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Inhibitory Function of $p21^{Cip1/WAF1}$ in Differentiation of Primary Mouse Keratinocytes Independent of Cell Cycle Control

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The cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$ has been implicated as an inducer of differentiation. However, although expression of p21 is increased in postmitotic cells immediately adjacent to the proliferative compartment, its expression is decreased in cells further along the differentiation program. Expression of the p21 protein was decreased in terminally differentiated primary keratinocytes of mice, and this occurred by a proteasome-dependent pathway. Forced expression of p21 in these cells inhibited the expression of markers of terminal differentiation at both the protein and messenger RNA levels. These inhibitory effects on differentiation were not observed with a carboxyl-terminal truncation mutant or with the unrelated cyclin-dependent kinase inhibitor $p16^{INK4a}$, although all these molecules exerted similar inhibition of cell growth. These findings reveal an inhibitory role of p21 in the late stages of differentiation that does not result from the effects of p21 on the cell cycle.

A precise coupling between cell growth and differentiation is required during embryonic development and in self-renewing tissues of the adult. The cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1/WAF1}$ inhibits cell growth and, in some circumstances, promotes differentiation (*1*). However, in many tissues, expression of p21 in vivo correlates with the onset but not necessarily the establishment of the terminally differentiated phenotype (*2*, *3*). For instance, in the intestine, p21 expression is induced in postmitotic cells adjacent to the proliferative compartments, but is down-modulated at later stages of differentiation. Similarly, in hair follicles of the skin, p21 is highly expressed in a narrow zone of postmitotic keratinocytes next to the proliferating cells of the lower bulb, whereas little or no p21 is expressed in the differentiated keratinocytes of the upper region (*2*).

We examined the functional meaning of decreased p21 expression at late stages of differentiation in mouse keratinocytes. Addition of calcium (1.2 to 2 mM) to cultures of primary keratinocytes triggers a terminal differentiation program that includes early structural changes, such as desmosome formation and reorganization of keratin cables, as well as later events, such as growth arrest and expression of markers of the upper differentiated layers of the epidermis (*4*). Increased expression of p21 occurs 4 to 8 hours after exposure to calcium, along with inhibition of CDK and block of the cell cycle at the G_1 phase (*5*, *6*). After 24 hours, despite a sustained increase in p21 mRNA expression, the p21 protein returns to basal amounts or less (*6*).

Primary keratinocytes cultured in low-calcium medium (0.05 mM calcium) consist of two populations: continuously proliferating attached cells and terminally differentiated detached cells (*7*). The p21 protein was present in the attached keratinocytes but was almost undetectable in the detached population (Fig. 1A). No change was detected in the amount of the closely related CDK inhibitor p27, nor of p53 or cyclin A, the amount of which decreases after detachment in other cell types (*8*) (Fig. 1A). The amounts of p21 mRNA in the detached and attached populations were similar (Fig. 1B). Human and mouse keratinocytes can be induced to terminally differentiate when they are artificially brought into suspension by trypsinization (*7*, *9*). Amounts of the p21 protein were decreased even under these conditions (Fig. 1C), with a time course similar to that of expression of terminal differentiation markers (*10*). Expression of p21 was similarly reduced in the detached versus attached populations of keratinocytes derived from p53 null mice (Fig. 1D).

