Reduced skin tumor development in cyclin D1-deficient mice highlights the oncogenic *ras* pathway in vivo

Ana I. Robles,[1,6] Marcelo L. Rodriguez-Puebla,[1] Adam B. Glick,[2] Carol Trempus,[3] Laura Hansen,[2] Piotr Sicinski,[4] Raymond W. Tennant,[3] Robert A. Weinberg,[5] Stuart H. Yuspa,[2] and Claudio J. Conti[1,7]

[1]The University of Texas, M.D. Anderson Cancer Center, Science Park–Research Division (SPRD), Smithville, Texas 78957 USA; [2]Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, Maryland 20892 USA; [3]Laboratory of Environmental Carcinogenesis and Mutagenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 USA; [4]Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115 USA; [5]The Whitehead Institute for Biomedical Research and Department of Biology, Massachusets Institute of Technology, Cambridge, Massachusetts 02142 USA

Cyclin D1 is part of a cell cycle control node consistently deregulated in most human cancers. However, studies with cyclin D1-null mice indicate that it is dispensable for normal mouse development as well as cell growth in culture. Here, we provide evidence that *ras*-mediated tumorigenesis depends on signaling pathways that act preferentially through cyclin D1. Cyclin D1 expression and the activity of its associated kinase are up-regulated in keratinocytes in response to oncogenic *ras*. Furthermore, cyclin D1 deficiency results in up to an 80% decrease in the development of squamous tumors generated through either grafting of retroviral *ras*-transduced keratinocytes, phorbol ester treatment of *ras* transgenic mice, or two-stage carcinogenesis.

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Research carried out over the last several years has revealed a distinct biochemical pathway regulating G_1 progression in mammalian cells. In this pathway, a cyclin D–cyclin-dependent kinase (CDK)4/6 protein complex phosphorylates the retinoblastoma protein (pRb) to promote progression through a late G_1 checkpoint (Weinberg 1995). Such process may be negatively regulated by a series of CDK inhibitors (CDKIs), including p15, p16, p21, and p27. Many of the proteins that participate in this regulatory circuit have been implicated in the causation of various types of human tumors (Sherr 1996). For example, several laboratories have reported that subsets of tumors of a particular type show loss of pRb or, alternatively, overexpression of cyclin D1 (Jiang et al. 1993; Bartkova et al. 1994; Schauer et al. 1994). Similarly, in yet other tumor types, loss of p16 and pRb appears to be mutually exclusive (Otterson et al. 1994; Aagaard et al. 1995; Shapiro et al. 1995). These observations have led to the hypothesis that inactivation of cyclin D1–CDK4/6–p16–pRb by means of either loss of suppressor activity of pRb or p16, or overexpression of cyclin D1, can promote tumor development (Weinberg 1995; Sherr 1996).

Several lines of evidence indicate that the cyclin D1–CDK4/6–p16–pRb regulatory circuit lies downstream of oncogenic *ras*. The tumor suppressor activity of p16 was demonstrated through its ability to inhibit *ras*-induced proliferation and cellular transformation (Serrano et al. 1995), and it was recently observed that cell cycle arrest following *ras* inactivation is dependent on functional pRb (Peeper et al. 1997). Moreover, cyclin D1 expression can be stimulated in vitro by oncogenic *ras* in epithelial (Filmus et al. 1994) as well as fibroblast (Liu et al. 1995) cell lines, apparently through activation of the MAP kinase cascade (Albanese et al. 1995; Liu et al. 1995). Data from experimental and human cancers also indicate that cyclin D1 mediates the proliferative response to oncogenic *ras*. Thus, mouse skin tumors induced by a two-stage carcinogenesis protocol, which contain a characteristic oncogenic mutation in codon 61 of the Ha-*ras* gene, overexpress cyclin D1 (Robles and Conti 1995), although they have a very low frequency of cyclin D1 gene amplification (Bianchi et al. 1993). Such amplification represents a common mechanism of cyclin D1 up-regulation in human tumors (Peters 1994). Similar results have been observed in carcinogen-induced rat mammary tumors (Sgambato et al. 1995), which also carry characteristic *ras* mutations (Zarbl et al. 1985). Recently, a strong positive correlation between cyclin D1 and c-Ki–*ras* immunoexpression was reported in human epithelial ovarian tumors (Hung et al. 1996). However, none of these reports demonstrates that cyclin D1 is necessary for oncogenic *ras*-mediated growth.

