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The downregulation of the pro-apoptotic protein Par-4 is critical for Ras-induced survival and tumor progression

Marta Barradas, Alicia Monjas[1], María T.Diaz-Meco[1], Manuel Serrano and Jorge Moscat[1,2]

Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología and [1]Laboratorio Glaxo Wellcome-CSIC de Biología Molecular y Celular, Centro de Biología Molecular 'Severo Ochoa' (Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

[2]Corresponding author
e-mail: jmoscat@cbm.uam.es

M.Barradas and A.Monjas contributed equally to this work

Inhibition of apoptosis is an important characteristic of oncogenic transformation. The *Par-4* gene product has recently been shown to be upregulated in cells undergoing apoptotic cell death, and its ectopic expression was shown to be critical in apoptosis. We demonstrate that expression of oncogenic Ras promotes a potent reduction of Par-4 protein and mRNA levels through a MEK-dependent pathway. In addition, the expression of permanently active mutants of MEK, Raf-1 or ζprotein kinase C but not of phosphatidylinositol 3-kinase (PI 3-kinase) is sufficient to decrease Par-4 levels. These effects are independent of p53, p16 and p19, and were detected not only in fibroblast primary cultures but also in NIH 3T3 and HeLa cells, indicating that they are not secondary to Ras actions on cell cycle regulation. Importantly, restoration of Par-4 levels to normal in Ras-transformed cells makes these cells sensitive to the pro-apoptotic actions of tumor necrosis factor-α under conditions in which PI 3-kinase is inhibited and also severely impairs colony formation in soft agar and tumor development in nude mice, as well as increases the sensitivity of these tumors to camptothecin. This indicates that the downregulation of Par-4 by oncogenic Ras is a critical event in tumor progression.

Keywords: apoptosis/Par-4/Ras/tumor progression

Introduction

Par-4 is the product of a gene initially identified by differential screening of prostate cells induced to undergo apoptosis, suggesting a potential role for this protein in programmed cell death (Sells *et al*., 1994). Consistent with this notion, the ectopic expression of Par-4 induces apoptosis in NIH 3T3 cells and sensitizes prostate cancer and melanoma cells to apoptotic stimuli (Diaz-Meco *et al*., 1996; Berra *et al*., 1997; Sells *et al*., 1997). In addition, the immunohistochemical analysis of normal rat tissues reveals that Par-4 is expressed in apoptotic and terminally differentiated cells (Boghaert *et al*., 1997). A more recent study indicates that Par-4 expression is upregulated in vulnerable neurons in the brains of individuals suffering from Alzheimer disease. The blockade of Par-4 expression was shown to inhibit dramatically the neuronal apoptosis induced by the amyloid β-protein and the withdrawal of trophic factors (Guo *et al*., 1998). Par-4 displays at its C-terminus a leucine zipper domain with a predicted coiled-coil secondary structure that has significant homology, at the amino acid level, to several death domains (Diaz-Meco *et al*., 1996). Through that region, Par-4 binds the atypical protein kinase C isoforms (aPKCs), which serves to inhibit their enzymatic activity (Diaz-Meco *et al*., 1996). This is of particular functional relevance because the ability of Par-4 to induce apoptosis is abrogated by the overexpression of those PKC isoforms (Diaz-Meco *et al*., 1996). Therefore, the inhibition of the aPKC activity by Par-4 may be a critical event in apoptotic cell death. In fact, the blockade of ζPKC (Diaz-Meco *et al*., 1996) or λ/ιPKC (Diaz-Meco *et al*., 1996; Murray and Fields, 1997) with dominant-negative mutants or antisense oligonucleotides is sufficient to promote apoptosis. All these results together suggest a model whereby the induction of Par-4 leads to the inhibition of the aPKCs, which emerges as one of the required events for apoptosis to proceed.

The Ras oncogene activates multiple effector pathways that give rise to different outputs depending on the cell context (Downward, 1998). Of particular relevance for cell survival are phosphatidylinositol 3-kinase (PI 3-kinase) (Khwaja *et al*., 1997; Marte and Downward, 1997; Marte *et al*., 1997) and the aPKCs (Diaz-Meco *et al*., 1994; Downward, 1998). In addition, ERK that is activated by Raf-1 and ζPKC also appears to trigger anti-apoptotic signals (Xia *et al*., 1995; Gardner and Johnson, 1996; Berra *et al*., 1997, 1998; Li *et al*., 1998; Erhardt *et al*., 1999). In this regard, recent data in *Drosophila* indicate that Ras promotes survival through the Raf–MEK–ERK pathway by downregulating the pro-apoptotic protein Hid (Bergmann *et al*., 1998, Kurada and White, 1998; Meier and Evan, 1998). A third pathway whereby oncogenic Ras promotes cell survival involves NF-κB, which is necessary for Ras-induced transformation by suppressing the p53-independent induction of apoptosis (Mayo *et al*., 1997). Collectively, these observations would be in keeping with the notion that cell transformation is associated with the activation of pathways that inhibit apoptosis. In this study, we have addressed the potential regulation of Par-4 expression by oncogenic Ras. We show here that expression of Ras^{V12} downregulates Par-4 through the MEK–ERK signaling pathway, in a manner that is independent of p16/p19 and p53. Consistent with the physiological implications of these findings is the fact that the ectopic expression of Par-4 in Ras-transformed cells to levels comparable to those observed in control

