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p19^{ARF}-independent induction of p53 and cell cycle arrest by Raf in murine keratinocytes

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In tumorigenesis of the skin, activated Ras co-operates with mutations that inactivate the tumour suppressor p53, but the molecular basis for this co-operation remains unresolved. Here we show that activation of the Raf/MAP kinase pathway in primary mouse keratinocytes leads to a p53 and p21^{Cip1}-dependent cycle arrest and to terminal differentiation. Raf activation in keratinocytes lacking *p53* or *p21^{Cip1}* genes leads to expression of differentiation markers, but the cells do not cease to proliferate. Thus, loss of p53 or p21^{Cip1} function is necessary to disable growth-inhibitory Raf/MAP kinase signalling. Activation of oncogenes, including *Ras*, has been reported to stabilize and activate p53 via induction of the tumour suppressor p19^{ARF}. However, the response to Raf in p19$^{ARF-/-}$ keratinocytes was indistinguishable from wild-type controls. Thus, p19^{ARF} is not essential for Raf-induced p53 induction and cell cycle arrest in keratinocytes, indicating that oncogenes engage p53 activity via multiple mechanisms.

INTRODUCTION

Tumorigenesis is a multi-step process and requires the co-operation of several distinct oncogenic mutations. Frequently two of these mutations are required to establish a signalling configuration promoting cell division. For example, activated Ras or Raf induce cell cycle arrest in various cell types prior to immortalization and at high signalling strength in immortalized fibroblasts (Hirakawa and Ruley, 1988; Ridley *et al.*, 1988; Lloyd *et al.*, 1997; Sewing *et al.*, 1997; Woods *et al.*, 1997). In non-immortalized fibroblasts this cell cycle arrest has been associated with premature senescence (Serrano *et al.*, 1997). Ras/Raf-induced cell cycle arrest can be engaged via multiple pathways. In Schwann cells, Ras and Raf induce rapid cell cycle arrest via p53-dependent induction of the cell cycle inhibitor p21^{Cip1} (Lloyd *et al.*, 1997). In fibroblasts, Ras/Raf activity can induce p21^{Cip1} expression and cell cycle arrest through a p19^{ARF} and p53-dependent (reviewed in Sherr and Weber, 2000) or a p53-independent mechanism (Sewing *et al.*, 1997; Woods *et al.*, 1997). To disable growth-inhibitory Ras/Raf/MAP kinase signalling, loss of p19^{ARF}, p53 or p21^{Cip1} function is required.

Most malignant forms of cancer are of epithelial origin and activation of the Ras–Raf–MAP kinase pathway can occur in conjunction with loss of p53 function. This is reflected in experimentally-induced tumours in the murine epidermis, one of the best studied models of multi-stage epithelial carcinogenesis (reviewed in Frame and Balmain, 2000). Treatment of mouse skin with the carcinogen DMBA results in the activation of the ras oncogene with p53 mutations appearing as a later event during progression to malignancy. Ras and loss of p53 have been directly shown to co-operate in the transformation of murine keratinocytes both *in vitro* and *in vivo* (Kemp *et al.*, 1993; Weinberg *et al.*, 1994). Similarly, activated Ras co-operates with loss of p21^{Cip1} function (Missero *et al.*, 1996; Philipp *et al.*, 1999; Topley *et al.*, 1999; Weinberg *et al.*, 1999). However, the molecular basis of the co-operation between these oncogenic defects has not been explored in epithelial cells. This is particularly relevant, as the interactions between Ras/Raf and p53/p21^{Cip1} differ between fibroblasts and Schwann cells. We thus have investigated the molecular mechanisms involved in the co-operation between Raf activation and p53, p19^{ARF} or p21^{Cip1} loss-of-function in cultures of murine keratinocytes.