Here, we provide biochemical and genetic evidence that cyclin D1 is a critical target for oncogenic *ras* in mouse skin. Cyclin D1, its association with CDK4, and CDK4 kinase activity are up-regulated in response to retroviral *ras*. In addition, *ras*-mediated skin tumorigenesis is substantially reduced in a cyclin D1-deficient background.

[*Key Words:* Knockout; Tg.AC; cell cycle; keratinocyte; two-stage carcinogenesis]

[6]Present address: Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20892 USA.
[7]Corresponding author.
E-MAIL SA83125@odin.mdacc.tmc.edu; FAX (512) 237-2444.

Results

Retroviral ras*-associated expression of cyclin D1 and cyclin E*

Retroviral introduction of a mutant *ras* oncogene into cultured murine keratinocytes results in an increased proliferation rate, resistance to Ca^{2+}-induced terminal differentiation, and development of benign tumors when grafted onto athymic nude mice (Roop et al. 1986). Because of their role as positive regulators of the cell cycle, G_1 cyclins were likely to mediate this response. Through total RNA Northern analysis, we found that among the G_1 cyclins, cyclins D1 and E were induced by v-Ha-*ras*, cyclin D2 remained unchanged, and cyclin D3 mRNA was almost undetectable and was not induced by *ras* (Fig. 1A; data not shown). Western blot analysis of whole cell extracts showed a predominant induction of cyclin D1, a modest increase in cyclin E, and no change in cyclin D3 (Fig. 1B). A polyclonal cyclin D1 antibody weakly cross-reactive with cyclin D2 confirmed cyclin D1 induction and showed an apparent increase in cyclin D2 level (Fig. 1B).

Certain growth factors such as epidermal growth factor (EGF) or keratinocyte growth factor (KGF) are known to induce a proliferative response in cultured keratinocytes (Dlugosz et al. 1994). Consistent with extensive data regarding the role of cyclin D1 in the regulation of G_1-phase transit in response to growth factors, EGF and KGF also stimulated cyclin D1 expression (Fig. 1C). The magnitude of the increase in cyclin D1 expression obtained by oncogenic *ras* was comparable to that induced by the continuous presence of EGF in the culture media (Fig. 1C). Other cell cycle proteins were affected only minimally by either v-Ha-*ras* infection or growth factor treatment, and p16 was undetectable (Fig. 1C; data not shown).

In many and perhaps all types of normal cells, inactivation of pRb by hyperphosphorylation during late G_1 phase of the cell cycle is a prerequisite for S-phase entry (Weinberg 1995). Upon infection with retroviral *ras*, cultured keratinocytes showed an increase in the proportion of high-molecular-weight bands that are characteristic of the hyperphosphorylated forms of pRb (Fig. 1C). This band shift indicative of a higher degree of phosphorylation is concomitant with cyclin D1 induction and coincides with the increase in keratinocyte proliferation that is observed after *ras* infection. As expected, EGF and KGF also stimulated pRb hyperphosphorylation (Fig. 1C).