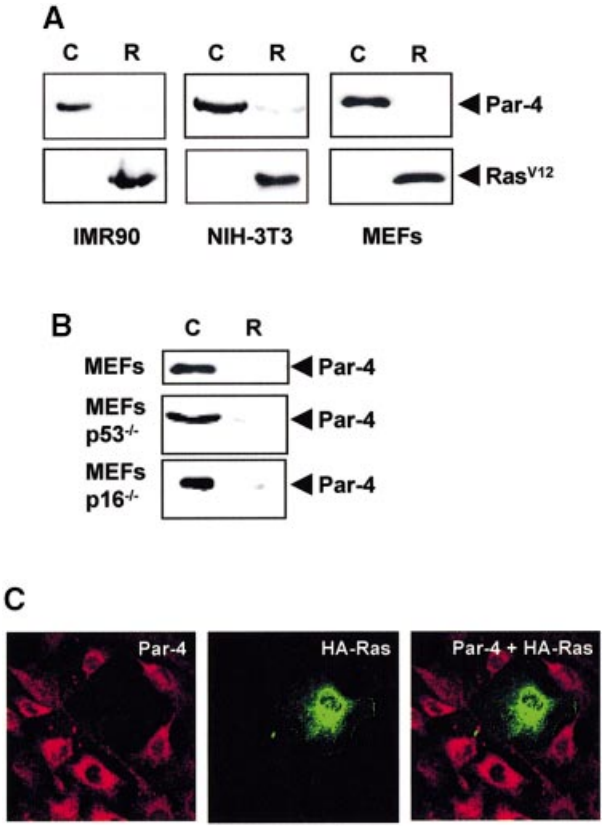

Fig. 1. Par-4 levels in H-Ras^{V12}-expressing cells. (**A** and **B**) Cell extracts from the indicated cell types transduced with empty vector (C) or H-Ras^{V12}-expressing retroviruses (R) were analyzed by immunoblotting with anti-Par-4 or anti-Ras antibodies. Similar results were obtained in two other independent experiments. (**C**) HeLa cells were transiently transfected with either control or an expression vector for HA-tagged H-Ras^{V12} and analyzed 24 h post-transfection by confocal laser scanning microscopy with a rabbit polyclonal affinity-purified anti-Par-4 antibody and tetramethylrhodamine tyramide (red fluorescence), to detect endogenous Par-4, and with the monoclonal anti-HA 12CA5 antibody and FITC-conjugated anti-mouse IgG (green fluorescence), to detect HA-tagged H-Ras^{V12}. Similar results were obtained in two other independent experiments.

cell lines severely impairs Ras-induced survival and tumor development.

Results

To investigate the potential regulation of Par-4 expression by oncogenic Ras, we initially introduced the H-Ras^{V12} mutant into primary cultures of either mouse embryo (MEFs) or human (IMR90) fibroblasts using a recombinant replication-deficient retrovirus. Afterwards, cell extracts were prepared and the levels of Par-4 were determined by immunoblot analysis with an affinity-purified anti-Par-4 antibody. The results in Figure 1A demonstrate a dramatic downregulation of Par-4 protein levels by the Ras mutant in both cell types. In mouse fibroblast primary cultures, the expression of oncogenic Ras leads to cell cycle arrest through the p16/Rb and the p19/p53 pathways, whereas in NIH 3T3 mouse fibroblasts it promotes proliferation and transformation (Serrano *et al*., 1997; Palmero *et al*., 1998). To determine whether the down-regulation of Par-4 may be secondary to growth arrest, oncogenic Ras was introduced into NIH 3T3 cells and

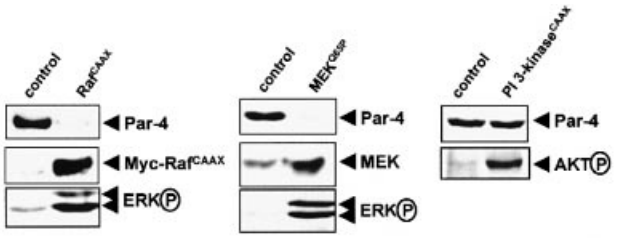

Fig. 2. Par-4 levels in cells expressing different Ras effectors. Cell extracts from NIH 3T3 fibroblasts transduced either with empty vector (control), Myc-Raf^{CAAX}, MEK^{Q65P} or Myc-PI 3-kinase^{CAAX} expressing retroviruses were analyzed by immunoblotting with anti-Par-4, anti-Myc, anti-MEK, anti-phospho-Akt or anti-phospho-ERK antibodies. Similar results were obtained in two other independent experiments.

Par-4 levels were determined as above. The results in Figure 1A demonstrate that the expression of oncogenic Ras downregulates Par-4 levels not only in fibroblast primary cultures but also in NIH 3T3 cells that are p16/p19 negative (Quelle *et al*., 1995), indicating that the downregulation of Par-4 by oncogenic Ras should be independent of both cell cycle regulators. To establish more definitively the independence of p16/p19 for oncogenic Ras to downregulate Par-4, fibroblast primary cultures from p16/p19 knockout mice were infected with the replication-deficient vector that expresses the oncogenic Ras. Consistently, the ability of Ras to downregulate Par-4 was not affected by the lack of p16/p19 (Figure 1B). The other critical molecule in the mechanism of growth arrest by Ras in primary fibroblasts is p53 (Serrano *et al*., 1997). The results in Figure 1B demonstrate that Ras is equally potent in downregulating Par-4 in p53-deficient primary fibroblasts.

In addition, the ability of Ras^{V12} to downregulate Par-4 was also determined in transiently transfected HeLa cells. Thus, an expression plasmid for hemagglutinin (HA)-tagged Ras^{V12} was transfected into HeLa cells, and 48 h post-transfection the levels of endogenous Par-4 in the Ras^{V12}-expressing cells were determined by confocal double immunofluorescence analysis. Par-4 was detected with a rabbit polyclonal affinity-purified antibody and tetramethylrhodamine tyramide (red fluorescence). Transfected HA-Ras^{V12} was detected with the monoclonal anti-HA antibody 12CA5 and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (green fluorescence). The results in Figure 1C demonstrate that the transient expression of oncogenic Ras almost completely depletes the levels of endogenous Par-4 in HeLa cells.

