+}$ keratinocytes (*bottom panel*) were suspended for 6 h. The *top panel* examines the composition of complex I, and the *bottom panel* examines the composition of complex II.

II (compare *lanes 2* and *3* with *lanes 5* and *6*). Both complexes were supershifted with antibodies to p107 and E2F4 but were unaffected by antibodies to Rb, the Rb-related protein p130, or other E2F family members (Fig. 7D). Thus, complex I contains E2F4, p107, and cyclin A, whereas complex II contains E2F4 and p107 but not cyclin A.

Discussion

Our data show that suspension reduces the abundance and associated activity of cyclin A in primary keratinocytes much more rapidly in the presence than in the absence of $p21^{Cip1}$. Suspension-induced decreases in cyclin A abundance apparently result (at least in part) from the transcriptional repression of the *cyclin A* gene. In wild-type keratinocytes, suspension reduced the abundance of the cyclin A transcript without affecting its half-life. This transcript also decayed with similar kinetics in $p21^{+/+}$ and $p21^{-/-}$ keratinocytes. Others have shown that suspension represses cyclin A expression in rodent fibroblasts by a process involving cdk2 inactivation and the consequent interaction of Rb with the cyclin A promoter (10, 14, 21, 22). In our studies, loss of cyclin A activity preceded loss of cyclin A protein. Thus, the primary target of $p21^{Cip1}$ in suspended keratinocytes is cyclin A-associated activity rather than cyclin A expression.

We show that $p21^{Cip1}$ inhibits cyclin A-associated activity in suspended keratinocytes by two mechanisms: it interacts with cyclin A-cdk2 complexes, and it promotes the accumulation of $p27^{Kip1}$. Suspension of $p21^{+/+}$ keratinocytes for 3 or 6 h increased the percentage of cyclin A complexes containing $p21^{Cip1}$. Such increases presumably result from the redistribution of $p21^{Cip1}$ from cyclin D1-cdk4 complexes to cyclin A-cdk2 complexes. First, as presented here, amounts of $p21^{Cip1}$ remained constant for 6–9 h after suspension of wild-type keratinocytes and then decreased. Second, as described previously, amounts of cyclin D1 declined within 3 h of suspension of wild-type keratinocytes and were essentially undetectable at 6 h (18). $p21^{Cip1}$ may interact with both cyclin A-cdk2 and cyclin E-cdk2 complexes in suspended keratinocytes; we have shown that suspension reduces cyclin E-associated activity and cyclin A-associated activity with similar kinetics (18). Others have shown that $p21^{Cip1}$ associates with cyclin E-cdk2 complexes in suspended NIH 3T3 cells and that this association correlates with decreases in cyclin D1 abundance rather than increases in $p21^{Cip1}$ abundance (9).

Amounts of $p27^{Kip1}$ increased within 6 h of suspension of wild-type keratinocytes and were substantially elevated at 9–24 h. $p21^{+/+}$ keratinocytes expressed much more $p27^{Kip1}$ than did $p21^{-/-}$ keratinocytes whether attached or suspended.

Consistent with these results, only a small percentage of cyclin A complexes contained $p27^{Kip1}$ in suspended $p21^{-/-}$ keratinocytes. Immunodepletion of $p21^{Cip1}$ from extracts of $p21^{+/+}$ cells suspended for 6 or 12 h partially removed cyclin A complexes, whereas immunodepletion of both $p21^{Cip1}$ and $p27^{Kip1}$ removed almost all cyclin A complexes. Thus, both CKIs contribute to the formation of inactive cyclin A-cdk2 complexes in suspended wild-type keratinocytes. In this regard, $p21^{Cip1}$ may be important at early times after suspension (*i.e.*, at times before $p27^{Kip1}$ increases in abundance), whereas $p27^{Kip1}$ is important at later times (*i.e.*, at times when $p27^{Kip1}$ is abundant and $p21^{Cip1}$ is not appreciably expressed). The data of Di Cunto *et al*. (36) show that enforced expression of $p21^{Cip1}$ blocks the terminal stages of keratinocyte differentiation. Thus, the "$p21^{Cip1}$ to $p27^{Kip1}$ switch" may represent a means of keeping cdk2 inactive while allowing the completion of differentiation.

How $p21^{Cip1}$ modulates $p27^{Kip1}$ abundance in keratinocytes is not known. $p21^{+/+}$ and $p21^{-/-}$ keratinocytes express comparable amounts of $p27^{Kip1}$ mRNA whether attached or suspended. cdk2 phosphorylates $p27^{Kip1}$, which targets $p27^{Kip1}$ for destruction in the preteasome (37–39). Thus, the greater amounts of $p27^{Kip1}$ in $p21^{+/+}$ than in $p21^{-/-}$ keratinocytes may be the indirect result of reduced amounts of cdk2 activity and consequent $p27^{Kip1}$ stabilization. On the other hand, Carrano and Pagano (16) found that suspension of cycling fibroblasts accelerated $p27^{Kip1}$ degradation in the absence of cdk2 activity; whether cdk2-independent $p27^{Kip1}$ destruction required $p21^{Cip1}$ was not determined.

