Efficient Down-Regulation of Cyclin A-Associated Activity and Expression in Suspended Primary Keratinocytes Requires p21<sup>Cip1</sup>

Paul Hauser,¹ Le Ma,¹ Deepak Agrawal,¹ Eric Haura,² W. Douglas Cress,¹ and W. Jackson Pledger¹

¹Molecular Oncology Program and ²Experimental Therapeutics Program, H. Lee Moffitt Cancer Center and Research Institute, Department of Interdisciplinary Oncology, University of South Florida School of Medicine, Tampa FL

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<sup>Cip1</sup>. 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<sup>Cip1</sup>. Neither suspension nor p21<sup>Cip1</sup> status affected the stability of cyclin A mRNA. Loss of p21<sup>Cip1</sup> reduced the capacity of suspended cells to growth arrest, differentiate, and accumulate p27<sup>Kip1</sup> (a second cdk2 inhibitor) and affected the composition of E2F DNA binding complexes. Cyclin A-cdk2 complexes in suspended p21<sup>Cip1</sup> cells contained p21<sup>Cip1</sup> or p27<sup>Kip1</sup>, whereas most of the cyclin A-cdk2 complexes in p21<sup>−/−</sup> cells lacked p27<sup>Kip1</sup>. Ectopic expression of p21<sup>Cip1</sup> allowed p21<sup>−/−</sup> keratinocytes to efficiently down-regulate cyclin A and differentiate when placed in suspension. These findings show that p21<sup>Cip1</sup> 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<sub>1</sub>, 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<sub>1</sub>, in late G<sub>1</sub>, and at G<sub>1</sub>-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<sup>Cip1</sup> and p27<sup>Kip1</sup> interact with and inhibit the activity of complexes containing cdk4 or cdk2 and their cyclin partners (6, 7). The overall abundance of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> varies during the cell cycle, and sequestration of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> 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<sup>Cip1</sup> or p27<sup>Kip1</sup> in suspended cells than in attached cycling cells (9, 19, 23, 24). p21<sup>Cip1</sup> and p27<sup>Kip1</sup> often accumulate in suspended cells, and in some instances, the absence of the D cyclins precludes the sequestration of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> by D cyclin-cdk4 complexes (9, 10, 12, 16, 19, 20, 23, 24). Thus, suspension increases the availability of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, which results

Received 9/15/03; revised 12/30/03; accepted 1/8/04.

Grant support: This work was supported by the Cortner-Couch Endowed Chair for Cancer Research (W. J. P.) and NIH Grants CA73780 (W. J. P.) and CA90489 (W. D. C.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: W. J. Pledger, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa FL 33612. Phone: (813) 979-3887; Fax: (813) 979-3393. E-mail: pledgerw@moffitt.usf.edu

Copyright © 2004 American Association for Cancer Research.
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 cell cycle progression and differentiation. We found that p27Kip1 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 p27Kip1 antisense oligonucleotides, and thus in the absence of p27Kip1, prevented 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 p27Kip1 promotes keratinocyte differentiation by actions unrelated to the inhibition of cdk2 activity. We also suggest that p27Kip1 (or p27Kip1 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 p21Cip1. We show that efficient down-regulation of cyclin A and cyclin A-associated activity in suspended keratinocytes requires p21Cip1 as do growth arrest, differentiation, and maximal accumulation of p27Kip1. 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 p21Cip1 for Efficient Down-Regulation of Cyclin A, Cyclin A Messenger RNA, and Cyclin A-Associated Activity in Suspended Keratinocytes

To assess the need for p21Cip1 for suspension-induced decreases in cyclin A expression and cyclin A-associated activity, primary keratinocytes derived from wild-type and p21Cip1−/− 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), p21Cip1 selectively inhibits the activity of cdk2-containing complexes (27). It is noted that p21Cip1 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 11–13). 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 p21Cip1 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 p21Cip1 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 p21Cip1 status accounts for the different expression patterns of cyclin A in wild-type versus p21Cip1−/− keratinocytes, we ectopically expressed p21Cip1 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 p21Cip1 (Ad-p21Cip1). 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 p21Cip1 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-p21Cip1 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 p21Cip1. It is unclear why Ad-p21Cip1 more completely reduces cyclin A abundance in suspended keratinocytes (lane 7) than in attached keratinocytes (lane 4).
Submaximal Accumulation of p27Kip1 in Suspended p21Cip1-Null Keratinocytes

We have shown that amounts of p27 Kip1 increase in suspended keratinocytes, as do amounts of p27Kip1-associated cyclin A-cdk2 complexes (18). As shown here, loss of p21Cip1 prevents the rapid decline in cyclin A-associated activity in suspended cells. Together, these findings indicate that p27Kip1 contributes to cdk2 inactivation in suspended cells but does not compensate for the absence of p21Cip1. As a possible explanation of this phenomenon, we examined p27Kip1 expression in p21+/+ and p21−/− keratinocytes in methylcellulose.

