p21\textsuperscript{WAF1/Cip1} Suppresses Keratinocyte Differentiation Independently of the Cell Cycle through Transcriptional Up-regulation of the IGF-I Gene\textsuperscript{*}\textsuperscript{,}\textsuperscript{†}\textsuperscript{,}\textsuperscript{‡}\textsuperscript{,}\textsuperscript{§}\textsuperscript{1}

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Vikram Devgan\textsuperscript{i}, Bach-Cuc Nguyen\textsuperscript{i}, Heysun Oh\textsuperscript{i}, and G. Paolo Dotto\textsuperscript{‡§1}

From the \textsuperscript{‡}Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129 and \textsuperscript{§}Department of Biochemistry, Lausanne University, CH-1066 Epalinges, Switzerland

p21 plays a dual role in keratinocyte growth and differentiation control. It restricts the number of keratinocyte stem cell populations while inhibiting the later stages of differentiation independently of the cell cycle. The molecular/biochemical mechanism for the differentiation suppressive function of p21 is unknown. Here we show that elevated p21 expression leads to activation of MAPK family members in a keratinocyte-specific and cell cycle-independent manner, and up-regulation of MAPK activity can explain the inhibitory effects of p21 on differentiation. p21 induces transcription of several genes with MAPK activation potential. Although several of these genes are induced by p21 in a MAPK-dependent manner, expression of IGF-I is induced upstream of MAPK activation. IGF-1 stimulation is by itself sufficient to cause MAPK activation and inhibit differentiation and suppression of IGF-1 signaling by knock down of the cognate receptor (IGF-R1), diminishing the ability of p21 to activate MAPK and suppress differentiation. Thus, in keratinocytes, the ability of p21 to suppress differentiation can be explained by cell type-specific activation of the MAPK cascade by transcriptional up-regulation of the IGF-I gene.

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\textsuperscript{†} The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

\textsuperscript{i} To whom correspondence should be addressed: Dept. of Biochemistry, University of Lausanne, Chemin de Bovaresses 155, CH-1066 Epalinges, Switzerland. Tel.: 41-21-692-5720; Fax: 41-21-692-5705; E-mail: Gian-Paolo.Dotto@unil.ch.

\textsuperscript{‡} The abbreviations used are: CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; MAPK, mitogen-activated protein kinase; IGF, insulin-like growth factor; GFP, green fluorescent protein; JNK, c-Jun NH\textsubscript{2}-terminal kinase; ERK, extracellular signal-regulated kinase; STAT, signal transducers and activators of transcription; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; IGF-R, IGF receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; IGF-BP, IGF-binding protein; m.o.i., multiplicity of infection.

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\textsuperscript{1} Down-modulation of p21 expression is not limited to the keratinocyte/epithelial cell systems as it has also been observed with differentiating osteoblasts, and even in these cells overexpression of p21 exerts inhibitory effects on differentiation (15). In retinoic acid-induced differentiation of acute promyelocytic leukemia cells, p21 has also been shown to play a necessary function, which is unlinked from its effects on the cell cycle (16). However, in this case, unlike in the keratinocyte and osteoblast systems, p21 appears to play a positive promoting role in differentiation. Finally an essential function of p21 in differentiation has also been demonstrated for the myoblast system by the analysis of mice with a double knock-out mutation of p21 and the related p57 gene (17). Thus, the emerging consensus is that p21 can play an important regulatory function in differentiation that is not directly linked to its effects on the cell cycle and that can be either negative or positive depending on cell type and specific stages of differentiation.
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In the present study, we explored the mechanism whereby persistently elevated p21 expression suppresses late stages of keratinocyte differentiation. We show that this cell cycle-independent function of p21 involves cell type-specific activation of MAPK family members through selective changes in gene expression specifically by induction of IGF-I gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Adenoviral Infection—Primary mouse keratinocytes were cultured in medium at low calcium concentration (0.05 mM) and induced to differentiate by addition of CaCl2 to the medium (2 mM) or suspension culture as described previously (14). All adenovirus infections were performed for 1 h in serum and epidermal growth factor-free-low calcium medium as described previously (14). Keratinocytes were then incubated in fully supplemented medium for 24 h prior to collection for further analysis. Use of adenoviruses expressing full-length and amino-terminal p21, p16, p27, and GFP (14) was described previously.