Ras triggers a number of effectors including activators of the MEK–ERK signaling cascade such as Raf-1 and the aPKCs, and PI 3-kinase (Diaz-Meco *et al*., 1994; Katz and McCormick, 1997; Liao *et al*., 1997; Downward, 1998). To determine which of these signaling events may be responsible for downregulating Par-4, we used permanently active mutants of either MEK or PI 3-kinase. Interestingly, the expression of activated MEK (Figure 2, middle panel) but not activated PI 3-kinase (Figure 2, right panel) is sufficient to downregulate Par-4 completely. The results in Figure 2 also demonstrate that the activated PI 3-kinase construct was fully capable of activating its downstream target Akt, as determined by immunoblotting with an anti-phospho-Akt antibody. In addition, as a

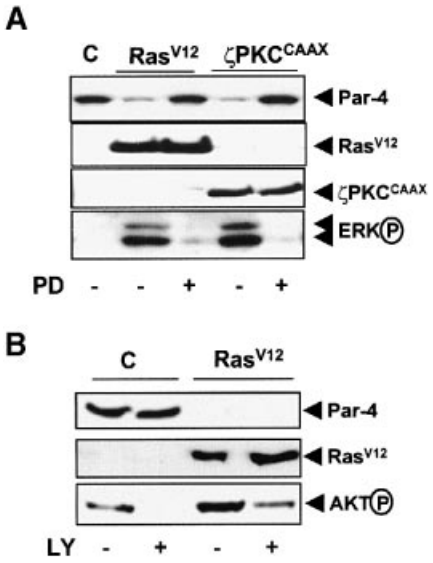

Fig. 3. Effect of MEK and PI 3-kinase inhibition on Ras-induced Par-4 downregulation. (**A**) Cell extracts from NIH 3T3 fibroblasts transduced either with empty vector (C), H-Ras^{V12} or ζPKC-expressing retroviruses and incubated either with or without PD98059 (PD) were analyzed by immunoblotting with anti-Par-4, anti-Ras, anti-ζPKC or anti-phospho-ERK antibodies. Similar results were obtained in two other independent experiments. (**B**) NIH 3T3 cells transduced with empty vector (C) or H-Ras^{V12}-expressing retroviruses were incubated either in the absence or the presence of the PI 3-kinase inhibitor LY294002 (LY). Cell extracts were then prepared and analyzed by immunoblotting with anti-Par-4, anti-Ras or anti-phospho-Akt antibodies. Similar results were obtained in two other independent experiments.

control it was determined by immunoblotting with an anti-phospho-ERK antibody that activated MEK stimulates ERK (Figure 2, middle panel). Ras activates MEK/ERK through at least two kinases, Raf-1 and ζPKC (Diaz-Meco *et al*., 1994; Bjorkoy *et al*., 1997; Liao *et al*., 1997; van Dijk *et al*., 1997; Downward, 1998; Schonwasser *et al*., 1998). To determine whether Raf and ζPKC activation are sufficient to downregulate Par-4, membrane-tagged active Raf (Raf^{CAAX}) or ζPKC (ζPKC^{CAAX}) mutants were used in the following series of experiments. Interestingly, the expression of Raf^{CAAX} (Figure 2, left panel) or ζPKC^{CAAX} (Figure 3A) provoked the downregulation of Par-4 to an extent comparable to that produced by oncogenic Ras (Figure 3A) or activated MEK (Figure 2, middle panel). Also demonstrated in Figures 2 and 3 is the ability of active Raf and ζPKC mutants to stimulate ERK as determined by immunoblotting with anti-phospho-ERK antibody. Consistent with the importance of MEK in the downregulation of Par-4 by oncogenic Ras is the finding that the presence of the MEK inhibitor PD98059 severely abrogated the ability of this oncogene to downregulate Par-4 (Figure 3A), indicating that the activation of MEK is not only sufficient but also necessary for the effects of Ras on Par-4 levels. It is of note that the inhibition of MEK abrogated not only the effect of Ras^{V12} on Par-4 levels, but also those of ζPKC (Figure 3A) and Raf-1 (not shown). As a control, Figure 3A also shows that the MEK inhibitor severely reduced ERK phosphorylation in response to oncogenic Ras and activated ζPKC. In addition, consistent with the lack of effect of the active PI 3-kinase mutant on Par-4 levels, the incubation of Ras-transformed cells with the PI 3-kinase inhibitor LY294002 did not alter the ability of oncogenic Ras to downregulate Par-4, although it completely abrogated the activation of Akt, as determined by immunoblot analysis with the anti-phospho-Akt antibody (Figure 3B). It should be noted that incubation of NIH 3T3 cells in the absence of fetal calf serum (FCS) induces a modest but reproducible increase in Par-4 levels (not shown). The re-addition of FCS to these quiescent cells reduces Par-4 to the levels found in proliferating cells in a manner that is inhibited by PD98059, suggesting that the state of activation of the Ras proto-oncogene also controls Par-4 content through MEK.

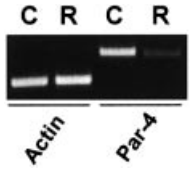

Fig. 4. Effect of oncogenic Ras expression on Par-4 mRNA levels. Approximately 1 μg of total RNA from human primary fibroblasts transduced either with empty vector (C) or H-Ras^{V12}-expressing retroviruses (R) was transcribed into first-strand cDNA. Either one-fifth or one-twentieth of the reverse transcription samples were used subsequently for PCR amplification with primers for Par-4 or actin, respectively, as described in Materials and methods. Essentially identical results were obtained in two other independent experiments.