RESULTS

Raf activation in primary mouse keratinocytes induces cell cycle arrest and differentiation

To investigate the response of primary mouse keratinocytes to the activation of Raf, we used a conditional Raf-oestrogen receptor

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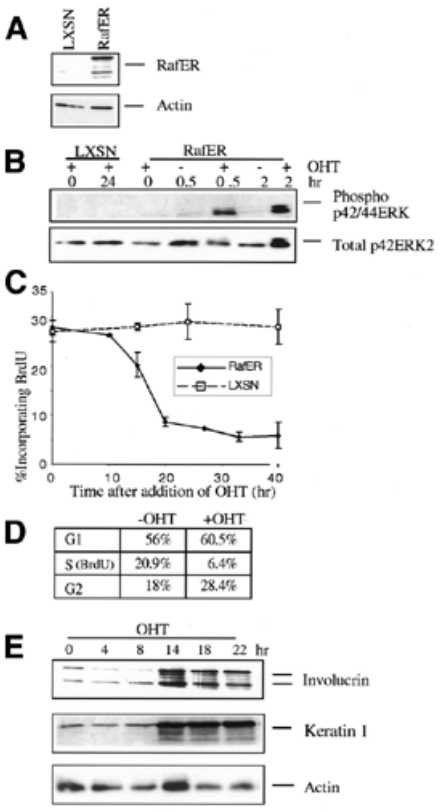

Fig. 1. Raf activation in primary mouse keratinocytes induces cell cycle arrest and differentiation. (**A**) Expression of RafER in primary mouse keratinocytes infected with RafER and empty vector (LXSN) retroviruses. Protein (20 μg) was separated on SDS-12% polyacrylamide gels and probed with with antibodies specific for ER or actin. (**B**) RafER or LXSN infected keratinocytes were lysed at the indicated time points following addition of 200 nM 4-hydroxytamoxifen (OHT). The lysates were normalized for protein content followed by western blotting using either an anti-phospho-MAP kinase or a p42ERK2 antibody. (**C**) RafER or LXSN infected keratinocytes were analysed for BrdU incorporation in triplicate via LSC at different time points after addition of OHT. (**D**) RafER infected keratinocytes were cultured in the absence or presence of OHT for 24 h. Cells were analysed for BrdU uptake and examined for DNA content, by propidium iodide staining. (**E**) Protein lysates were prepared from RafER infected keratinocytes after addition of OHT at the time points indicated. These were analysed by western blotting with antibodies specific for involucrin, keratin 1 and actin. Multiple forms of murine involucrin are detected (Li *et al*., 2000).

chimera (RafER) (Lloyd *et al*., 1997). The chimeric protein could be detected in keratinocytes infected with RafER retrovirus but not in control cells (Figure 1A). RafER was activated in response to 4-hydroxytamoxifen (OHT), as monitored by the phosphorylation of MAP kinase (Figure 1B). Activation of RafER in pools of infected keratinocytes led to inhibition of DNA synthesis (Figure 1C) with similar kinetics as observed in Schwann cells and fibroblasts (Lloyd *et al*., 1997; Sewing *et al*., 1997), due to an arrest in both G_1 and G_2 phases of the cell cycle (Figure 1D). Raf activation also resulted in a dramatic change in keratinocyte morphology within 30 h. As compared with control-infected cells or cells with inactive RafER, keratinocytes containing activated RafER became enlarged, highly spread and then detached from the dish (not shown). This alteration in cell shape and cell density is characteristic of differentiating keratinocytes and suggests that Raf may be inducing differentiation.

Involucrin and keratin 1 are suitable markers for keratinocyte differentiation *in vitro* (reviewed in Yuspa, 1994). To determine whether RafER can cause differentiation in primary keratinocytes the expression of involucrin and keratin 1 in response to Raf activation was measured. Western blot analysis revealed an induction of involucrin and keratin 1 in RafER-keratinocytes 14–16 h after OHT treatment (Figure 1E), an effect not seen in keratinocytes infected with control virus (not shown). Thus, RafER activation induces keratinocyte differentiation *in vitro*.

Raf induces p53 and p21^{Cip1}

To determine whether the RafER induced cell cycle arrest in keratinocytes is linked to cell cycle inhibitors, cell lysates were prepared at various time points after RafER activation and analysed by immunoblotting. Activation of RafER led to an increase in the levels of p53 and its target p21^{Cip1} between 8 and 14 h after addition of OHT (Figure 2A and B and data not shown). In contrast, RafER activation has no effect on p16^{INK4A} levels or on p19^{ARF} expression (Figure 2B and C). Cyclin E, Cdk2 and Cdk4 expression levels remain relatively constant (Figure 2B). However, cyclin E-dependent kinase activity is reduced almost to background levels in the arrested cells (Figure 2D), presumably due to the increase in p21^{Cip1} levels, as demonstrated in Schwann cells and fibroblasts (Lloyd *et al*., 1997; Sewing *et al*., 1997; Woods *et al*., 1997). Cyclin A expression and cyclin D1 and D2 levels are downregulated in Raf-arrested keratinocytes.