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IGF-I ELISA Analysis—Concentrations of IGF-I in serum-free conditioned medium were measured by ELISA using a commercial kit specific for mouse IGF-I (OCTEIA® rat/mouse IGF-I ELISA kit, Immunodiagnostic Systems Inc., Fountain Hills, AZ) according to the manufacturer’s protocol. Briefly primary mouse keratinocytes were infected with adenoviruses expressing p21 or GFP. After 24 h, conditioned medium was collected and concentrated using a UFV2BCC10 filter device (Millipore, Bedford, MA). Concentrated medium was pretreated for 10 min with a releasing reagent to denature IGF-BPs. Samples were mixed with a diluent and were aliquoted in duplicate into microtiter strip wells coated with a polyclonal IGF-I antibody. A monoclonal IGF-I antibody labeled with horseradish peroxidase was added to the wells and incubated for 2 h at room temperature. Following the incubation, wells were washed three times prior to addition of enzyme substrate. Samples were then incubated for 30 min. The resulting yellow acid dye at 450 nm with a reference filter of 620 nm was measured by a plate reader. Serial dilutions of recombinant mouse IGF-I were used to calibrate the assay and ensure that experimental samples were within the linear range of detection.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP analysis was carried out as described previously (9). Briefly ~6 × 10⁶ primary mouse keratinocytes were fixed with formaldehyde and lysed in SDS lysis buffer (chromatin immunoprecipitation assay kit; Upstate Biotechnology). DNA in the cross-linked chromatin preparations was fragmented by sonication to an average size of 500 bp to 1 kb. Samples were precleared with salmon sperm DNA/protein A-agarose (50% slurry). Antibodies and fresh protein A-agarose were added and incubated overnight at 4 °C. Non-immune controls were performed by incubating parallel samples with non-immune IgG/non-immune serum and/or coated beads alone with similar background levels being obtained in all cases. Precipitated chromatin complexes were removed from the beads through 30-min incubation with 50 µl of elution buffer (1% SDS, 0.1 M NaHCO₃). Finally the protein-DNA cross-links were reversed by overnight incubation at 65 °C, and immunoprecipitated DNA was analyzed by real time PCR. Primers for this analysis are indicated in supplemental Table S1.

siRNA Transfection and Analysis—Primary mouse keratinocytes were transfected with 200 nM siRNAs for mouse IGF-R1 (sense, CCCAAGCGUGUGUGCUCCGAAUUU; antisense, AAAAAUGGAGACACACAGCUUGGG; Stealth RNAi, Invitrogen) and control siRNAs (Stealth RNAi negative control, Invitrogen) using Lipofectamine 2000 following the manufacturer’s recommendations as we described previously (18). Cells were infected with adenoviruses 48 h after transfection. Subsequently 24 h after infections, cells were analyzed by Western blot as indicated.

RESULTS

The Negative Effect of p21 on Keratinocyte Differentiation Is Mediated by MAPK Activation—We previously showed that the suppressing effects of p21 on keratinocyte differentiation occur at the level of gene transcription (14). However, the underlying biochemical mechanism(s) was not established. MAPK family members have been connected with control of broad aspects of cell physiology, including chromatin rea-
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p16\textsubscript{Ink4a} CDK inhibitor, both of which cause growth arrest without effects on differentiation (14). Activation levels of the ERK1/2, JNK, and p38 kinases were significantly elevated by overexpression of full-length p21 (Fig. 1A), whereas little or no up-regulation was caused by overexpression of the p21 amino-terminal domain or p16\textsubscript{Ink4a} (Fig. 1A). These findings were confirmed by immunoblotting analysis showing a dose-dependent increase of the phosphorylated active form of ERK1/2 as a function of increased p21 expression (Fig. 1B). To assess whether p21 induces MAPK activation in a cell-type specific manner, we performed a parallel analysis of primary keratinocytes and dermal fibroblasts infected with the above adenoviruses. Although overexpression of full-length p21 induced substantial increase of the phosphorylated activated forms of ERK1/2, JNK1/2, and p38 kinases in primary keratinocytes, no such effect occurred in the dermal fibroblasts (Fig. 1C).

The TSG101 protein, a ubiquitin ligase-related protein involved in large multilamellar body cell sorting (21), binds the p21 protein and causes its stabilization in keratinocytes, thereby suppressing differentiation (22). To determine whether stabilization of endogenous p21 by TSG101 overexpression also results in MAPK activation, primary keratinocytes derived from wild p21\textsuperscript{+/+} versus p21\textsuperscript{−/−} mice were infected with a recombinant TSG101-expressing adenovirus. In parallel with increased levels of endogenous p21 (Fig. 2A), TSG101 overexpression induced activity of the ERK1/2, JNK, and p38 kinases only in wild type keratinocytes but not p21\textsuperscript{−/−} cells (Fig. 2, B and C).