The induction of Par-4 during apoptosis has been shown to correlate with a specific increase in its mRNA levels (Sells *et al*., 1997). In order to determine whether the downregulation of Par-4 by oncogenic Ras correlates with a decrease on its mRNA content, total RNA from either control or oncogenic Ras-expressing human primary fibroblasts was extracted and the Par-4 mRNA levels were determined by RT–PCR analysis. The results in Figure 4 demonstrate that oncogenic Ras induces a profound depletion of Par-4 mRNA but not that of actin (used as a negative control), indicating that the decreased Par-4 protein levels in Ras-expressing cells are most probably due to a decrease in its mRNA. It is noteworthy that the basal levels of the Par-4 mRNA must be very low as they are below the level of detection by Northern blotting. However, it should be stressed that the PCR analysis is quantitative, as different routine controls demonstrate that the reactions shown in Figure 4 are not outside the linear range. Oncogenic Ras has recently been demonstrated to induce the downregulation of Fas at the mRNA level through the PI 3-kinase/Akt signaling pathway and involving a DNA methylation-dependent mechanism (Peli *et al*., 1999). The downregulation of Par-4 by oncogenic Ras does not involve PI 3-kinase, but rather MEK (see above). However, in order to determine whether a similar DNA methylation mechanism could be implicated in the downregulation of Par-4 by Ras^{V12}, either control or Ras^{V12}-transformed fibroblasts were treated or not with 5-aza-2′-deoxycytidine (aza), an inhibitor of DNA methylation (Peli *et al*., 1999), and the levels of Par-4 and Fas were determined by immunoblotting with the corresponding antibodies. The results shown in Figure 5 demonstrate that whereas the treatment with aza abolishes the downregulation of Fas by oncogenic Ras, as previously reported by Peli *et al*. (1999), it has no effect on the Ras^{V12}-induced downregulation of Par-4.

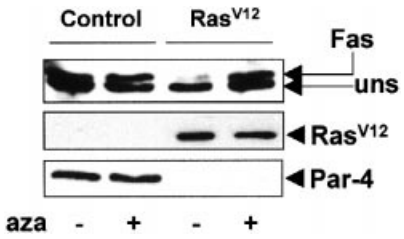

Fig. 5. Inhibition of DNA methylation blocks the downregulation of Fas but not of Par-4 by oncogenic Ras. NIH 3T3 cells, either control or expressing oncogenic Ras, were treated with aza for 2 days, after which Fas and Par-4 levels were determined by immunoblotting with the corresponding antibodies. Essentially identical results were obtained in two other independent experiments.

This indicates that the mechanisms whereby Ras inhibits both pro-apoptotic proteins are clearly different. Taken together all these results demonstrate that the expression of oncogenic Ras produces a potent signal, through the Raf/ζPKC–MEK pathway, for the downregulation of the pro-apoptotic protein Par-4 in a manner that is independent of both p53 and p16/p19.

The ectopic expression of Par-4 potentiates apoptotic cell death in several cell systems (Diaz-Meco *et al*., 1996; Berra *et al*., 1997; Sells *et al*., 1997; Guo *et al*., 1998). Ras oncogenic transformation activates not only mitogenic pathways but also cell survival, which is important for its full transforming capability (Downward, 1998). Ras inhibits apoptosis by activating the PI 3-kinase/Akt route (Downward, 1997; Khwaja and Downward, 1997; Khwaja *et al*., 1997; Marte and Downward, 1997) and by down-modulating pro-apoptotic proteins such as Hid in *Drosophila*, through the Raf–MEK–ERK cascade (Bergmann *et al*., 1998; Kurada and White, 1998), or by activating NF-κB, which is a potent pro-survival transcription factor (Finco *et al*., 1997; Mayo *et al*., 1997). Therefore, it was of interest to determine whether the downregulation of Par-4 is important for Ras to protect cells from apoptosis. To address this possibility we generated a Ras^{V12} cell line that ectopically expresses Par-4 to levels comparable to those of control cell lines (Figure 6A). The ectopic expression of Par-4 induces apoptosis in control NIH 3T3 cells (Diaz-Meco *et al*., 1996). The reasons why we succeeded here in obtaining clones that ectopically express Par-4 are probably two-fold: on the one hand, the expression levels of the ectopic Par-4 did not exceed the amount of endogenous protein in control NIH 3T3 cells (Figure 6A); on the other hand, the simultaneous expression of oncogenic Ras activates the PI 3-kinase-dependent survival signaling cascade that would make the expression of Par-4 compatible with cell viability. If the downregulation of Par-4 by oncogenic Ras is important for Ras-induced survival, one can predict that cells which simultaneously express Ras^{V12} and Par-4 would be more vulnerable to inducers of apoptosis than cells expressing Ras^{V12} alone. The inactivation of NF-κB has been shown to be a key step in the sensitization of cells to tumor necrosis factor-α (TNF-α)-induced apoptosis. We recently have shown that the expression of Par-4 in NIH 3T3 cells potentiates the ability of TNF-α to induce apoptosis by inhibiting the activation of NF-κB (Diaz-Meco *et al*., 1999). Because of the activation of the PI 3-kinase survival pathway by oncogenic Ras, the