Raf-induced cell cycle arrest, but not differentiation, is p53 dependent

To determine the role of p53 in the RafER-induced arrest, p53$^{-/-}$, p53$^{-/+}$ and wild-type (wt) primary mouse keratinocytes derived from littermates were infected with RafER. Wt and p53$^{-/+}$ cells arrest equally well in response to Raf. In contrast, following Raf activation, p53$^{-/-}$ keratinocytes remain in cycle (Figure 3A), although MAP kinase activation occurs to a similar extent in p53$^{-/-}$ and control cells (Figure 3B). Hence, similar to Schwann cells, the RafER-induced arrest in mouse keratinocytes is p53-dependent. The induction of the differentiation markers involucrin and keratin 1 by Raf is p53-independent and still occurs in the absence of cell cycle arrest with the same kinetics as in cells with functional p53 (Figures 3B and 1E). Thus, Raf-induced differentiation can be uncoupled from cell cycle arrest.

The induction of p21^{Cip1} by Raf is lost in p53$^{-/-}$ cells but is maintained in p53$^{-/+}$ cells (Figure 3B) Conversely, the loss of a single copy of the *p53* gene renders cyclin D expression responsive to Raf activation in keratinocytes, as observed in wt fibroblasts and Schwann cells. Similarly, the downregulation of cyclin A expression following Raf activation in wt keratinocytes is partially relieved even by loss of a single copy of the *p53* gene (Figure 3B). Taken together these observations suggest a role for p21^{Cip1} in the Raf-induced cell cycle arrest (see also below), while downregulation of cyclin A or cyclin D levels appear not essential in this context.

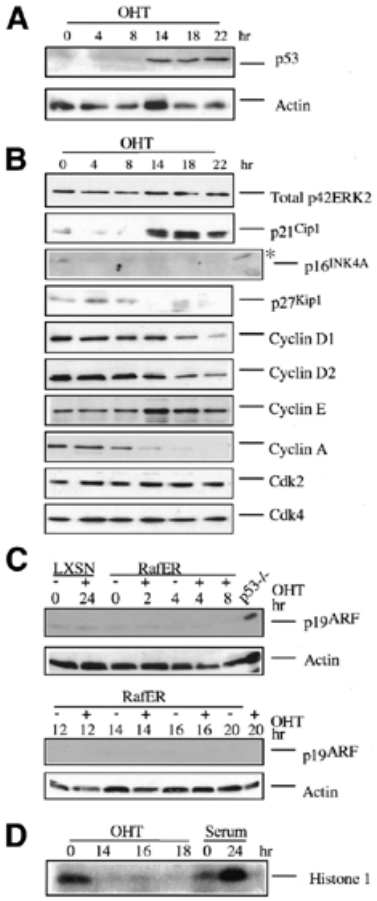

Fig. 2. Raf induces p53 and p21^{Cip1}. (**A**–**C**) Protein lysates were prepared from RafER infected keratinocytes at the time points indicated after addition of OHT and were analysed by western blotting with antibodies specific for the indicated proteins. (**D**) RafER infected keratincytes were cultured in the presence of OHT for the indicated time points. Lysates (200 µg) were immunoprecipitated with a cyclin E antibody and then assayed for histone H1 kinase activity (*). The positive p16^{INK4A} control was from RafER-infected p21$^{Cip1-/-}$ mouse embryo fibroblasts (MEFs) in which RafER had been activated for 7 days.

Induction of p53 and cell cycle arrest by Raf is p19^{ARF}-independent

In wt mouse embryo fibroblasts, Ras induced growth arrest, induction of p53, and senescence all depend on the presence of the tumour suppressor p19^{ARF} (reviewed in Sherr and Weber, 2000). However, in mouse primary keratinocytes no induction of p19^{ARF} protein can be seen after Raf induction (Figure 2B). To test the role of p19^{ARF} in Raf-mediated cell cycle arrest we introduced RafER into p19$^{ARF-/-}$ keratinocytes. Upon activation, RafER induces proliferation arrest (Figure 4A) preceeded by p53 induction (Figure 4B) in these cells with similar efficiency as in wt cells. Induction of differentiation markers and alterations in cell morphology also are indistinguishable from wt controls (not shown). Thus, RafER induction of p53 is p19^{ARF}-independent in this epithelial cell type.