We have shown that wild-type keratinocytes withdraw from the cell cycle regardless of whether they are in G_1, S, or G_2-M at the time of suspension (29). Results of experiments using BrdUrd to tag S phase cells show that more $p21^{-/-}$ cells exit S phase when placed in suspension than do $p21^{+/+}$ cells. Thus, loss of $p21^{Cip1}$ appears to increase the capacity of keratinocytes to proliferate in suspension. Using a similar method, others (40) showed that ectopic expression of $p21^{Cip1}$ prevented SAOS-2 cells from exiting S phase. $p21^{Cip1}$ may arrest suspension-cultured keratinocytes in S phase arrest by inhibiting the activity of cdk2, as was shown in the SAOS-2 study (40), and/or by directly interfering with DNA replication (41).

$p21^{Cip1}$-null mice develop normally (42), thus indicating that $p21^{Cip1}$ is not required for keratinocyte differentiation during development. However, our data suggest that loss of $p21^{Cip1}$ impedes differentiation to some extent in suspension-cultured keratinocytes. We show that suspended $p21^{-/-}$ keratinocytes express much less K1, a marker of differentiation, than do suspended $p21^{+/+}$ keratinocytes or $p21^{-/-}$ keratinocytes ectopically expressing $p21^{Cip1}$. A second differentiation marker, loricrin, increased in suspended $p21^{+/+}$ keratinocytes but not in $p21^{-/-}$ keratinocytes. The differentiation-defective phenotype of $p21^{-/-}$ keratinocytes may reflect their impaired capacity to growth arrest in suspension and/or to accumulate $p27^{Kip1}$. We have shown that keratinocytes treated with $p27^{Kip1}$ antisense oligonucleotides or derived from $p27^{Kip1}$-null mice do not differentiate when placed in suspension (18, 43). Similar to our results, Missero *et al*. (44) found that loss of $p21^{Cip1}$ reduced the expression of a set of differentiation markers in calcium-treated mouse keratinocytes.

E2F-p107 complexes associate with cyclin E-cdk2 and cyclin A-cdk2 complexes in G_1 and S, respectively (31–33, 45). Our data show that suspension of wild-type keratinocytes results in the removal of cyclin A from E2F-p107 DNA binding complexes, whereas suspension of $p21^{Cip1}$-null keratinocytes does not. Similarly, others have shown that cyclin E and cdk2 associate with E2F-p107 complexes in attached but not suspended NIH 3T3 cells (10, 19) and that $p21^{Cip1}$ releases cyclin-cdk2 complexes from E2F-p107 complexes *in vitro* (34, 35). The physiological relevance of cyclin-cdk2 interaction with E2F-p107 complexes is not known at present (46). However, it is clear that among its other effects, $p21^{Cip1}$ modulates the composition of E2F-p107 complexes in primary keratinocytes.

Although the $p21^{Cip1}$ gene is rarely mutated in human tumors (47), $p21^{Cip1}$ may be expressed at subnormal amounts or dysfunctional in some human tumors (48, 49). Importantly, inefficient expression of $p21^{Cip1}$ is indicative of short survival times for patients with acute lymphoblastic leukemia or colorectal cancer (48, 50). We have shown that irradiated $p21^{Cip1}$-null mice develop significantly more metastatic tumors than do irradiated wild-type mice (51). A key step in the metastatic process is the loss of adherent-dependent growth control (52). Thus, loss of $p21^{Cip1}$ may contribute to tumor progression by increasing the capacity of cells to grow in suspension.

Materials and Methods

Cell Culture

$p21^{Cip1}$-null mice (from Dr. Tyler Jacks; 53) and $p21^{Cip1}$-wild type mice (The Jackson Laboratory, Bar Harbor, ME), both from the same mixed C57BL/6 and 129 genetic background, were used in our study. Keratinocytes were isolated from the epidermis of newborn mice as described previously (25, 54) and cultured in keratinocyte basal medium (KBM; Clonetics Corp., San Diego, CA) supplemented with 0.05 mM calcium, 0.5 μg/ml insulin, 0.05 μg/ml hydrocortisone, 1.5 ng/ml epidermal growth factor, 7.5 μg/ml bovine pituitary extract, and 2% dialyzed FCS. Confluent 3-day-old keratinocytes were refed with KBM containing the above ingredients 12–15 h before suspension. Cells were suspended in 50-ml conical tubes in KBM containing 1.45% methylcellulose (Sigma, St. Louis, MO) and all of the above ingredients except epidermal growth factor.