The p21 protein is a substrate for ubiquitination and proteasome-dependent degradation (*11*). Primary keratinocytes were treated for 24 hours with two chemically unrelated proteasome inhibitors, *N*-acetyl-leucinyl-leucinyl-norleucinal (LLnL) and lactacystin (*12*), or with an inhibitor of cysteine proteinases devoid of any inhibitory activity toward the proteasome, L-*trans*-epoxysuccinic acid (E64) (*13*). The latter compound had no effect on the amount of p21 in either attached or spontaneously detached keratinocytes (Fig. 1E). By contrast, the amount of p21 was increased in attached keratinocytes from cultures treated with either LLnL or lactacystin, at concentrations as low as 3 μM. Similar large amounts of p21 protein were also present in the corresponding detached populations. Moreover, in extracts of keratinocytes treated with proteasome inhibitors, but not in those with E64, antibodies to p21 (anti-p21) recognized specific bands of larger molecular size that are likely to correspond to ubiquitinated forms of p21 (Fig. 1E). Treatment with proteasome inhibitors led to no increase in expression of p21 mRNA, but rather a slight decrease (Fig. 1F). Proteasome inhibitors also counteracted the decrease in the amount of p21 protein that occurred when keratinocytes were artificially brought into suspension by trypsinization (*10*). Thus, a specific proteasome-mediated pathway contributes to decreased expression of p21 in differentiated keratinocytes.

To assess the biological meaning of decreased p21 expression in differentiation, we infected primary keratinocytes with a control adenovirus expressing a bacterial β-galactosidase gene (Ad-lac z) and an adenovirus expressing full-length human p21 (Ad-p21) (*14*). Infection of cells with the Ad-p21 virus caused inhibition of DNA synthesis by 24 hours after infection; no

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such effect was observed with Ad-lac z (Fig. 2A) (*15*). Infection with Ad-p21 caused decreased expression of terminal differentiation markers in the spontaneously detached keratinocyte population, including keratin 1, involucrin, loricrin, and (pro)-filaggrin (Fig. 3A). Unlike these markers of the differentiated layers of the epidermis, expression of keratin 5, a marker of the basal proliferative layer constitutively expressed in cultured keratinocytes, was not inhibited by the Ad-p21 virus. Infection with an adenovirus expressing the unrelated CDK inhibitor p16 (Ad-p16) (*15*) caused growth inhibition similar to that caused by Ad-p21 (Fig. 2A), but Ad-p16 had no effect on expression of differentiation markers (Fig. 3A). An adenovirus was also tested that encodes a truncated p21 mutant with a COOH-terminal deletion and an intact cyclin-CDK binding domain [Ad-p21$^{\Delta C}$, Ad-p21WAF-341 in (*16*)]. Infection of keratinocytes with Ad-p21$^{\Delta C}$ and infection with the virus expressing intact p21 had similar growth-inhibitory effects (Fig. 2A). The two viruses expressed similar amounts of the corresponding p21 proteins and did not alter the amount of endogenous p21 (Fig. 2B). However, infection with Ad-p21$^{\Delta C}$ caused little or no decrease in expression of differentiation markers (Fig. 3A). The same specificity of effects of the virus expressing intact p21 was found to occur at both the protein and mRNA levels (Fig. 3B). The inhibitory effects of p21 were confirmed by cotransfection of primary keratinocytes with