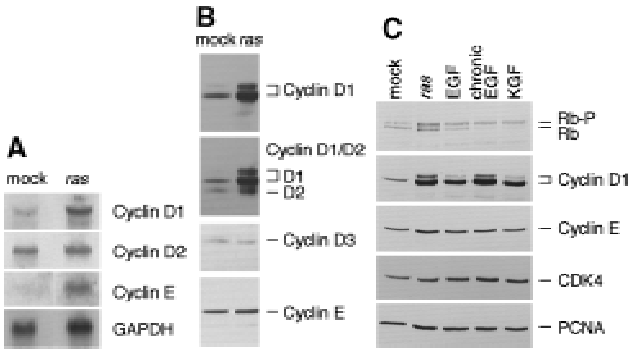

Figure 1. Expression of G_1 cyclins mRNA (*A*) and proteins (*B*) in mock and v-Ha-*ras*-infected murine primary keratinocytes. (*C*) Comparative induction of cyclin D1 protein expression and pRb hyperphosphorylation in mock-infected (mock), v-Ha-*ras*-infected (*ras*), and after single-dose EGF (EGF), continuous presence of EGF in the media (chronic EGF), and single-dose KGF (KGF) treatments of primary murine keratinocytes.

*Association of cyclin D1 with CDK4 and CDK4 kinase activity are stimulated by v-Ha-*ras

In CDK immunoprecipitates, cyclin D1 was found associated exclusively with CDK4 and not with CDK2 or CDK6 in normal and *ras*-infected keratinocytes (Fig. 2A), although these three CDKs are expressed in murine keratinocytes (data not shown). This interaction was confirmed by immunoprecipitation of cyclin D1 followed by CDK4 immunoblotting (Fig. 2B). In both cases, we observed that the amount of CDK4-bound cyclin D1 increased after *ras* infection proportionally to the increase in cyclin D1 in whole cell extracts. As expected, cyclin E was found associated with CDK2 in both normal and *ras*-infected keratinocytes (data not shown). Binding of the CDKI p21 and p27 to cyclin–CDK complexes is known to regulate their catalytic activity in response to antimitogens (Sherr and Roberts 1995). However, a possible function of these CDKIs in the assembly of active cyclin–CDK complexes has been suggested recently (LaBaer et al. 1997). We therefore studied the presence of these CDKIs in cyclin and CDK immunoprecipitates to determine if the pattern of binding changed after v-Ha-*ras* infection. We found that p21 and p27 were associated with CDK2 and CDK4 even after v-Ha-*ras* infection (Fig. 2A). Both inhibitors were also present in cyclin D1 immunoprecipitates (Fig. 2B). Surprisingly, the expression of p21 increased after *ras* transduction; however, the in vitro kinase activity of CDK4 toward pRb was clearly enhanced by retroviral *ras* (Fig. 2C), confirming the association among oncogenic *ras*, cyclin D1 expression, and pRb phosphorylation.

Absence of cyclin D1 impairs ras*-associated tumor development*

Immunohistochemical analysis of cyclin D1 expression in archival tumor samples generated by grafting in vitro-initiated primary murine keratinocytes into nude mice showed that in all cases, cyclin D1 was strongly expressed in basal undifferentiated keratinocytes from tumors and absent in adjacent normal skin (data not shown). This indicated a correlation between oncogenic *ras* and cyclin D1 expression in vivo. However, the question remained whether cyclin D1 is an essential mediator of keratinocyte transformation by oncogenic *ras* or is independently induced as a nonessential consequence of the proliferative response generated by *ras* transformation. To address these possibilities, we studied *ras*-mediated tumorigenesis in cyclin D1-deficient mice.

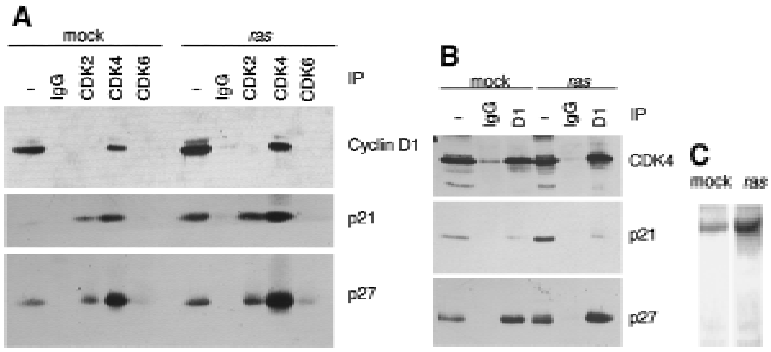

Figure 2. Analysis of G_1 cyclin–CDK complex formation in mock and v-Ha-*ras*-infected murine primary keratinocytes. Whole cell extracts and CDK (*A*) or cyclin D1 (*B*) immunoprecipitates were subjected to immunoblot analysis with cyclin D1, p21, and p27 (*A*), or CDK4, p21, and p27 (*B*). (*C*) Phosphorylation of pRb by CDK4 immunocomplex.