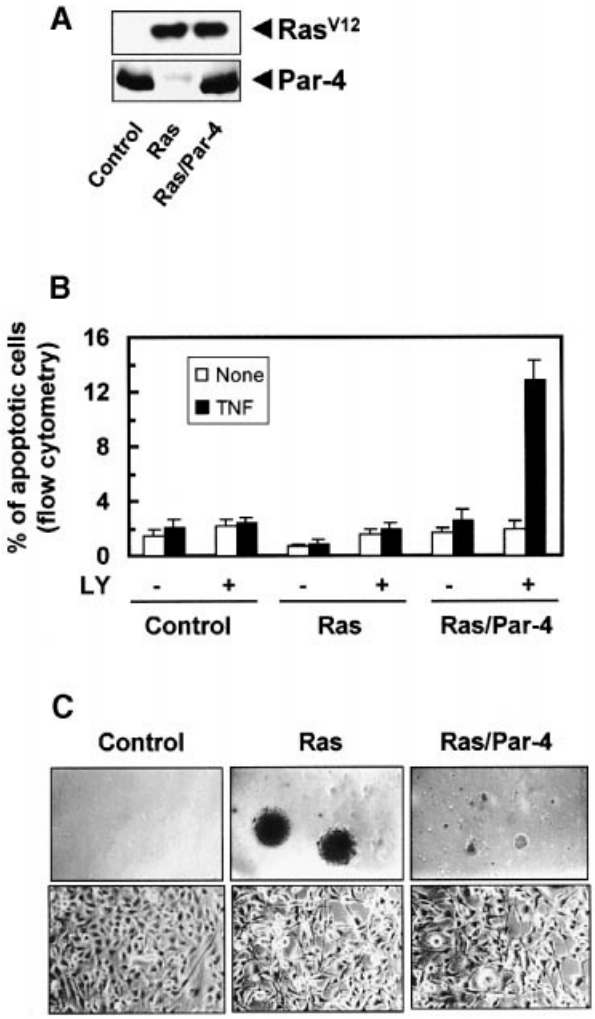

Fig. 6. Effect of ectopic Par-4 expression on Ras-induced cell survival and colony formation in soft agar. (**A**) Representative immunoblot analysis of the expression of Ras^{V12} and Par-4 in the different NIH 3T3 cell lines transduced with Ras^{V12} either alone (Ras) or together with Par-4 (Ras/Par-4) retroviral expression vectors. (**B**) NIH 3T3 cells, either control or expressing Ras^{V12} (Ras) or Ras^{V12} and ectopic Par-4 (Ras/Par-4), were treated with TNF-α (100 ng/ml) for 24 h, either in the absence or the presence of LY294002 (20 μM), after which the percentage of apoptotic cells (with sub-G_1 DNA content) was determined by flow cytometry analysis. (**C**) Upper panels: aliquots containing 2×10^4 NIH 3T3 cells, either control or expressing Ras^{V12} alone (Ras) or in combination with ectopic Par-4 (Ras/Par-4), were plated in 0.3% agar containing DMEM plus 10% FCS. Photographs of representative experiments were taken after 20 days of growth. Lower panels: representative photomicrographs of the above cell lines plated and grown as monolayer cultures in DMEM plus 10% FCS.

vulnerability of the Ras^{V12} and Par-4 double expressers would be more apparent in the presence of LY294002. In order to investigate this hypothesis, NIH 3T3 cells, either controls or expressing Ras^{V12} or Ras^{V12} and ectopic Par-4 (Ras/Par-4), were treated with TNF-α for 24 h, either in the absence or presence of LY294002, after which the percentage of apoptotic cells (with sub-G_1 DNA content) was determined by flow cytometry analysis. According to the data of Figure 6B, the exposure of control NIH 3T3 cells to either LY294002 or TNF-α, or a combination of both, did not affect cell viability appreciably. The Ras-transformed cell line displayed a