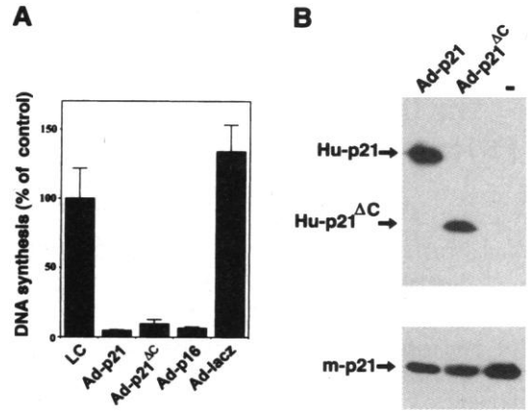

Fig. 2. Growth inhibition of keratinocytes infected with recombinant adenoviruses expressing wild-type p21, p21 with a COOH-terminal deletion, or wild-type p16. (**A**) Primary keratinocytes plated in low-calcium medium were either left uninfected (LC) or infected in triplicate wells with recombinant adenoviruses expressing an intact p21 protein (Ad-p21), a p21 protein with a deletion of its COOH-terminal part (Ad-p21$^{\Delta C}$), a p16^{INK4a} protein (Ad-p16), or a bacterial β-galactosidase protein (Ad-lac z). After 24 hours, DNA synthesis was measured by a 1-hour pulse labeling with [^{3}H]thymidine (*6*). Values are expressed as percentage of the DNA synthesis in the uninfected control. Error bars indicate SD. (**B**) Primary keratinocytes were either uninfected (–) or infected with the Ad-p21 or Ad-p21$^{\Delta C}$ viruses at a similar multiplicity of infection (100). Total cell extracts were prepared 24 hours after infection. Equal amounts of proteins (15 μg) were resolved by SDS-PAGE (12.5% gel) and immunoblotted with monoclonal antibody CP36 (*21*), which specifically recognizes an NH_2-terminal epitope of the virally encoded human p21 (upper panel). The same blot was stripped and reprobed with antibodies specific for endogenous mouse p21 (lower panel). The position of the intact (~21 kD) and truncated (~12 kD) p21 proteins is indicated.