Primary keratinocytes were isolated from newborn cyclin D1 knockout, heterozygous, and wild-type siblings and transduced with retroviral v-Ha-*ras*. In vitro growth and morphology of cyclin D1-deficient keratinocytes was similar to those of their wild-type counterparts before and after retroviral infection (data not shown). This observation is consistent with that of normal in vitro growth of cyclin D1-deficient mouse embryo fibroblasts (Fantl et al. 1995). The growth of tumors generated by grafting v-Ha-*ras* infected keratinocytes was followed for several weeks after removal of the grafting chamber, and the combined results of two independent experiments are shown in Figure 3A. Tumors obtained by chamber grafting of *ras*-infected cyclin D1-deficient primary keratinocytes were at least 50% smaller throughout the experiment than those from isogenic wild-type and heterozygous controls. However, cyclin D1-deficient papillomas presented a morphology and histopathology indistinguishable from that of the controls, except for expression of cyclin D1 (Fig. 3B,C).

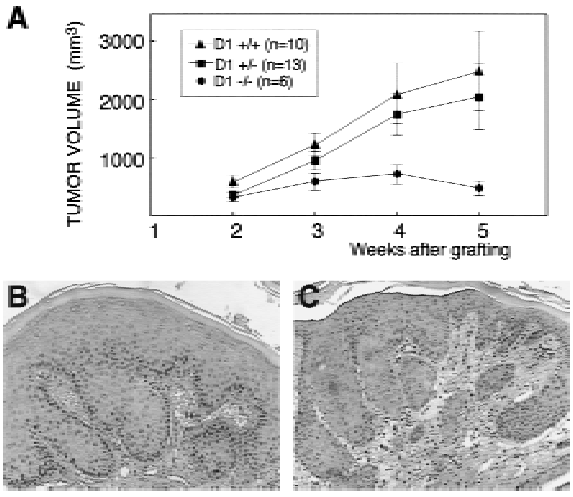

Figure 3. (*A*) Primary keratinocytes from each cyclin D1 genotype were infected with v-Ha-*ras* retrovirus and grafted onto nude mice. Approximate tumor volume was calculated as tumor height × length × width in mm. Error bars, S.E.M. Immunohistochemical staining of cyclin D1 in a cyclin D1 heterozygous (*B*) and a cyclin D1 knockout (*C*) graft.

A second confirmatory study of tumor susceptibility in the presence of exogenous *ras* involved the development of benign skin tumors in the v-Ha-*ras* transgenic Tg.AC mouse strain in response to treatment with the phorbol ester TPA (Spalding et al. 1993). Upon treatment with TPA, the *ras* transgene is expressed in preneoplastic focal follicular hyperplasias and in papillomas, but not in non-tumor-bearing Tg.AC mouse skin (Hansen and Tennant 1994a,b). Consistently, we found that cyclin D1 expression was up-regulated in papillomas and even in follicular hyperplasias of Tg.AC mice but not in TPA-treated uninvolved skin (data not shown). Genetically matched female mice were generated that carried a v-Ha-*ras* transgene ($Tg.AC^{+/-}$) and were cyclin $D1^{-/-}$ ($n = 7$) or cyclin $D1^{+/-}$ ($n = 5$). This cross also yielded v-Ha-*ras* transgene-negative ($Tg.AC^{-/-}$) cyclin $D1^{-/-}$ ($n = 10$) or cyclin $D1^{+/-}$ ($n = 5$) females that were used as controls (Fig. 4A). As expected, in the absence of v-Ha-*ras* transgene no tumors appeared in response to TPA treatment, regardless of cyclin D1 status. A multitude of papillomas developed on the backs of $Tg.AC^{+/-}$ mice as a result of TPA application. Papilloma multiplicity was up to sixfold higher in $Tg.AC^{+/-}$ cyclin $D1^{+/-}$ mice compared to $Tg.AC^{+/-}$ cyclin $D1^{-/-}$ siblings. Tumor appearance was delayed by 1 week in cyclin D1-deficient Tg.AC mice, but the fraction of mice with papillomas at the end of the experiment was similar in both groups