Preparation of Cell Extracts and Western Blotting

Adherent keratinocytes were trypsinized from the plates, collected by centrifugation, and washed with KBM containing 5% serum to inactivate the trypsin. Keratinocytes suspended in methylcellulose were diluted 1/10 with PBS and collected by centrifugation. Cells were rinsed with PBS, resuspended in immunoprecipitation buffer [50 mM HEPES (pH 7.2), 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 1 mM EGTA, 0.1 mM orthovanadate, 0.5 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, and 1 mM DTT], and sonicated for 5 s. Insoluble material was removed by centrifugation. Cell extracts (25–50 μg protein) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were

blocked in PBS containing 0.1% Tween 20 and 5% instant milk and incubated with primary antibody in PBS containing 0.1% Tween 20 for 1 h at room temperature. Proteins recognized by the primary antibody were detected by enhanced chemiluminescence using a horseradish peroxidase-coupled secondary antibody as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ).

In Vitro Kinase Assays

Cell extracts (20–40 μg of protein) were incubated with antibody to cyclin A for 2–10 h at 4°C and subsequently with protein A-agarose beads for 30 min at 4°C. Immune complexes were collected by centrifugation and washed twice with immunoprecipitation buffer and once with kinase buffer [50 mM HEPES (pH 7.5) and 10 mM $MgCl_2$]. Washed complexes were incubated for 5 min at 30°C in kinase buffer containing 0.5 μCi [γ-^{32}P]ATP, 1 μM ATP, and 100 μg/ml histone H1. Reaction mixtures were applied to SDS-polyacrylamide gels, and phosphoproteins were visualized by autoradiography.

Detection of Cells Labeled With BrdUrd

Cells received 4 μM BrdUrd for 2 h. Rinsed cell pellets were fixed in 60% cold ethanol and incubated in 0.04% pepsin (5 ml in 0.1 N HCl) for 40 min at 37°C. Cells were centrifuged, resuspended in 3 ml of 2 N HCl, and incubated for 20 min at 37°C. Cells were pelleted after addition of 6 ml of 0.1 N sodium borate to the samples, washed with PBS containing 0.1% Tween 20, and incubated in the dark for 1 h at room temperature in 0.2 ml of PBS containing 0.1% Tween 20 and 0.4 μl of BrdUrd-FITC antibody (Boehringer-Mannheim, Indianapolis, IN). Cells were washed twice with PBS containing 0.1% Tween 20 and incubated for 25 min at room temperature in PBS containing 0.1% Tween 20, 0.05% BSA, 20 μg/ml propidium iodide, and 50 μg/ml RNase. Cell cycle distributions were determined using a Becton Dickinson FACScan.

Electrophoretic Mobility Shift Assays

E2F DNA-binding assays were performed essentially as described by Ikeda *et al*. (55). Cell pellets were incubated on ice for 30 min in a buffer containing 50 mM HEPES (pH 7.9), 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP40, 0.4 mM NaF, 0.4 mM vanadate, 10% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml leupeptin. After removal of insoluble material, extracts (20 μg of protein) were incubated for 30 min at room temperature in binding buffer [20 mM HEPES (pH 7.9), 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1% NP40, 30 mg/ml BSA, and 500 ng/ml sonicated salmon sperm DNA] containing a ^{32}P-labeled DNA fragment (0.1 ng) corresponding to residues −103 to −23 of the dihydrofolate reductase promoter in a final volume of 50 μl. DNA-protein complexes were separated on 5% non-denaturing polyacrylamide gels (75:1 acrylamide/bisacrylamide) in Tris-borate with 5% glycerol and visualized by autoradiography. For supershifts, extracts were incubated with antibodies for 30 min before addition of the radiolabeled DNA probe.

Adenovirus Infection

Stocks of adenovirus encoding green fluorescent protein with and without $p21^{Cip1}$ were produced, purified, and titered as described previously (56, 57). Adenoviruses were provided by Dr. Joseph Nevins at Duke University.

Messenger RNA Analysis

Total mRNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). For RNase protection assays, mRNA (20 μg) was hybridized overnight at 56°C with ^{32}P-labeled probes (10^5 cpm) from a custom-made probe set (PharMingen, San Diego, CA). Samples were digested with RNase T1 and RNase A for 45 min at 30°C and proteinase K for 15 min at 37°C. Samples were extracted with phenol/chloroform, collected by sodium acetate/ethanol precipitation, denatured at 90°C for 3 min, and electrophoresed on a 5% polyacrylamide gel. Gels were dried and exposed to X-ray film.

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