To assess whether the induction of MAPK activity by p21 can account for its suppressive effects on differentiation, primary keratinocytes were infected with control versus p21-expressing adenoviruses followed by treatment with PD98059, a MAPK kinase (MEK) inhibitor, or the unrelated protein kinase C inhibitor GF109203X. PD98059 treatment was sufficient to reverse suppression of differentiation marker expression caused by p21 overexpression, whereas this reversion of effects was not observed after treatment with GF109203X (Fig. 3A).

A primary mouse keratinocytes were either uninfected (Ctrl) or infected with adenoviruses expressing GFP (Ad-GFP), full-length p21 protein (Ad-p21F), the p21 amino-terminal domain (Ad-p21N), and p16\textsubscript{Ink4a} (Ad-p16) for 24 h. Total cell extracts were immunoprecipitated with immobilized anti-phospho-ERK1/2 or anti-phospho-p38 antibodies or with c-Jun-conjugated beads, and kinase activities were measured by in vitro assays with ELK-1, ATF-2 and c-Jun as exogenous substrates. The same extracts were normalized for MAPK expression by immunoblotting with the corresponding antibodies. The right panel shows quantification of the results by densitometry after normalization for total level of each MAPK. B, primary keratinocytes were infected with Ad-GFP at 100 multiplicity of infection (m.o.i.) or Ad-p21F at the indicated m.o.i. for 24 h. Total cell extracts were analyzed by immunoblotting with antibodies specific for p21, the activated phosphorylated forms of ERK1/2, and c-Jun (as equal loading control). The lower panel shows quantification of the results by densitometry after normalization for c-Jun levels. C, primary mouse keratinocytes and primary dermal fibroblasts were infected with the same adenoviruses as in A for 24 h. Total cell extracts were analyzed by immunoblotting with antibodies specific for the activated phosphorylated forms of ERK1/2, JNK1/2, and p38 in parallel with antibodies recognizing the total proteins. The Ad-p21F virus was expressed at similar levels in keratinocytes and dermal fibroblasts as verified by immunoblotting with anti-p21 antibodies (data not shown). p-, phospho-; Ctrl, control.
FIGURE 2. Endogenous p21 can induce activation of MAPK. A, primary keratinocytes were infected with Ad-GFP or an adenovirus expressing the TSG101 protein (Ad-TSG101) at the indicated m.o.i. for 24 h. Total cell extracts were analyzed by immunoblotting with antibodies specific for the p21 and γ-tubulin. The upper panel shows quantification of the results by densitometry after normalization for γ-tubulin levels. B and C, primary keratinocytes derived from p21+/− and p21−/− mice of the same genetic background (Sencar) were infected with Ad-GFP at 50 m.o.i. or Ad-TSG101 at the indicated m.o.i. for 24 h. Total cell extracts were analyzed by immunoblotting with antibodies specific for the phosphorylated activated forms of MAPKs as well as with antibodies recognizing the total proteins. C, quantification of the results by densitometry after normalization for total level of each MAPK. WT, wild type; KO, knock-out; p-, phospho-

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To evaluate whether increased MAPK activity is by itself sufficient to suppress differentiation, primary keratinocytes were transfected with expression plasmids for the ERK1, JNK1, or p38 kinases together with a trace amount of a GFP expression vector. The transfected GFP-positive cells were purified by sorting. Increased ERK1 activity, as achieved by overexpression of this kinase, was sufficient to totally suppress differentiation, whereas lesser inhibitory effects were exerted by JNK1 or p38 (Fig. 3B).

p21 Induces IGF-I Expression at the Transcription Level Independently of the Cell Cycle and Upstream of MAPK Activation—Several studies have established a possible connection between p21 and control of transcription (9–11, 23). Therefore, an attractive possibility is that the keratinocyte-specific activation of MAPK by p21, with consequent suppression of differentiation, is mediated by the induction of genes with MAPK activation potential. As a part of our more general studies on the role of p21 in keratinocytes, we have used global analysis of gene expression to identify genes that are modulated by p21 in these cells (9, 23). Using this approach, a restricted number of genes were identified with a known MAPK activation potential, including three genes encoding diffusible factors, IGF-I (24, 25), IGF-BP5 (26), GRO-1 (27), and a gene for a tyrosine kinase receptor, epithelial cell kinase (28). To assess whether these genes are indeed under p21 control in a manner not directly linked to the cell cycle, primary mouse keratinocytes were infected with adenoviruses expressing full-length 21 (p21F) or the amino-terminal domain (p21N), which is capable of causing cyclin/CDK inhibition and growth arrest without suppressing differentiation (14), in parallel with a GFP control. Real time RT-PCR showed that these genes are induced in keratinocytes by expression of the full-length p21 protein but not by the p21 amino-terminal domain (Fig. 4, A–D). Although some of these genes may be primary targets of p21 up-regulation, others might be induced as a secondary consequence of increased expression of other genes and/or MAPK activation. In fact, the ability of p21 to induce endogenous IGF-BP5, GRO-1, and epithelial cell kinase expression was countered substantially by treatment with the MEK inhibitor PD98059 (Fig. 4, B–D). In contrast, induction of the endogenous IGF-I gene by p21 was not blocked by the MEK inhibitor; rather it was superinduced (Fig. 4A).