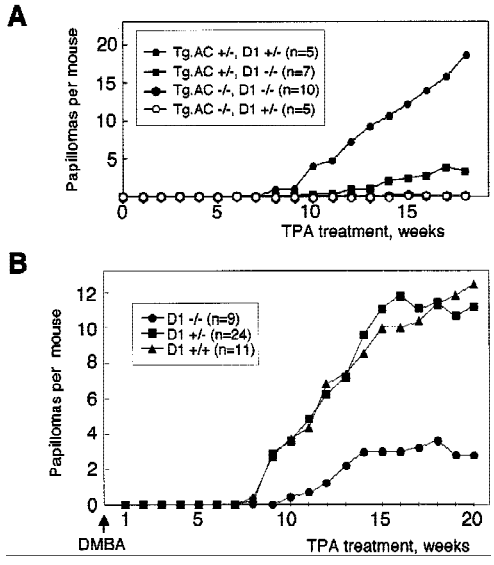

Figure 4. (*A*) Papilloma development in isogenic female mice generated through backcrossing of the Tg.AC transgene onto cyclin D1 knockout background and treated with TPA. (*B*) Papilloma development in isogenic cyclin D1 knockout (−/−), heterozygous (+/−), and wild-type (+/+) mice subjected to a two-stage carcinogenesis protocol using DMBA as initiator and TPA as promoter.

(3/5 for Tg.AC$^{+/-}$ cyclin D1$^{+/-}$ and 5/7 for Tg.AC$^{+/-}$ cyclin D1$^{-/-}$).

To determine if skin tumor development mediated by mutation of endogenous Ha-*ras* was also dependent on cyclin D1 expression, a third experimental approach consisting of a two-stage protocol of skin carcinogenesis was undertaken next. Such protocol involves the application of a single dose of the genotoxic compound 9,10-dimethyl-1,2-benz[*a*]anthracene (DMBA), followed by multiple applications of TPA, and results in the development of benign skin lesions. These papillomas contain a characteristic oncogenic mutation in codon 61 of the Ha-*ras* gene and overexpress cyclin D1 (Robles and Conti 1995). In this experiment tumor appearance was delayed by 2 weeks in cyclin D1$^{-/-}$ mice, and the fraction of mice with papillomas at the end of the experiment (20 weeks) was 56% (5/9) in cyclin D1$^{-/-}$, 96% (23/24) in cyclin D1$^{+/-}$, and 100% (11/11) in cyclin D1$^{+/+}$ mice. Papilloma multiplicity in cyclin D1$^{-/-}$ mice was 25% of that of cyclin D1$^{+/-}$ mice and 22% of that of cyclin D1$^{+/+}$ mice (Fig. 4B).

Taken together, the results of these three protocols of experimental carcinogenesis indicate that cyclin D1 has a unique role in promoting *ras*-mediated growth in mouse skin and that *ras*-mediated tumor development is dependent on pathways that favor cyclin D1 expression.