Amounts of p27Kip1 increased substantially when p21+/+ keratinocytes were placed in suspension (Fig. 3A); (18). Increases occurred within 6 h of suspension and amounts of p27Kip1 remained elevated for at least 24 h. p27Kip1 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 p27Kip1 mRNA were similar in attached and suspended p21+/+ and p21−/− keratinocytes (Fig. 3B). These findings show that p21Cip1 elicits events that increase the

**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 culture 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 p27Kip1 in Suspended p21Cip1-Null Keratinocytes

We have shown that amounts of p27Kip1 increase in suspended keratinocytes, as do amounts of p27Kip1-associated cyclin A-cdk2 complexes (18). As shown here, loss of p21Cip1 prevents the rapid decline in cyclin A-associated activity in suspended cells. Together, these findings indicate that p27Kip1 contributes to cdk2 inactivation in suspended cells but does not compensate for the absence of p21Cip1. As a possible explanation of this phenomenon, we examined p27Kip1 expression in p21+/+ and p21−/− keratinocytes in methylcellulose.

Amounts of p27Kip1 increased substantially when p21+/+ keratinocytes were placed in suspension (Fig. 3A); (18). Increases occurred within 6 h of suspension and amounts of p27Kip1 remained elevated for at least 24 h. p27Kip1 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 p27Kip1 mRNA were similar in attached and suspended p21+/+ and p21−/− keratinocytes (Fig. 3B). These findings show that p21Cip1 elicits events that increase the

**FIGURE 2.** Amounts of cyclin A in suspended p21−/− keratinocytes ectopically expressing p21Cip1. Proliferating p21−/− keratinocytes were infected with adenovirus without insert (Ad) or Ad-p21Cip1 (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 p21Cip1 and cyclin A abundance by Western blotting. Uninfected p21+/+ and p21−/− keratinocytes were also suspended in methylcellulose for 19 h. The p21Cip1 antibody used for Western blotting recognizes ectopic human p21Cip1 but not endogenous (mouse) p21Cip1. A, attached cells; S, suspended cells.
Increased Association of CDK Inhibitors With Cyclin A Complexes in Suspended Wild-Type Keratinocytes

In contrast to p27Kip1, p21Cip1 did not accumulate in suspended p21+/+ keratinocytes (Fig. 4A). In fact, p21Cip1 decreased in abundance 6–9 h after suspension and was not detected at 24 h. Although total amounts of p21Cip1 were constant for at least 6 h after suspension, amounts of p21Cip1 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 p21Cip1-associated cyclin A and p21Cip1-free cyclin A in p21+/+ keratinocytes at various times after suspension. Cell extracts were immunoprecipitated with antibody to p21Cip1, and p21Cip1 immune complexes were Western blotted with antibody to cyclin A (p21Cip1-associated cyclin A). p21Cip1-immunodepleted extracts were immunoprecipitated and Western blotted with antibody to cyclin A (p21Cip1-free cyclin A). Western blots using p21Cip1 antibody confirmed that immunodepleted extracts contained little if any p21Cip1 (data not shown).

Suspension of p21+/+ keratinocytes for 3 or 6 h resulted in an increase in the amounts of p21Cip1-associated cyclin A and a decrease in the amounts of p21Cip1-free cyclin A (Fig. 4B, compare lanes 2 and 3 with lanes 7 and 8). Thus, a greater percentage of cyclin A complexes contain p21Cip1 in suspended keratinocytes than in attached keratinocytes. After 6 h, amounts of p21Cip1-associated cyclin A declined (lanes 4 and 5), most likely because of reductions in p21Cip1 abundance (see Fig. 4A). Although suspension reduced amounts of p21Cip1-free cyclin A, it did not eliminate p21Cip1-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 p21Cip1 and p27Kip1 (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 p21Cip1 from cyclin D1 complexes to cyclin A complexes and thus in the inhibition of cyclin A-cdk2 activity in wild-type keratinocytes. Redistribution of p27Kip1 and/or increases in p27Kip1 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 p27Kip1, as do most of the cyclin A-ck2 complexes in attached p21−/− cells. This finding is consistent with our data showing subnormal amounts of p27Kip1 in p21−/− keratinocytes (Fig. 3A).
Reduced Capacity of p21<sup>−/−</sup> Keratinocytes to Growth Arrest and Differentiate in Suspension