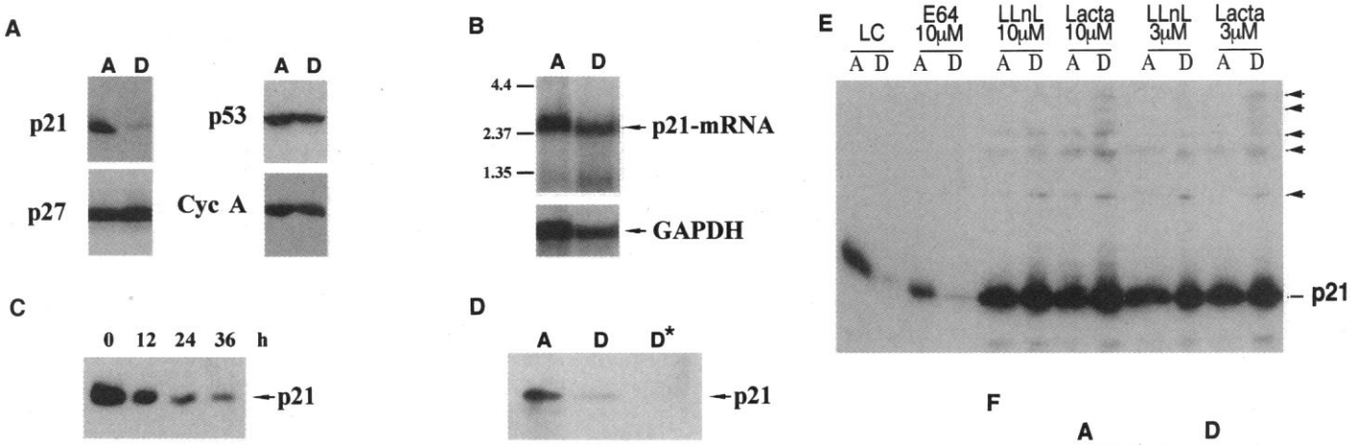

Fig. 1. Decreased expression of p21 in terminally differentiated keratinocytes. (**A**) Decreased expression of the p21 protein in spontaneously detached keratinocytes grown in low-calcium medium. Total cell extracts (15 μg) from attached (A) and detached (D) keratinocytes were resolved by 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted with antibodies specific for p21, p27, p53, and cyclin A (Cyc A) (*6*). (**B**) Constant amounts of p21 mRNA in differentiated keratinocytes. Total RNA (20 μg) from attached and detached keratinocytes was blotted with a radioactive p21 cDNA from mouse (*5*). Equal loading was verified by blotting the same filter to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Molecular size markers (in kilobases) are indicated. (**C**) Decreased expression of the p21 protein in keratinocytes cultured in suspension for the indicated times (*10*). Cell extracts (15 μg) were analyzed by immunoblotting with anti-p21 as in (A). (**D**) Decreased expression of the p21 protein in the detached population of keratinocytes derived from p53 null mice (*5*). Cell extracts (30 μg) from attached (A) and spontaneously detached (D) keratinocytes, and from keratinocytes cultured in suspension for 24 hours (D*), were analyzed by immunoblotting with anti-p21 as in (A). (**E**) Sustained expression of p21 in the presence of proteasome inhibitors. Keratinocytes in low-calcium medium were exposed to dimethyl sulfoxide (DMSO) solvent alone (LC) or to medium with E64, LLnL, or lactacystin (Lacta) at the indicated concentrations (10 μM and 3 μM). Attached and detached keratinocytes were harvested 24 hours after treatment. Total cell extracts (15 μg) were analyzed by immunoblotting with anti-p21. Immunoreactive products of large molecular mass are indicated by arrowheads. (**F**) Effects of proteasome inhibitors on p21 mRNA. Keratinocytes in low-calcium medium were exposed to DMSO solvent alone (LC) or to medium with 10 μM E64, LLnL, or lactacystin (Lacta) for 24 hours. Total RNA (20 μg) prepared from attached and detached primary keratinocytes was blotted with a radioactive p21 cDNA (upper panel). The same blot was then probed with a GAPDH cDNA and the p21/GAPDH ratio was calculated (lower panel).

an expression plasmid for the green fluorescent protein together with a plasmid coding for full-length p21 or a p21 mutant with the COOH-terminal deletion, followed by immunofluorescence analysis with antibodies to loricrin. At 72 hours after transfection, 50% of detached keratinocytes transfected with the truncated p21 mutant coexpressed the loricrin marker; this is similar to the frequency of loricrin-positive cells in the untransfected population. By contrast, loricrin expression was detected in less than 5% of cells transfected with full-length p21 (*17*).