To further confirm that the p21-mediated induction of IGF-I mRNA levels is not an indirect consequence of its CKI activity, primary keratinocytes were infected in parallel with adenoviruses expressing the unrelated CKIs p16Ink4a or p27Kip1, which have CKI activity but no effect on differentiation (14). Expres-
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FIGURE 4. Increased p21 protein level up-regulates IGF-I expression independently of MAPK activation and cell cycle control. A–D, primary keratinocytes were infected with Ad-GFP (GFP), Ad-p21N (p21N), and Ad-p21F (p21F) with or without concomitant treatment with the MAPK inhibitor PD98059 (25 or 50 μM) for 24 h. mRNA levels for IGF-I (A), IGF-BP5 (B), GRO-1 (C), and ECK (D) were quantified by real time RT-PCR. Values are expressed as relative arbitrary units after normalization for glyceraldehyde-3-phosphate dehydrogenase mRNA levels. E, primary keratinocytes were infected with Ad-GFP (GFP), Ad-p21F (p21F), Ad-p16Ink4a (p16), and Ad-p27Kip1 (p27) for 24 h followed by IGF-I mRNA quantification as before. F, primary keratinocytes were infected with Ad-GFP (GFP) and Ad-p21F (p21) and cultured in serum-free medium for 24 h. Conditioned medium was subjected to ELISA according to the manufacturer's instruction to measure the levels of IGF-I protein. G, mouse L-B10.BR melanocytes were infected with Ad-GFP (GFP) and Ad-p21F (p21) for 24 h. Adenoviral-mediated expression of p21 was verified by immunoblotting (data not shown). mRNA levels of IGF-I was measured by real time RT-PCR as before. ECK, epithelial cell kinase.

The above results showed that induction of IGF-I expression by p21 is independent of MAPK activation. This raised the possibility that induction of IGF-I might itself account for the ability of p21 to trigger MAPK activation and suppress differentiation. To assess this possibility, we analyzed at first the effect of IGF-I stimulation on MAPK activity in primary mouse keratinocytes. Consistent with previous reports in other cell types (25), IGF-I stimulation resulted in significant ERK1/2 activation with a similar but milder effect on p38 and JNK (Fig. 6A). In parallel with these effects, IGF-I stimulation was sufficient to reproduce the inhibitory effects of p21 on differentiation as assessed by immunoblotting of primary mouse keratinocytes upon induction of differentiation by increased extracellular calcium (Fig. 6B).

To assess whether endogenous IGF-I signaling mediates p21-dependent MAPK activation and suppression of differentiation, we knocked down expression of its cognate active receptor, IGF-R1, by transfection with corresponding specific siRNA oligomers. This approach caused very substantial suppression of IGF-R1 protein levels (Fig. 6, C and D). Concomitantly we found that increased p21 levels, as achieved by adenoviral infection, triggered little or no MAPK activation in keratinocytes with IGF-R1 knock down relative to the controls (Fig. 6C). Likewise knock down of
IGF-R1 significantly diminished the ability of p21 to suppress differentiation (Fig. 6D).

To assess whether IGF-I is under p21 control in vivo, in a manner that may be inversely related with differentiation, we measured levels of IGF-I and K1 mRNA expression in the epidermis of p21−/− versus p21+/+ mice. As shown in Fig. 7, there was significant down-regulation of IGF-I expression in the epidermis of p21−/− mice that was accompanied by a concomitant up-regulation of K1 expression.

DISCUSSION

Earlier work has shown that down-modulation of p21 expression is required for later stages of keratinocyte differentiation to occur and that persistent p21 expression under these conditions suppresses differentiation (13, 14). We have shown here that the differentiation suppressive function of p21 in this context can be attributed to the cell type-specific activation of the MAPK cascade through transcriptional up-regulation of the IGF-I gene.