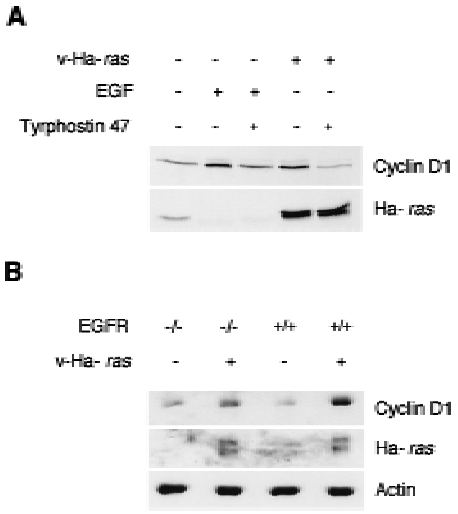

Figure 5. (*A*) v-Ha-*ras*-infected primary murine keratinocytes were treated with 50 μM of tyrphostin 47 or DMSO for 18 hr. As a control, primary murine keratinocytes were treated with 10 ng/ml EGF for 18 hr in addition to tyrphostin. (*B*) Expression of cyclin D1 and v-Ha-*ras* in mock- and v-Ha-*ras*-infected primary murine keratinocytes isolated from EGFR knockout mice and wild type. Expression of actin was used for equal loading correction.

Possible mechanism of v-Ha-ras *stimulation of cyclin D1 expression in keratinocytes*

Cyclin D1 induction by activated *ras* genes has been reported previously in a number of established fibroblast cell lines (Albanese et al. 1995; Liu et al. 1995; Winston et al. 1996) and in intestinal epithelial cells (Filmus et al. 1994). In fibroblasts, cyclin D1 seems to be directly induced by the *ras*-signaling cascade. Because this induction is insensitive to growth factor receptor blockade, it appears that it is not dependent on an extracellular autocrine-signaling loop (Winston et al. 1996). However, autocrine stimulation has proven to be necessary for development of oncogenic *ras*-associated epithelial tumors, as indicated by the fact that EGF receptor (EGFR)-deficient keratinocytes form smaller tumors than their wild-type counterparts when transformed by retroviral *ras* and subsequently grafted onto nude mice (Dlugosz et al. 1997). In view of our data from tumorigenic assays indicating a role for cyclin D1 in the proliferation of *ras*-transformed keratinocytes, we hypothesized that in epithelial cells, cyclin D1 induction by oncogenic *ras* might also be dependent on autocrine stimulation. Consistently, we found that exposure of cultured keratinocytes to the protein tyrosine kinase inhibitor Tyrphostin 47, which is known to block EGFR signaling (Dvir et al. 1991), resulted in down-regulation of cyclin D1 in both EGF-treated as well as v-Ha-*ras*-infected keratinocytes (Fig. 5A). It has been demonstrated previously that at the dosage used in this experiment (50 μM), Tyrphostin 47 is not cytotoxic but cytostatic for cultured keratinocytes (Dvir et al. 1991), and its effect can be reversed by culturing in fresh media devoid of tyrphostin (data not shown). However, although tyrphostin preferentially inhibits the EGFR-associated kinase, it may inhibit other kinases as well. Therefore, to test our hypothesis in a system that was specifically deficient in EGFR, we analyzed cyclin D1 protein expression before and after *ras* transduction in keratinocytes derived from EGFR wild-type and null newborn mice and grown in basal media (Dlugosz et al. 1997). With equal total protein loading and equal expression of v-Ha-*ras*, expression of cyclin D1 was four-fold higher in EGFR wild-type keratinocytes than in their EGFR null siblings upon retroviral transduction of v-Ha-*ras* (Fig. 5B).

Discussion

In view of the critical role of cyclin D1 in G_1 progression it was surprising that cyclin D1-deficient mice developed with few very specific tissue defects (Fantl et al. 1995; Sicinski et al. 1995). Nonetheless, the observation of defective proliferation of cells from the retina and mammary gland confirmed the functional importance of cyclin D1 in vivo and was consistent with the role of cyclin D1 in mammary gland proliferation and carcinogenesis (Peters 1994).