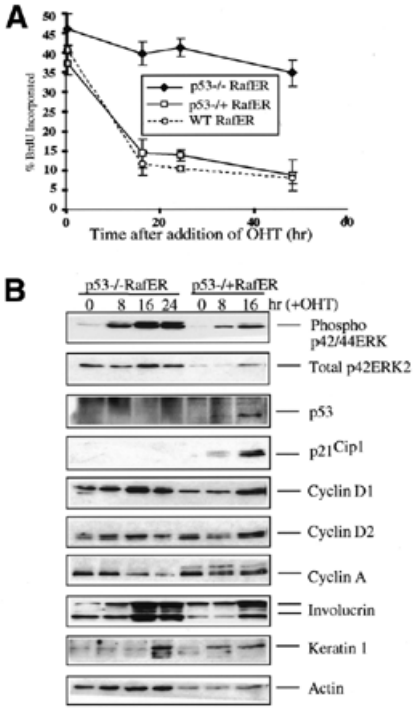

Fig. 3. Raf-induced cell cycle arrest, but not differentiation, is p53-dependent. (**A**) RafER-infected littermate wt, p53$^{-/+}$ or p53$^{-/-}$ keratinocytes were analysed for BrdU incorporation by LCS in triplicate at different time points after addition of OHT. (**B**) Protein lysates were prepared from RafER infected p53$^{-/+}$ or p53$^{-/-}$ keratinocytes at the time points indicated after addition of OHT and were analysed by western blotting for expression of the specified proteins. Multiple independently derived littermate-controlled cell batches gave consistent results.

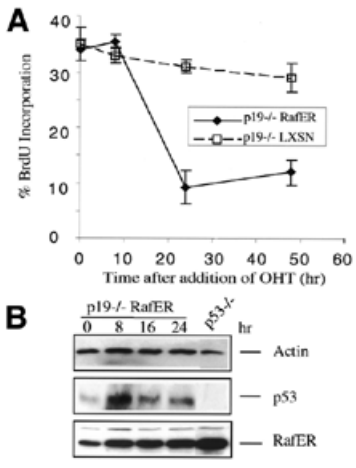

Fig. 4. Induction of p53 and cell cycle arrest by RafER is p19^{ARF}-independent. (**A**) RafER or LXSN-infected p19$^{ARF-/-}$ keratinocytes were analysed for BrdU incorporation in triplicate on the LCS at different time points after addition of OHT. (**B**) Protein lysates were prepared from RafER infected p19$^{ARF-/-}$ keratinocytes after addition of OHT at the time points indicated and were analysed by western blotting with antibodies specific for p53, ER or actin. Two independently derived littermate-controlled cell batches produced consistent results.

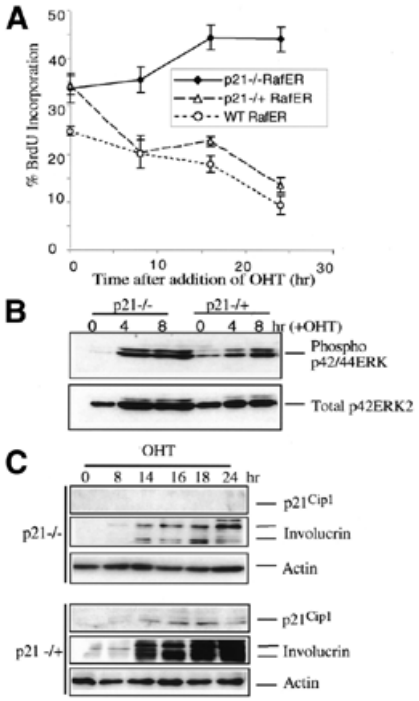

Fig. 5. The RafER arrest is p21^{Cip1}-dependent. (**A**) RafER-infected littermate wt, p21$^{Cip1-/+}$, and p21$^{Cip1-/-}$ keratinocytes were analysed for BrdU incorporation in triplicate at different time points after addition of OHT. (**B**) RafER infected wt, p21$^{Cip1-/+}$, and p21$^{Cip1-/-}$ keratinocytes were lysed at the indicated time points following addition of 200 nM OHT. The lysates were analysed as described in Figure 1B. (**C**) RafER infected p21$^{Cip1-/+}$, and p21$^{Cip1-/-}$ keratinocytes were lysed at the indicated time points following addition of OHT. The lysates were analysed by western blotting with antibodies specific for p21^{Cip1}, involucrin or actin.