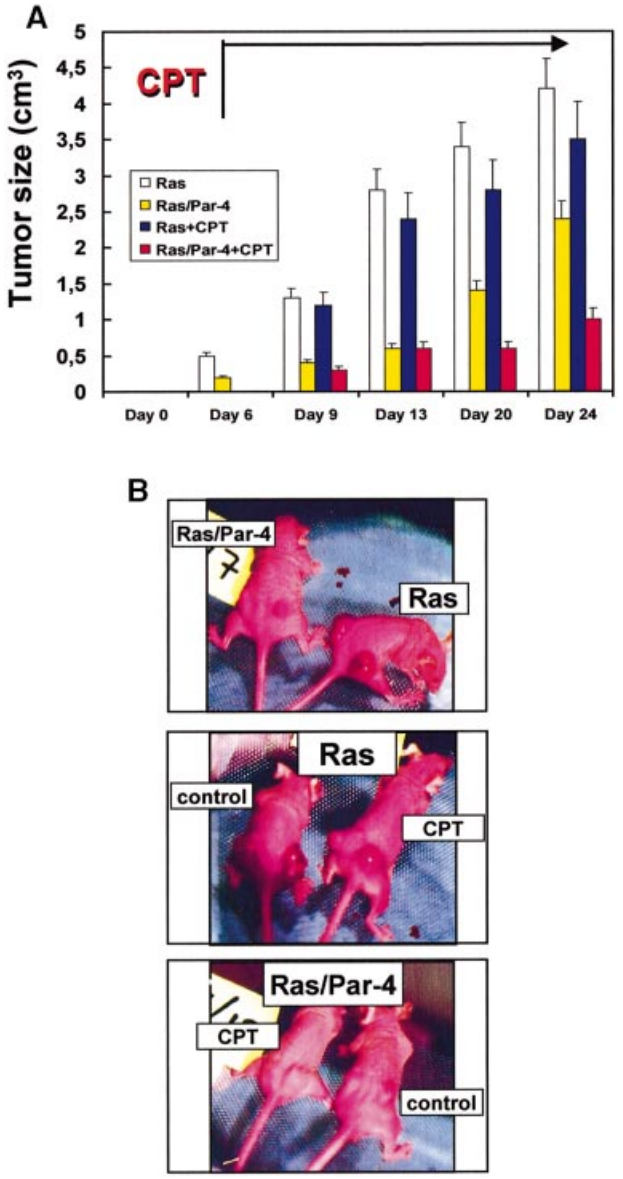

Fig. 7. The ectopic expression of Par-4 inhibits tumor development of Ras- transformed cells *in vivo*. Suspensions of Ras or Ras/Par-4 cell lines were injected intradermally into each flank of nude mice, and tumors were allowed to develop for 24 days. Camptothecin (CPT) or PBS were administered to the mice described above at day 6 after injection of the two cell lines. The size of the tumor was determined as described in Wang *et al.* (1999). The results shown in (**A**) represent the mean ± SD of five mice in each group in one experiment and is representative of another two experiments with similar results. (**B**) Upper panel: two representative mice injected with either Ras or Ras/Par-4 cells are shown at 24 days of tumor development. The middle and lower panels show representative animals injected with Ras or Ras/Par-4 cell lines, respectively, which have been treated with PBS (control) or CPT.

reduced basal level of apoptosis compared with that of the control cell line. The presence of the PI 3-kinase inhibitor returned the basal levels of apoptosis to those of the control cell line, indicating that the survival pathway that controls cell viability in Ras-transformed cells can be accounted for by the activation of PI 3-kinase by this oncogene (Figure 6B). More importantly, the incubation of the double expresser cell line (Ras/Par-4) with TNF-α in the presence of LY294002 provoked a reproducible increase in apoptosis that was not observed when this cell line was incubated with TNF-α or the PI 3-kinase inhibitor alone (Figure 6B). Collectively, these results suggest that the downregulation of Par-4 is important for Ras^{V12} to induce survival in TNF-α-treated cells and that this pathway is apparent under conditions in which the PI 3-kinase survival signaling cascade is impaired. Therefore, the downregulation of Par-4 by oncogenic Ras may be critical for cells to survive during the induction of the transforming phenotype. If this notion is correct, the ectopic expression of Par-4 should reduce the ability of oncogenic Ras to induce cell colonies in soft agar, or to produce tumors in nude mice. Consistent with this hypothesis, the colony-forming activity of the Ras-transformed NIH 3T3 cell line is inhibited dramatically by the ectopic expression of Par-4 (Figure 6C, upper panels). Therefore, all these results suggest that the down-regulation of Par-4 is a required event for the proliferative potential of Ras-transformed cells. Interestingly, the ectopic expression of Par-4 does not affect the morphological phenotype of the Ras-expressing cells (Figure 6C, lower panels), indicating that Par-4 controls the proliferative/survival signals activated by oncogenic Ras but not those involved in the control of the morphological changes associated with oncogenic transformation.

In order to determine whether the ectopic expression of Par-4 would reduce the tumorigenic potential of Ras-transformed cells *in vivo*, we injected cell suspensions of the Ras or Ras/Par-4 cell lines described above intradermally into each flank of nude mice, and tumors were allowed to develop for 24 days. The results shown in Figure 6A and B demonstrate that the Ras/Par-4 cell line was much less efficient in inducing tumors *in vivo* than the Ras cell line, especially at early times of tumor development, which is in keeping with its lower proliferative potential *in vitro* (Figure 6C). The expression of Par-4 has recently been shown to inhibit NF-κB in response to TNF-α in normal cells, as well as the synergistic activation of this transcription factor by oncogenic Ras in TNF-α-treated cells (Diaz-Meco *et al.*, 1999). Previous observations in Baldwin's laboratory have demonstrated that tumor cells with an impaired NF-κB response are particularly sensitive to the anti-cancer actions of the chemotherapeutic compound camptothecin (Wang *et al.*, 1999). Because the inhibition of the aPKCs by Par-4 has a measurable impact in the activation of NF-κB, not only in normal cells but also in Ras transformants (Diaz-Meco *et al.*, 1999), we reasoned that the tumors derived from the Ras/Par-4 cell line would be more sensitive to camptothecin than the tumors developed by the Ras cell line. To address this hypothesis, camptothecin or phosphate-buffered saline (PBS) were administered to the mice described above at day 6 after injection of the two cell lines. Interestingly, camptothecin treatment has little effect on the tumors derived from the Ras cell line, whereas it completely abrogated the growth of the tumors derived from the Ras/Par-4 cell line (Figure 7A and B). Together, these results suggest that the depletion of Par-4 promoted by oncogenic Ras is critical for tumor development.

Discussion

Ras activates a number of effectors that play important roles in cell cycle progression and survival (Katz and

McCormick, 1997; Downward, 1998). The activation of the MEK–ERK signaling cascade has been reported to provide an important anti-apoptotic signal in several cell systems (Xia *et al*., 1995; Gardner and Johnson, 1996; Berra *et al*., 1997, 1998; Parrizas *et al*., 1997; Erhardt *et al*., 1999). In addition, oncogenic Ras activates PI 3-kinase and Akt, which blocks apoptosis by phosphorylation of at least two targets. One of these may be the pro-apoptotic protein BAD whose phosphorylation by Akt enables its inactivation by interaction with 14-3-3 (Datta *et al*., 1997; del Peso *et al*., 1997). The other substrate is caspase-9, whose activity is reduced by Akt phosphorylation (Cardone *et al*., 1998). The mechanism whereby ERK induces cell survival is less clear, but recent results demonstrate that in *Drosophila* ERK inactivates the pro-apoptotic protein Hid possibly by direct phosphorylation (Bergmann *et al*., 1998; Kurada and White, 1998). Here we present evidence of a potential novel mechanism whereby oncogenic Ras promotes cell survival. Thus, we demonstrate that the expression of Ras^{V12} induces the downregulation of the pro-apoptotic protein Par-4, through the MEK–ERK signaling pathway. We also show that active mutants of both Raf and ζPKC are able to mimic the actions of Ras, consistent with the proposed bifurcation of cascades downstream of Ras towards both protein kinases (Diaz-Meco *et al*., 1994; Bjorkoy *et al*., 1995, 1997).