Although suspension typically arrests cells in G1 (1), suspended primary mouse keratinocytes arrest in G1, S, and G2-M (29). To determine whether cell cycle arrest requires p21<sup>Cip1</sup>, p21<sup>+/+</sup> and p21<sup>−/−</sup> 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<sup>+/+</sup> and p21<sup>−/−</sup> populations, respectively (Fig. 5). Moreover, a greater percentage of p21<sup>−/−</sup> cells were in G1 at this time than were p21<sup>+/+</sup> cells. These data suggest that p21<sup>−/−</sup> cells cycle for longer periods of time when placed in suspension than do p21<sup>+/+</sup> cells. In this respect, p21<sup>−/−</sup> keratinocytes mimic MK cells, which are immortalized mouse keratinocytes (30) and which express little if any p21<sup>Cip1</sup> (data not shown).

To assess the need for p21<sup>Cip1</sup> for differentiation, we placed suspension-defective phenotype of p21<sup>Cip1</sup> 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<sup>+/+</sup> keratinocytes. Increases in the abundance of loricrin were especially pronounced. Amounts of loricrin did not increase in suspended p21<sup>−/−</sup> 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<sup>−/−</sup> keratinocytes than in p21<sup>+/+</sup> keratinocytes (data not shown).

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

E2F Complexes in Wild-Type and p21<sup>Cip1</sup>-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). Therefore, as another potential consequence of p21<sup>Cip1</sup> loss, we monitored E2F DNA binding activity in p21<sup>+/+</sup> and p21<sup>−/−</sup> keratinocytes before and after suspension. As demonstrated by electrophoretic mobility shift assays, attached keratinocytes (wild-type and p21<sup>Cip1</sup>-null) expressed an activity that interacted with an E2F binding site; this DNA/protein complex is designated complex I (Fig. 7A, lanes 1–3). In vivo both <i>in vivo</i> (21, 34, 35). Thus, as another potential consequence of p21<sup>Cip1</sup> loss, we monitored E2F DNA binding activity in p21<sup>+/+</sup> and p21<sup>−/−</sup> keratinocytes before and after suspension. As demonstrated by electrophoretic mobility shift assays, attached keratinocytes (wild-type and p21<sup>Cip1</sup>-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<sup>+/+</sup> 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<sup>−/−</sup> keratinocytes, although amounts of complex I decreased somewhat (lanes 6–8). Infection of p21<sup>−/−</sup> keratinocytes with Ad-p21<sup>Cip1</sup> 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<sup>Cip1</sup>-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
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 p21Cip1. 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 p21Cip1 in suspended keratinocytes is cyclin A-associated activity rather than cyclin A expression.

We show that p21Cip1 inhibits cyclin A-associated activity in suspended keratinocytes by two mechanisms: it interacts with cyclin A-ckd2 complexes, and it promotes the accumulation of p27Kip1. Suspension of p21+/− keratinocytes for 3 or 6 h increased the percentage of cyclin A complexes containing p21Cip1. Such increases presumably result from the redistribution of p21Cip1 from cyclin D1-ckd4 complexes to cyclin A-ckd2 complexes. First, as presented here, amounts of p21Cip1 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). p21Cip1 may interact with both cyclin A-ckd2 and cyclin E-ckd2 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 p21Cip1 associates with cyclin E-ckd2 complexes in suspended NIH 3T3 cells and that this association correlates with decreases in cyclin D1 abundance rather than increases in p21Cip1 abundance (9). Amounts of p27Kip1 increased within 6 h of suspension of wild-type keratinocytes and were substantially elevated at 9–24 h. p21+/− keratinocytes expressed much more p27Kip1 than did p21−/− keratinocytes whether attached or suspended.