Using three diverse models of *ras*-mediated tumorigenesis of mouse skin in a cyclin D1-deficient background, we have provided genetic evidence of the role of cyclin D1 as a downstream mediator of oncogenic *ras*. This is in agreement with previous biochemical evidence of signaling between *ras* and cyclin D1 and *ras* and pRb (Peeper et al. 1997). We found that although lack of cyclin D1 did not completely inhibit cellular transformation, it significantly reduced the efficiency of tumor development. During preliminary studies of protein ex-

pression we have observed no compensatory up-regulation of either cyclins D2 or D3 in the tumors that developed in a cyclin D1-deficient background (M.L. Rodríguez-Puebla and C.J. Conti, in prep.). This indicates that cyclin D1 is a preferential target of oncogenic *ras* and underscores the functional uniqueness of each D-type cyclin. It is also consistent with previous observations in vitro using cyclin D1-deficient fibroblasts (Fantl et al. 1995). Other evidence supporting this hypothesis is that cyclin D1 did not behave as an oncogene when expressed in stratified epithelia (Robles et al. 1996) and did not synergize with oncogenic *ras* in the development of squamous tumors (A.I. Robles, M.L. Rodríquez-Puebla, and C.J. Conti, unpubl.).

We have extended our observations to show that oncogenic *ras*-mediated expression of cyclin D1 is also dependent on autocrine stimulation through the EGFR. Our data are consistent with previous reports on the importance of autocrine stimulation through the EGFR in epithelial tumors (Dlugosz et al. 1997; Gangarosa et al. 1997) and is in conflict with the observation that in *ras*-transformed fibroblasts cyclin D1 induction is insensitive to growth factor receptor blockade (Winston et al. 1996). This difference might be explained by a response that is cell-type specific. Both v-H-*ras* and v-H-raf can transform NIH-3T3 fibroblasts, and cyclin D1 is up-regulated in each case (Liu et al. 1995). In contrast, in rat intestinal epithelial cells a *raf*-independent EGFR autocrine loop is necessary for *ras* transformation (Gangarosa et al. 1997). It would appear that the crosstalk between cyclin D1 and autocrine stimulation through the EGFR is unique to oncogenic *ras* in epithelial cells, and its implications warrant further investigation.

Materials and methods

Cell culture

Primary keratinocytes were isolated from newborn mice, cultured, and infected with retroviral v-Ha-*ras* as described previously (Dlugosz et al. 1995). All cultures were harvested at day 8 of culture for RNA or protein extraction. Growth factors were added to the basic media at 10 ng/ml where indicated. In one set of experiments, 50 μM of the protein tyrosine kinase inhibitor Tyrphostin 47 (RG-50864 or AG 213, Sigma) or its vehicle (DMSO) alone were added to the cultures for 18 hr before harvesting for protein extraction.

Northern blotting

Total RNA was extracted from mock- and v-Ha-*ras*-infected primary keratinocytes with Trizol (GIBCO BRL). Total RNA (20 μg) was separated on a 0.8% agarose gel, blotted onto a nylon membrane (Amersham), and hybridized with ^{32}P-labeled cDNAs. Human cyclin D1 cDNA was provided by A. Arnold (Massachusetts General Hospital, Boston, MA), murine cyclin D2 and murine cyclin D3 cDNAs were provided by C. Sherr (St. Jude Children's Research Hospital, Memphis, TN), and murine cyclin E cDNA was provided by J.A. DeLoia (University of Pittsburgh, PA). GAPD (ATCC) was used as a control for equal loading. Hybridizations were performed with QuickHyb (Stratagene) followed by autoradiography.

Immunoblotting and immunoprecipitation

Cultures were washed in ice-cold PBS and lysed in 50 mM Tris (pH 7.4) containing 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 2 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, and 25 μg/ml aprotinin. One hundred micrograms was electrophoresed through 10% or 7.5% SDS-PAGE and transferred to Hybond-ECL membranes (Amersham). Immunoblots were incubated for 1 hr at room temperature in blocking solution (TBS with 5% milk and 0.05% Tween 20) followed by the primary antibody diluted in blocking solution for 1–2 hr. The following primary antibodies were used: mouse monoclonal antibodies against cyclin D1 (Zymed), Rb (Pharmingen), Ha-*ras* (Transduction Labs), and actin (clone C4, Boehringer Mannheim), and rabbit polyclonal antibodies against cyclin D1 (Upstate Biotechnology), weakly cross-reactive with cyclin D2, and against cyclin D3 (C-16), cyclin E (M-20), PCNA, CDK2, CDK4, CDK6, p21, and p27 (Santa Cruz Biotechnology). The immunoblots were developed with a horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence (Amersham).