In addition, the findings reported here unveil the existence of an activation loop for the regulation of ζPKC activity. Thus, the aPKCs are activated by Ras (Diaz-Meco *et al*., 1994; Überall *et al*., 1999) and inhibited by Par-4 (Diaz-Meco *et al*., 1996). Interestingly, the activation of ζPKC decreases the levels of Par-4, which probably serves to activate the atypical PKCs further. This stimulatory loop may be important for survival during cell transformation in light of the role of the aPKCs as anti-apoptotic kinases (Diaz-Meco *et al*., 1996; Berra *et al*., 1997; Murray and Fields, 1997). In this regard, we have recently shown that at least one of the mechanisms whereby Par-4 induces apoptosis is by inhibiting NF-κB through its ability to block the aPKC–IKK axis (Diaz-Meco *et al*., 1999). NF-κB has been shown to be necessary for Ras-induced transformation (Finco *et al*., 1997; Mayo *et al*., 1997). Thus, the expression of a mutant of IκBα that cannot be phosphorylated by the IKKs and subsequently hydrolyzed by the proteasome severely inhibits the Ras focus-forming activity (Finco *et al*., 1997). This is particularly interesting because we have shown recently that Ras-induced transformation has a measurable impact on TNF-α signaling towards NF-κB activation (Diaz-Meco *et al*., 1999). Thus, Ras transformation promotes by itself a slight but significant translocation of NF-κB to the nucleus, consistent with previously published results (Mayo *et al*., 1997), and synergistically cooperates with TNF-α to activate NF-κB (Diaz-Meco *et al*., 1999). When Par-4 is ectopically expressed along with transforming Ras, not only is the activation of NF-κB by TNF-α inhibited, but also the synergism between Ras and TNFα is abrogated (Diaz-Meco *et al*., 1999). Altogether, these results would be consistent with a model whereby the downregulation of Par-4 by oncogenic Ras serves to sensitize cells for the activation of NF-κB that, together with the stimulation of the PI 3-kinase–Akt pathway, contributes to the survival of these transformed cells, a necessary event for tumor development. Here we have succeeded in obtaining a Ras-transformed cell line in which the level of Par-4 has been restored to normal values. This cell line displays an impaired ability to grow in colonies in soft agar as well as to develop tumors in nude mice. Interestingly, when PI 3-kinase is inhibited, TNF-α promotes apoptosis in the Ras/Par-4 double expresser but not in the control or in the Ras-transformed cell line. This indicates that inhibition of NF-κB by Par-4 (Diaz-Meco *et al*., 1999) is essential along with the blockade of PI 3-kinase in order for Ras-transformed cells to undergo apoptosis in response to TNF-α. This is particularly relevant from the point of view of potential therapeutic intervention. Thus, the chemotherapeutic compound camptothecin is, like TNF-α, a potent activator of NF-κB (Wang *et al*., 1999), and its ability to promote tumor regression is greatly increased in cell lines in which NF-κB is inhibited (Wang *et al*., 1999), indicating that the activation of NF-κB by this drug is a drawback for its pharmacological efficiency. Interestingly, we show here that camptothecin is very inefficient in promoting the regression of tumors derived from Ras-transformed NIH 3T3 fibroblast but that it does block the development of tumors from the Ras/Par-4 cell line effectively. This strongly suggests that the ability of normal levels of Par-4 in Ras transformants to make these cells more sensitive to apoptotic activation may have important therapeutic consequences.

Apoptosis and cell cycle progression are two tightly controlled and interconnected processes that ensure genetic fidelity during cell proliferation. Both phenomena are subverted in cancer in which anti-apoptotic proteins are overexpressed (Ambrosini *et al*., 1997; Li *et al*., 1998; Deveraux and Reed, 1999). Interestingly, recent data from human tumors demonstrate a dramatic decrease of Par-4 levels in renal cell carcinomas (Cook *et al*., 1999). Also, for example, Peli *et al*. (1999) demonstrated the downregulation of Fas by oncogenic Ras as one of the important mechanisms whereby this oncogene promotes cell survival. However, while the downregulation of Fas is mediated by the PI 3-kinase–Akt signaling cascade (Peli *et al*., 1999), that of Par-4 is channeled through MEK (this study). Therefore, it seems that two independent branches of Ras signaling share the ability to downregulate pro-apoptotic proteins. Oncogenic transformation will favor the survival of cancer cells not only by upregulating anti-apoptotic proteins but also by down-modulating pro-apoptotic proteins.

Materials and methods

Cell culture

NIH 3T3 cells, HeLa cells and human primary fibroblasts (IMR90) were obtained from the American Type Culture Collection (ATCC). Normal diploid human IMR90 fibroblasts expressing the murine ecotropic receptor and primary MEFs derived from wild-type, $p53^{-/-}$ and $p16/p19^{-/-}$ day 13.5 embryos were prepared as described previously (Serrano *et al*., 1997). NIH 3T3 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, penicillin G (100 μg/ml) and streptomycin (100 μg/ml) (Flow). HeLa cells were maintained in minimum Eagle's essential medium supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium piruvate and 10% FCS.