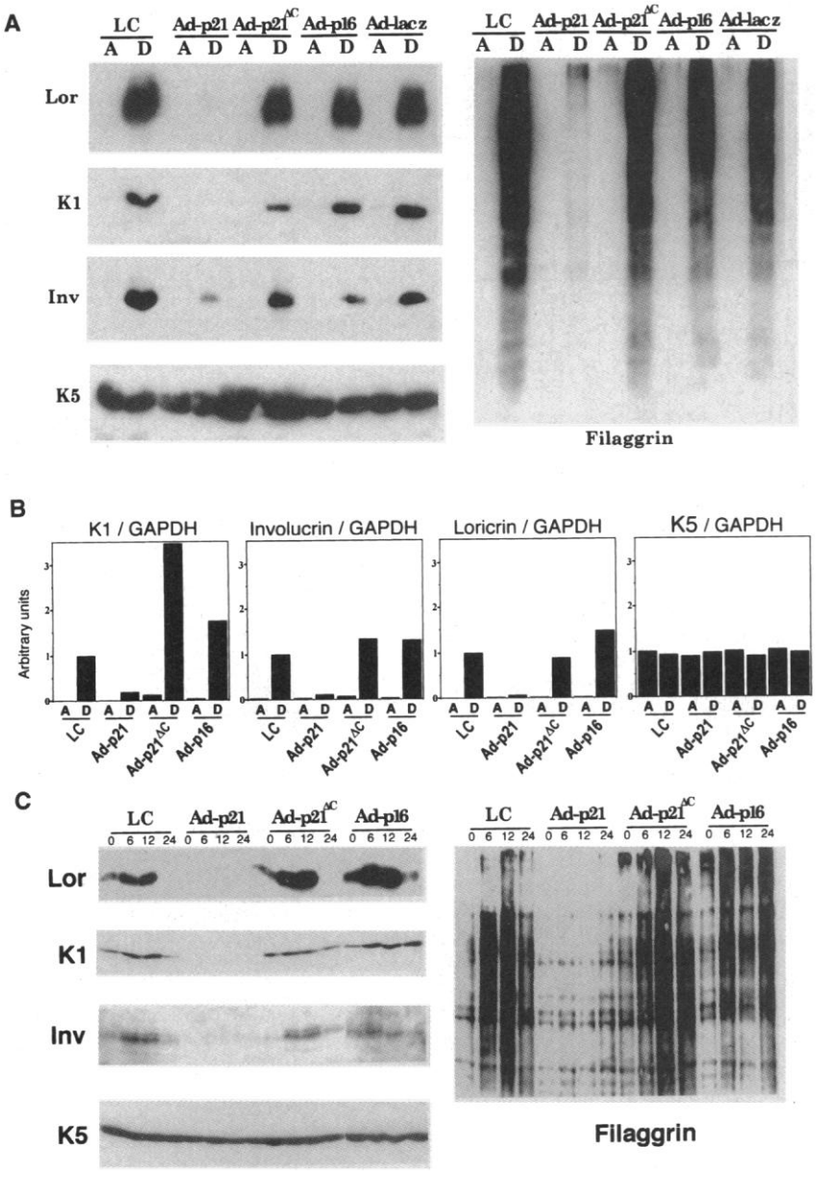

Fig. 3. Decreased expression of differentiation markers as a consequence of increased p21 expression. (**A**) Expression of differentiation marker proteins in attached (A) and detached (D) keratinocytes after adenovirus infection. Primary keratinocytes were infected with recombinant adenoviruses (*15*). Total cell extracts were prepared from attached and detached cell populations 24 hours after infection. Equal amounts of proteins (15 μg) were resolved by SDS-PAGE (7.5% gel) and sequentially immunoblotted with antibodies specific for the indicated differentiation markers (6). (**B**) Amounts of differentiation marker mRNA in adenovirally infected keratinocytes. Primary keratinocytes were infected with recombinant adenoviruses. Total RNA was extracted from attached and detached populations and analyzed by sequential blotting with cDNA probes specific for keratin 1, involucrin, loricrin, and keratin 5. The same blot was then probed with a GAPDH cDNA, and the ratios of the amount of differentiation marker mRNA to that of GAPDH were calculated. (**C**) Expression of differentiation marker proteins in attached keratinocytes cultured in the presence of low or high concentrations of calcium. Primary keratinocytes were infected with recombinant adenoviruses. After 24 hours, cells were either kept in low-calcium medium for an additional 24 hours (0) or exposed to higher calcium concentrations (2 mM) for the indicated times. Total cell extracts were prepared, and proteins (50 μg) were separated by SDS-PAGE (7.5% gel) and sequentially immunoblotted with antibodies specific for the indicated differentiation markers.

Decreased expression of p21 is not limited to detached keratinocytes but also occurs in attached cultures after several hours of exposure to increased calcium concentrations (6). A small fraction of keratinocytes starts to express terminal differentiation markers even in the presence of low calcium concentrations, before detaching from the dish. About 3 to 5% of keratinocytes express involucrin under these conditions (*10*). To assess differentiation marker expression in these populations, we increased the sensitivity of our analysis by immunoblotting larger amounts of total cell extracts (50 μg, rather than 15 μg as in the previous experiments). Expression of intact p21 by the Ad-p21 virus inhibited expression of the terminal differentiation markers under these conditions as well (Fig. 3C).