**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−/−-null keratinocytes were infected with control adenovirus (Ad) or Ad-p21Cip1 (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.
Consistent with these results, only a small percentage of cyclin A complexes contained p27kip1 in suspended p21+/− keratinocytes. Immunodepletion of p21Cip1 from extracts of p21+/+ cells suspended for 6 or 12 h partially removed cyclin A complexes, whereas immunodepletion of both p21Cip1 and p27kip1 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, p21Cip1 may be important at early times after suspension (i.e., at times before p27kip1 increases in abundance), whereas p27kip1 is important at later times (i.e., at times when p27kip1 is abundant and p21Cip1 is not appreciably expressed). The data of Di Cunto et al. (36) show that enforced expression of p21Cip1 blocks the terminal stages of keratinocyte differentiation. Thus, the “p21Cip1 to p27kip1 switch” may represent a means of keeping cdk2 inactive while allowing the completion of differentiation.

How p21Cip1 modulates p27kip1 abundance in keratinocytes is not known. p21+/+ and p21−/− keratinocytes express comparable amounts of p27kip1 mRNA whether attached or suspended. cdk2 phosphorylates p27kip1, which targets p27kip1 for destruction in the preteasome (37–39). Thus, the greater amounts of p27kip1 in p21+/+ than in p21−/− keratinocytes may be the indirect result of reduced amounts of cdk2 activity and consequent p27kip1 stabilization. On the other hand, Carrano and Pagano (16) found that suspension of cycling fibroblasts accelerated p27kip1 degradation in the absence of cdk2 activity; whether cdk2-independent p27kip1 destruction required p21Cip1 was not determined.

We have shown that wild-type keratinocytes withdraw from the cell cycle regardless of whether they are in G1, S, or G2–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 p21Cip1 appears to increase the capacity of keratinocytes to proliferate in suspension. Using a similar method, others (40) showed that ectopic expression of p21Cip1 prevented SAOS-2 cells from exiting S phase. p21Cip1 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).

p21Cip1−/−null mice develop normally (42), thus indicating that p21Cip1 is not required for keratinocyte differentiation during development. However, our data suggest that loss of p21Cip1 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 p21Cip1. 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 p27kip1. We have shown that keratinocytes treated with p27kip1 antisense oligonucleotides or derived from p27kip1−/−null mice do not differentiate when placed in suspension (18, 43). Similar to our results, Missero et al. (44) found that loss of p21Cip1 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 G1 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 p21Cip1−/− 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 p21Cip1 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, p21Cip1 modulates the composition of E2F-p107 complexes in primary keratinocytes.

Although the p21Cip1 gene is rarely mutated in human tumors (47), p21Cip1 may be expressed at subnormal amounts or dysfunctional in some human tumors (48, 49). Importantly, inefficient expression of p21Cip1 is indicative of short survival times for patients with acute lymphoblastic leukemia or colorectal cancer (48, 50). We have shown that irradiated p21Cip1−/−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 p21Cip1 may contribute to tumor progression by increasing the capacity of cells to grow in suspension.

Materials and Methods

Cell Culture

p21Cip1-null mice (from Dr. Tyler Jacks; 53) and p21Cip1−/−, 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 7.9), 250 mM KCl, 0.1 mM EDTA, 0.1% NP40, 40 mM KCl, 6 mM MgCl2, 1 mM EGTA, 1 mM DTT, 0.1% NP40, 30 mg/ml BSA, and 500 ng/ml sonicated salmon sperm DNA] containing a 32P-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 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% 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 MgCl2, 1 mM EGTA, 1 mM DTT, 0.1% NP40, 30 mg/ml BSA, and 500 ng/ml sonicated salmon sperm DNA] containing a 32P-labeled DNA fragment (0.1 ng) 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<sup>Cip1</sup> were produced, purified, and titrated 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 32P-labeled probes (10<sup>5</sup> 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 electroforesed on a 5% polyacrylamide gel. Gels were dried and exposed to X-ray film.

**Acknowledgments**

The authors thank Dr. Tyler Jacks for the p21<sup>Cip1</sup>-null mice, Dr. Joseph Nevins for the adenoviruses, and Dr. Nancy Olashaw for manuscript preparation. We also acknowledge the helpful service of the Molecular Biology, Molecular Imaging, and Flow Cytometry Core Laboratories at the Moffitt Cancer Center.

**References**

15. Carstens CP, Kramer A, Fahl WE. Adhesion-dependent control of cyclin


Molecular Cancer Research

Efficient Down-Regulation of Cyclin A-Associated Activity and Expression in Suspended Primary Keratinocytes Requires p21Cip1

This work was supported by the Cortner-Couch Endowed Chair for Cancer Research (W. J. P.) and NIH Grants CA73780 (W. J. P.) and CA90489 (W. D. C.)

Paul Hauser, Le Ma, Deepak Agrawal, et al.