Retroviral-mediated gene transfer

H-RasV12 and MEK^{Q65P} were expressed using the pBabe-Puro vector as described previously (Serrano *et al*., 1997; Lin *et al*., 1998).

Myc-Raf^{CAAX} and ζPKC^{CAAX} were subcloned into the same vector. PI3K^{CAAX} (p110α^{CAAX}) in pSG5 (Marte *et al*., 1997) was subcloned into pLPC (Serrano *et al*., 1997).

Retroviral gene transfer was performed as described (Serrano *et al*., 1997) using high-titer retroviral stocks generated by transient transfection of the Bosc23 ecotropic packaging line (Pear *et al*., 1993). Infected cell populations were selected by culture in puromycin (2 μg/ml, 3 days) to eliminate uninfected cells. In all experiments, cells infected with empty vector were used as control.

Immunoblot analysis

Cells extracts were prepared as described (Serrano *et al*., 1997). In some experiments, cells were treated for 24 h with PD98059 (50 μM; Calbiochem) or LY294002 (20 μM; Calbiochem). The inhibitors were added every 12 h. The inhibition of DNA methylation was achieved by culturing the cells in 10 μM 5-aza-2′-deoxycytidine (Sigma) for up to 36 h. Cellular extracts were resolved in SDS–polyacrylamide (10%) gels. Afterwards, they were transferred electrophoretically onto a nitrocellulose membrane (Hybond ECL, Amersham International) and incubated with the specific antibodies. The bands were visualized with the ECL system (Amersham International). The following antibodies were used: rabbit polyclonal affinity-purified anti-Par-4 (Diaz-Meco *et al*., 1996), monoclonal anti-Ras (Calbiochem), polyclonal anti-MEK (Transduction Laboratories), polyclonal anti-phospho-Akt (phosphoSer473; New England Biolabs), monoclonal anti-phospho-ERK and polyclonal anti-ζPKC, anti-Myc and anti-Fas antibodies (Santa Cruz Biotechnologies).

RT–PCR analysis

RNA was extracted using the RNAzol procedure (Cinna/Biotecx Laboratories, Inc.). Total RNA (1 μg) was analyzed by RT–PCR with Superscript II reverse transcriptase (Life Technologies) and AmpliTaq DNA polymerase (Perkin Elmer). Par-4 and actin detection were carried out with the following primers: 5′-ATCCTCTACCTGGTCGGC-TGACCCACAACTTT-3′ and 5′-GCCGCAGAGTGCTTAGA-3′ (for Par-4); 5′-GCATGGAGTCCTGTGGCATCCACG-3′ and 5′-GGTGTA-ACGCAACTAAGTCATAG-3′ (for actin). The PCR was performed using different inputs of the reverse transcription reaction and different numbers of cycles to verify that the PCR was not outside the linear range.

Immunofluorescence

HeLa cells were grown on glass coverslips in growth media. Subconfluent cells were transfected by the calcium phosphate method with 5 μg of HA-H-Ras^{V12} (White *et al*., 1995). At 24 h post-transfection, cells were washed twice rapidly in ice-cold PBS, and fixed in 4% formaldehyde for 15 min at room temperature. Cells were washed four times with PBS and permeabilized with 0.1% Triton X-100. Free aldehyde groups were quenched with 50 mM NH_4Cl. Endogenous peroxidase activity was quenched by treatment with 1% H_2O_2 in PBS for 15 min. The fixed cells were incubated in blocking solution. Cells were incubated with the different antibodies for 1 h at 37°C. Transfected HA-tagged Ras was visualized with the monoclonal 12CA5 anti-HA (Boehringer Mannheim) and an FITC-conjugated goat anti-mouse antibody (Cappel). Endogenous Par-4 was detected with a rabbit polyclonal affinity-purified anti-Par-4 antibody (Diaz-Meco *et al*., 1996) and the tetramethylrhodamine tyramide TSA-Direct amplification system (NEN Life Science Products). Glass coverslips were mounted on Mowiol and were examined with an MRC 1024 Bio-Rad confocal system (Bio-Rad, Richmond, CA) mounted on a Zeiss Axiovert 135 microscope (Zeiss, Oberkochen, Germany).

Flow cytometry

Apoptosis was determined by flow cytometry according to the following protocol. Briefly, cells were seeded at 10^6 cells per 100 mm culture dish in DMEM containing 10% FCS. After different treatments according to the experiments, medium was collected and cells were trypsinized after washing with PBS. The cell medium and the trypsinized cells were centrifuged and the pellet was resuspended in 1 ml of a solution containing 50 μg/ml of propidium iodide, 20 μg/ml of RNase, 0.6% NP-40 and 0.1% sodium citrate, and incubated for 20 min at 37°C. Cells were then analyzed in an EPICS XL flow cytometer (Coulter Electronics Inc., Hialeah, FL) by recording the propidium iodide staining in the red channel. The percentage of apoptotic cells was determined by calculating the fraction of cells with sub-G_1 DNA content.

In vivo tumor growth

Suspensions of Ras or Ras/Par-4 cell lines (8×10^5/100 μl) were injected intradermally into each flank of female athymic 4- to 6-week-old nu/nu mice, and tumors were allowed to develop for 24 days. Camptothecin (33 mg/kg in 100 μl of PBS) or PBS alone were administered to the mice described above at day 6 after injection of the two cell lines. The size of the tumor was determined as described in Wang *et al*. (1999).

Colony growth assays

To determine the ability of different cell lines to grow in soft agar, 2×10^4 cells were suspended in 0.3% agar in DMEM plus 10% FCS and overlaid on 0.5% agar in the same medium. Cells were re-fed with 10% FCS-containing medium every 5 days.

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