We explored whether the inhibitory effects of p21 on expression of differentiation markers occurs at the level of transcriptional regulation, and whether these effects are dependent on an intact p21 protein. The involucrin promoter was linked to a luciferase reporter gene and transiently transfected into primary keratinocytes (*5*), and the cells were subsequently infected with the Ad-p21, Ad-p16, and Ad-lac z adenoviruses. The activity of the involucrin promoter was suppressed after infection with Ad-p21 in attached keratinocytes in low-calcium medium (Fig. 4A). Strong inhibition of the involucrin promoter by Ad-p21 expression was also observed in keratinocytes induced to differentiate by calcium, as well as in the detached cell populations (Fig. 4A). By contrast, little or no inhibition of promoter activity was observed in cells infected with the Ad-p16 or Ad-lac z adenoviruses. Expression of the Ad-p21 adenovirus had no effects on the activity of a similarly transfected minimal metallothionein promoter (Fig. 4A). The activity of the involucrin promoter, but not that of the metallothionein promoter, was also suppressed in a dose-dependent manner by cotransfection with an expression plasmid coding for full-length p21 (Fig. 4B). Less or no inhibition was caused by expression vectors encoding p21 mutants with a deletion of the COOH-terminal domain, a deletion of the NH_2-terminal domain, or a deletion of the CDK2 binding domain (Fig. 4B) (*18*).

A positive role for p21 in keratinocyte differentiation was previously indicated by the substantial down-modulation of selective terminal differentiation markers in p21 knockout keratinocytes (6). However, this phenotype is likely to be related to an intrinsically altered balance between growth

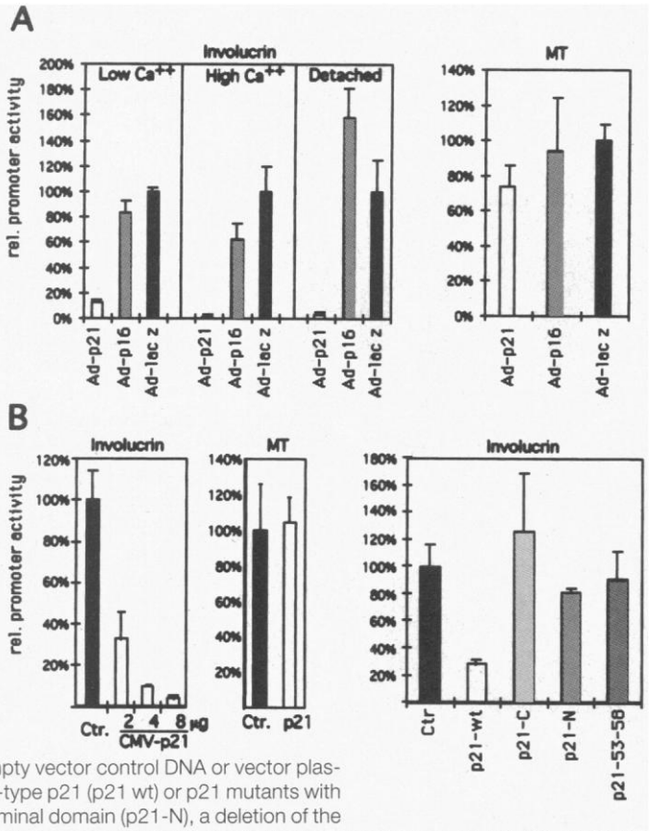

Fig. 4. Inhibition of involucrin promoter activity in keratinocytes expressing p21. (**A**) Primary keratinocytes were transfected with either an involucrin promoter or minimal metallothionein promoter (MT) reporter plasmids (1 µg per well) (*17*). After 24 hours, cells were infected with the indicated adenoviruses and maintained in low-calcium medium for an additional 48 hours, or switched to medium with high calcium concentrations for the last 24 hours of the incubation time. Detached keratinocytes from cultures in low-calcium medium were also collected for promoter activity determinations. (**B**) Primary keratinocytes were transfected with the involucrin or metallothionein promoter reporter plasmids as indicated, together with empty vector control DNA or vector plasmids encoding human wild-type p21 (p21 wt) or p21 mutants with a deletion of the COOH-terminal domain (p21-N), a deletion of the NH_2-terminal domain (p21-C), or a deletion of the CDK2 binding domain (p21-53-58) (*18*). In the left panel, increasing amounts of p21 vector DNA were transfected together with decreasing amount of control plasmid, so that in each case the amount transfected was 8 µg per well. In the center panel, the amount of control and p21 plasmids was 4 µg per well; in the right panel, various expression plasmids were used at 2 µg per well. Promoter activity is expressed as percentage relative to controls. Error bars represent SD; each condition was tested in triplicate wells. Results are representative of at least three independent experiments.