We found that increased p21 expression in keratinocytes activates MAPKs of all three families, ERK1/2, JNK, and p38. Of these, ERK1/2 activation exerted the strongest suppressive effects on differentiation, and ERK1/2 inhibition was sufficient to counteract the suppressive effect of p21 on differentiation. p21 participates in a number of protein-protein interactions that can directly affect activity of MAPK family members (8). In particular, it was found to bind directly to JNK/stress-activated protein kinase and not only inhibit its intrinsic catalytic activity but also block its phosphorylation and activation by the upstream MKK4 kinase (31). p21 could also inhibit activity of p38 kinase, whereas it had little if any inhibitory effect on ERK1/2 (31). Besides direct inhibition of JNK and p38 kinases, p21 has the potential of associating directly with an upstream regulator, the apoptosis signal-regulated kinase 1 (ASK1/MEKK5), with consequent suppression of the downstream JNK cascade (6, 32). However, such direct inhibitory mechanisms cannot account for our finding that increased p21 expression induces MAPK activation specifically in keratinocytes and not dermal fibroblasts, which is consistent with a previous report that p53 but not p21 induces MAPK in 293 cells (33). Rather the keratinocyte-specific activation of MAPK by p21 can be explained by its capability to affect transcription. We have shown previously that p21 can negatively regulate expression of specific genes in keratinocytes, such as Wnt4, involved in control of stem cell potential, and glucocorticoid-induced tumor necrosis factor receptor, important for the keratinocyte UVB...
In the present studies we found a restricted number of genes with MAPK activation potential that are induced rather than suppressed by increased p21 expression in keratinocytes. As in our previous studies, modulation of these genes by p21 was independent of effects on the cell cycle. Importantly of these genes only IGF-I was induced by p21 independently and upstream of MAPK activation. In mammalian cells, control of IGF-I expression occurs predominantly at the level of transcription (34). Consistent with this mode of regulation, increased p21 expression enhanced the recruitment of RNA polymerase II to the proximal region of the IGF-I promoter 1, which is the major promoter for the gene, located 5′ to exon 1 (35). Recent evidence from our laboratory as well as others has shown that p21 can function as a transcripational co-regulator by binding to promoters of genes in association with specific DNA-binding proteins, such as E2F-1 and estrogen receptor (9, 10). The IGF-I promoter 1 contains binding sites for E2F-1 and STAT3 (Fig. 4A). However, by chromatin immunoprecipitation we found that neither the E2F-1 nor p21 protein bind to this promoter, although there was constitutive binding of STAT3 that was unaffected by p21 levels. It is thus likely that the enhanced recruitment of RNA polymerase II to IGF-I promoter and its increased transcription are a more indirect result of the changes in gene expression triggered by p21 in a cell cycle-independent and keratinocyte-specific fashion (5–7).

IGF-I is mitogenic for both mouse and human keratinocytes (36, 37) and contributes to hair follicle morphogenesis and differentiation (38). Although some reports indicate that IGF-I is not expressed in keratinocytes (39), others have pointed to IGF-I expression in these cells and its possible role in autocrine control of differentiation (40). In agreement with a previous study (24) we found that IGF-I functions as a negative regulator of keratinocyte differentiation in a manner that is linked to MAPK activation. Importantly knock down of IGF-R1 abolished the ability of p21 to activate MAPK and suppress differentiation, demonstrating that IGF-I signaling is the mediator of the differentiation suppressive function of p21 in keratinocytes. The in vivo significance of these findings is supported by the decrease of IGF-I expression, together with a concomitant increase of K1 expression, in the epidermis of p21-null mice versus wild type controls. Our results are fully consistent with a recent report on the negative role of IGF-I signaling in keratinocyte differentiation in vivo. In particular, conditional deletion of the IGF-R1 gene in the skin results in abnormal epidermal differentiation with up-regulation of keratin 1 and 10 expression linked to a decrease in ERK1/2 activity (41).

In human conditions of keratinocyte hyperproliferation, like oral squamous cell carcinomas and psoriasis, p21 expression...
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FIGURE 7. p21 regulates expression of IGF-I and K1 in mouse epidermis. Total RNA was prepared from the separated epidermis of two p21+/− mice and two genetically matched p21+/+ controls, and for each mouse, levels of IGF-I and K1 expression were determined by real time RT-PCR as before. The epidermis was separated from the underlying dermis by a brief heat treatment as we described previously (18). Values are expressed as arbitrary units after glyceraldehyde-3-phosphate dehydrogenase normalization.

can be paradoxically increased (42, 43). As with keratinocytes of murine origin, we found that even in primary human keratinocytes and oral squamous carcinoma cells increased p21 levels lead to induction of IGF-I expression (data not shown), pointing to a possibility, even in a clinical setting, of a dual role of p21 as both a negative and positive growth regulator and/or enhancer of cell survival.

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