The RafER arrest is p21^{Cip1}-dependent

To investigate the role of p21^{Cip1} in RafER-induced arrest, RafER was introduced into littermate p21$^{Cip1-/-}$, p21$^{Cip1-/+}$ and wt keratinocytes and the cells were analysed for BrdU incorporation after addition of OHT (Figure 5A). Activation of RafER in the p21$^{Cip1-/+}$ and wt infected cells resulted in inhibition of DNA synthesis with similar kinetics. In contrast, the p21$^{Cip1-/-}$ cells did not undergo a cell cycle arrest in response to Raf, although MAP kinase activation was similar in all genotypes analysed (Figure 5B). The extent of p21^{Cip1} induction was similar in both p21$^{Cip1-/+}$ cells and wt cells, and p21^{Cip1} protein is not detectable in the p21$^{Cip1-/-}$ cells (Figure 5C). However, as in p53$^{-/-}$ cells, involucrin (Figure 5C) and keratin 1 (not shown) are still induced in the p21$^{Cip1-/-}$ keratinocytes, albeit to a lesser degree than in p21$^{Cip1-/+}$ and wt cells. Thus, as in fibroblasts, p21^{Cip1} is essential for Raf-induced cell cycle arrest, but p21^{Cip1} is not a prerequiste for keratinocyte differentiation.

Similar to Raf, Ras can inhibit proliferation and induce differentiation

Murine keratinocytes expressing Ha-Ras^{V12} have been reported to become hyperproliferative, resistant to Ca^{2+}-induced terminal differentiation and exhibit an extended lifespan relative to

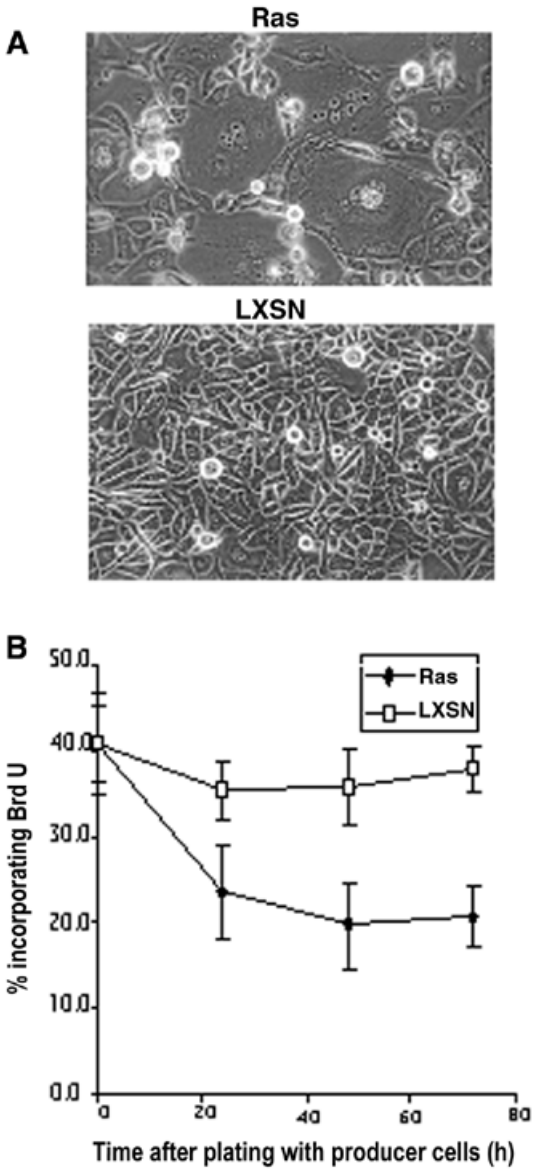

Fig. 6. Ras in primary mouse keratinocytes induces a morphological change and a cell cycle arrest. (**A**) Phase-contrast micrographs of keratinocytes infected with Ras or control virus (LXSN) for 48 h. (**B**) Ras or LXSN-infected keratinocytes were analysed for BrdU incorporation. The ratio of BrdU-positive and Hoechst 33258-stained nuclei was determined in triplicate cultures at the indicated time points.