and differentiation, which resulted in increased susceptibility to *ras* transformation, rather than to an impaired progression through differentiation, as triggered by exogenous stimuli (6). The present findings indicate that, besides playing a positive role in the commitment to differentiate, p21 can be involved in the progression of cells through differentiation, but in this case this CDK inhibitor plays a suppressing rather than favoring function. The up-regulation of p21 expression at early stages of differentiation may be part of a negative feedback mechanism that must be inactivated before later stages can proceed.

The p21 protein is composed of two functional domains, one involved in cyclin-CDK association and the other in direct proliferating cell nuclear antigen (PCNA) binding (*19*) as well as modulation of PCNA–DNA methylase interactions (*20*). In addition, p21 serves as an assembly factor for cyclin D–CDK complexes (*21*) and as a noncompetitive inhibitor of stress-activated protein kinases (SAPKs) (*22*). In keratinocytes, the capacity of p21 to inhibit the activity of the involucrin promoter was reduced in mutants deficient in cyclin-CDK binding or lacking the COOH-terminal domain but retaining CDK binding. Because the COOH-terminus of p21 was also not sufficient to inhibit the involucrin promoter, the most plausible explanation is that, for p21 to function in this context, it may have to bind both to cyclin-CDK complexes and to other, as yet unidentified, partners with which its COOH-terminus region interacts. An attractive model for future studies is that p21 may function as a specific bridge between signaling complexes (such as cyclin-CDK complexes and SAPKs) and other multiprotein apparatuses, such as the transcription machinery involved in differentiation.

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10. F. Di Cunto and G. P. Dotto, unpublished data. Primary keratinocytes derived from newborn mice (Sencar) were cultivated in minimal essential medium at low calcium concentrations (0.05 mM $CaCl_2$; low-calcium medium), supplemented as described (*5*). For suspension-induced differentiation, confluent cultures of primary mouse keratinocytes were harvested by trypsinization and suspended in low-calcium medium containing 1% methyl cellulose at a density of 2×10^6 cells/ml. Samples (4 ml) of the suspension were then plated in 60-mm petri dishes and incubated for the indicated times.
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15. With the Ad-p21 virus, a multiplicity of infection of 100 was required for a 90% inhibition of keratinocyte growth at 24 hours after infection. Similar doses were used with the other viruses. The adenovirus expressing human $p16^{INK4a}$ is based on a vector identical to that used for the p21 adenovirus (*14*). The third adenovirus expressing the p21 protein with a COOH-terminal truncation was developed by another laboratory (*16*) but has a backbone very similar to that of the other viruses [G. W. G. Wilkinson and A. Akrigg, *Nucleic Acids Res.* **20**, 2233 (1992)]. In all cases, infection was performed for 1 hour in serum- and epidermal growth factor–free low-calcium medium; keratinocytes were then incubated in fully supplemented low-calcium medium (*5*) for 24 hours and collected (for the studies of attached versus detached populations) or switched to high-calcium medium (2 mM) for the indicated times.
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17. Primary keratinocytes were cotransfected (*5*) with a green fluorescent protein expression plasmid together with a plasmid encoding either human wild-type p21 or a p21 mutant with a deletion of the COOH-terminal domain (*18*). Transfected cells were incubated in low-calcium medium for 72 hours, and the spontaneously detached cells were analyzed by immunofluorescence with antibodies to loricrin (*6*). Promoter activity assays were as described [J. L. Brissette, J. Lee, J. Kamimura, D. Lee, G. P. Dotto, *Genes Dev.* **10**, 2212 (1996)].
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