One milligram of total cell extract was used for coimmunoprecipitation followed by immunoblotting. Extracts were incubated with 1 μg of antibody, and the immunocomplexes were precipitated with protein G–Sepharose (GIBCO BRL), washed, separated through 10% SDS-PAGE, and transferred to nitrocellulose. Immunoblots were treated as above.

Primary keratinocytes derived from EGFR wild-type and null newborns (Dlugosz et al. 1997) were lysed in 50 mM Tris (pH 7.4) containing 1% NaDOC, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 10 mM NaF, 200 μM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Twenty-five micrograms of protein was electrophoresed through a 12% SDS–polyacrylamide gel followed by Western blotting.

CDK4 kinase assay

Mock- and v-Ha-*ras*-infected newborn primary keratinocyte cultures were lysed in 50 mM HEPES (pH 7.5) containing 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1 % Tween 20, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM NaF, 0.1 mM Na_3VO_4, and 10 mM β-glycerophosphate. The cell lysate was frozen and stored at −70°C. CDK4-associated kinase activity was measured as described (Matsushime et al. 1994) against recombinant pRb using polyclonal CDK4 antibody (Santa Cruz Biotechnology) to pull down the active kinase complex.

Tumorigenicity assays

*Grafting of v-H-*ras*-infected keratinocytes* Crossing cyclin D1 heterozygous siblings generated genetically matched cyclin D1 knockout, heterozygous, and wild-type newborn mice. After genotyping (Sicinski et al. 1995) skins with equal genotype were pooled for keratinocyte isolation, plated, and infected with the v-Ha-*ras*-encoding retrovirus. Five days after infection they were trypsinized, combined with wild-type primary fibroblasts, and transferred to a grafting chamber surgically implanted on the back of nude mice (Dlugosz et al. 1995). The chamber was removed 1 week after grafting, and tumor development was measured as tumor height × length × width.

*TPA treatment of v-Ha-*ras *transgenic Tg.AC mice* Female homozygous v-Ha-*ras* transgenic Tg.AC mice were mated with a cyclin D1 knockout male, and the F_1 offspring was backcrossed with the cyclin D1-deficient parent. Female (2–5 weeks old) mice were initially treated with 2.5 μg of TPA (Sigma) applied topically in 200 μl of acetone twice a week for 5 weeks. Treatment was then reduced to 1 μg of TPA twice a week for 13 weeks. Mice were housed in cages independent of genotype, and the number of lesions was scored weekly by palpation.

Two-stage carcinogenesis For chemical carcinogenesis studies, cyclin D1-deficient mice were backcrossed into a Sencar background. Genetically matched cyclin D1 knockout, heterozygous, and wild-type newborns were initiated at day 1 after birth by application of 50 μg of DMBA in 100 μl of acetone. At day 7, animals received 2.5 μg of TPA in 100 μl of acetone twice a week for 2 weeks. The TPA regimen was subsequently changed to 5 μg of TPA in 200 μl of acetone twice a week for 8 weeks and back to 2 μg of TPA in 200 μl of acetone for a total of 20 weeks.

Histology and immunohistochemistry

Tissues were fixed in 10% buffered formalin or 4% paraformaldehyde, and embedded in paraffin. Sections of all tissues were stained with hematoxylin and eosin for histopathological evaluation. Detection of cyclin D1 expression by immunostaining of formalin-fixed paraffin-embedded tissues has been described previously (Robles and Conti 1995), as well as detection of *ras* transgene by mRNA in situ hybridization using ^{35}S-labeled riboprobe (Hansen and Tennant 1994a).

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