control uninfected keratinocytes in culture (Yuspa *et al.*, 1983; Roop *et al.*, 1986; Dotto *et al.*, 1988). However, the cells used in these experiments were derived after cultivating retrovirally infected cells expressing constitutively activated Ras for 1 week. We examined the effect of activated Ras, on primary keratinocytes only 24 and 48 h after virus exposure. Similar to the response of keratinocytes to Raf activation, Ras-infected keratinocytes showed cell enlargement and incorporated BrdU to a much lower degree than cultures treated with control virus (Figure 6). Moreover, the Ras-infected cells also show induced

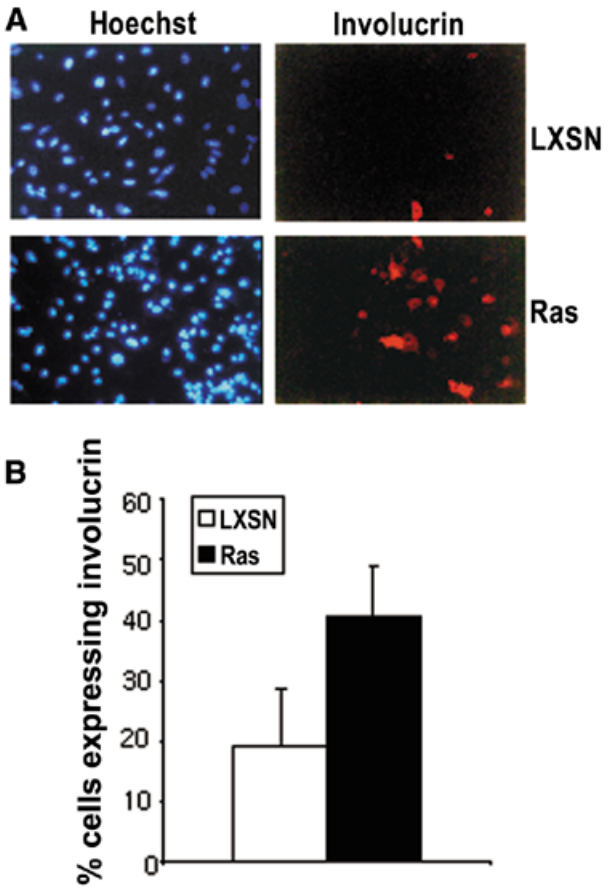

Fig. 7. Ras induces the differentiation marker involucrin. (**A**) Ras and LXSN-infected keratinocytes were analysed for involucrin expression by indirect immunofluorescence. (**B**) The ratio of involucrin-positive and Hoechst 33258-stained nuclei was determined in triplicate cultures 48 h after infection.

expression of involucrin, as measured by indirect immunofluorescence (Figure 7). Thus, Ras, like Raf, can inhibit proliferation and induce differentiation in primary mouse keratinocytes. We therefore suspect that the outgrowth of Ras^{V12}-positive keratinocytes is a consequence of selecting cells expressing Ras at low levels.

DISCUSSION

Raf, as well as Ras, can induce cell cycle arrest through multiple mechanisms. While in non-immortalized fibroblasts this arrest is associated with cellular senescence (Serrano *et al.*, 1997), NIH 3T3 cells or normal Schwann cells arrest in response to Ras or Raf without indication of phenotypical senescence (Lloyd *et al.*, 1997; Sewing *et al.*, 1997). Here we show that Raf and Ras activation in primary keratinocytes leads to cell cycle arrest and the induction of differentiation. Similar to Schwann cells (Lloyd *et al.*, 1997), keratinocytes lacking p53 or p21^{Cip1} do not arrest in response to Raf, although still inducing expression of keratinocyte differentiation-specific genes.

p19^{ARF} has been shown to function as an essential link between activation of a variety of oncogenes, including Ras and the induction of p53-dependent cell cycle arrest in fibroblasts (reviewed in Sherr and Weber, 2000). To our surprise, p19$^{ARF-/-}$ keratinocytes responded to Raf in the same way as wt cells, demonstrating that Raf can induce p53 expression at the protein level independently of p19^{ARF}. PML also has been implicated in Ras-mediated p53-dependent growth arrest (Ferbeyre *et al.*, 2000; Pearson *et al.*, 2000), although induction of p53 and p21^{Cip1} levels by Ras appear not to be affected in PML$^{-/-}$ cells (Pearson *et al.*, 2000). Analysis of p53 RNA levels in keratinocytes after Raf activation shows that p53 is not induced at the RNA level (data not shown), suggesting that Raf is regulating p53 at the level of protein stabilization. This is reminiscent of p53 activation in response to ionizing radiation which also has been shown to be p19^{ARF}-independent (Kamijo *et al.*, 1997).

Our experiments provide insight into the molecular mechanisms that underlie oncogene co-operation in epithelial cells. Activated Raf induces cell cycle arrest in conjunction with terminal differentiation in keratinocytes. The cell cycle arrest is mediated via induction of the tumour suppressor p53 and its target p21^{Cip1}. This explains why loss of p53 or p21^{Cip1} function is required for keratinocyte proliferation in presence of Raf activity. Surprisingly, Raf activation of p53 does not require the tumour suppressor p19^{ARF}, indicating that oncogenes can engage p53 activity via multiple mechanisms.

METHODS

Isolation and culture of primary mouse keratinocytes. Primary keratinocyte preparations were derived from pools of 3-day-old mice as described (Hennings *et al.*, 1980) and cultured in low calcium FAD (0.05 mM $CaCl_2$) + 8% chelex treated, charcoal stripped FBS + HICE (Carroll *et al.*, 1995). For all experiments, cells were used within the first 3 weeks after explantation. Genetically unmodified keratinocytes were derived from Balb/c mice. p53 (ICRF colony) and p19^{ARF} mutations and respective littermate controls were kept in C57BL/6 background, the p21^{Cip1} mutation in 129/SVEV (Weinberg *et al.*, 1999). Keratinocytes from p19$^{ARF-/-}$, p16$^{INK4A+/+}$ mice (Kamijo *et al.*, 1997) were received from Drs Sherr and Roussel.

Retroviral vectors and infection. The retroviral vector LXSN or its derivative LXSN-RafER were used as described (Lloyd *et al.*, 1997). Keratinocyte cultures were infected by co-cultivation, at a 1:2 ratio, with the virus producer cells pre-treated for 3 h with 20 µg/µl of mitomycin C (Sigma). Three days after plating the cultures were transferred into selective medium containing 200 µg/ml G418 (GIBCO). Drug-resistant keratinocyte colonies were pooled and passaged once or twice at a 1:2 ratio, while being maintained in absence of 4OH-tamoxifen.

Proliferation assay. Cells were incubated for 1 h with 10 µM BrdU (Sigma) followed by fixation in 4% paraformaldehyde for 10 min at room temperature. The cells were stained for BrdU, and BrdU incorporation and DNA content was analysed by a laser scanning cytometer (LSC; CompuCyte) (Rew *et al.*, 1998). The propidium iodide signal was used as a contouring parameter. Up to 5000 events were collected for each sample using proprietary WinCyte software. The results were confirmed by FACS analysis (Lloyd *et al.*, 1997) or by counting BrdU incorporation versus nuclear staining with Hoechst 33258.

Cell extracts, western blotting and kinase assay. Cells were scraped in cold PBS and centrifuged. The cell pellet was resus-

pended in lysis buffer A (50 mM Tris pH 7.4 containing 150 mM NaCl, 0.5% NP-40, 1% Aprotinin, 10 mM NaF, 1 mM Na_3VO_4, 100 µg/ml PMSF and 1 mM DTT). Protein concentrations were measured by either the Bio-Rad or BCA protein assays and 20–40 µg of protein was resolved by SDS–PAGE and electroblotted onto immobilon P membranes (Millipore). The following antibodies were used: ER (sc-543; Santa Cruz), actin (AC-20; Sigma), Phospho p42/44ERK (M-8159; Sigma), Total ERK 2 (sc-1647), involucrin [received from Elizabeth Li (Li *et al.*, 2000)], keratin 1 (PRB-165P; BAbCO), $p21^{Cip1}$ (sc-6246), $p16^{INK4A}$ (DPAR 14; Gordon Peters), $p27^{Kip1}$ (sc-1641), cyclin D1 (sc-450), cyclin D2 (sc-593), cyclin E (sc-481), cyclin A (sc-596), Cdk2 (sc-163), Cdk4 (sc-260), p53 (pAb421; ICRF), $p19^{ARF}$ (R562; Abcam). Reactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Pierce) and visualized using ECL detection (Amersham). Cyclin E/Cdk2-specific kinase activity was determined as described (Lloyd *et al.*, 1997).

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