

Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway

Asim Khwaja, Pablo Rodriguez-Viciana, Stefan Wennström, Patricia H. Warne and Julian Downward¹

Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

¹Corresponding author
e-mail: downward@icrf.icnet.uk

Upon detachment from the extracellular matrix, epithelial cells enter into programmed cell death, a phenomenon known as anoikis, ensuring that they are unable to survive in an inappropriate location. Activated *ras* oncogenes protect cells from this form of apoptosis. The nature of the survival signals activated by integrin engagement and usurped by oncogenic Ras are unknown: here we show that in both cases phosphoinositide 3-OH kinase (PI 3-kinase), but not Raf, mediates this protection, acting through protein kinase B/Akt (PKB/Akt). Constitutively activated PI 3-kinase or PKB/Akt block anoikis, while inhibition of PI 3-kinase abrogates protection by Ras, but not PKB/Akt. Inhibition of either PI 3-kinase or PKB/Akt induces apoptosis in adherent epithelial cells. Attachment of cells to matrix leads to rapid elevation of the levels of PI 3-kinase lipid products and PKB/Akt activity, both of which remain high in Ras-transformed cells even in suspension. PI 3-kinase acting through PKB/Akt is therefore implicated as a key mediator of the aberrant survival of Ras-transformed epithelial cells in the absence of attachment, and mediates matrix-induced survival of normal epithelial cells.

Keywords: Akt/apoptosis/integrin/phosphatidylinositol 3-kinase/Ras

Introduction

Many mammalian cell types are dependent on adhesion to the extracellular matrix for their continued survival. Upon detachment from the matrix, endothelial (Meredith *et al.*, 1993) and epithelial cells (Frisch and Francis, 1994) enter into programmed cell death; this cell detachment-induced apoptosis has been referred to as 'anoikis', from the Greek for homelessness (Frisch and Francis, 1994). This mechanism ensures that these types of cells do not normally survive in the absence of the correct interaction with extracellular matrix proteins and therefore are unable to proliferate in inappropriate sites or to survive in the absence of attachment, for example in the bloodstream. Some adherent cell types with a specialized tissue invasive function, such as some fibroblasts, do not undergo anoikis, although they do become quiescent in the absence of contact with the matrix (Folkman and Moscona, 1978; Ben-Ze'ev *et al.*, 1980).

The interaction of cells with the extracellular matrix is mediated by the integrin family of cell surface proteins. Upon binding to matrix proteins, integrins transmit an 'outside-in' signal to the cell; this triggers a large array of intracellular signalling events and rearrangement of cytoskeletal architecture (Parsons, 1996). A number of signal transduction pathways normally associated with the binding of soluble growth factors to their receptors are also activated by integrin engagement. For example, plating fibroblasts on fibronectin has been reported to induce activation of the MAP kinase cascade (Chen *et al.*, 1994; Schlaepfer *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995); however, others find that integrin engagement is neither necessary nor sufficient for activation of this pathway (Hotchin and Hall, 1995).

Transformed cells are very frequently characterized by their ability to grow in the absence of contacts with a solid extracellular matrix (Stoker *et al.*, 1968). Transformation of a wide range of adherent cells, including fibroblasts and epithelial cells, by cytoplasmic oncogenes such as *ras* or *src* is accompanied by the ability to grow in suspension. Cell detachment-induced apoptosis does not occur in epithelial cells expressing activated *src* or *ras* oncogenes (Frisch and Francis, 1994): these oncogenes must therefore be able to provide constitutively a signal normally emanating from matrix-occupied integrins, or in some other way overcome the anoikis machinery. Given the likely importance of growth and survival in suspension for the maintenance of the transformed phenotype, and particularly for metastatic spread of tumour cells within the body, an understanding of the signal transduction pathways by which oncogenes such as *ras* and *src* prevent this form of apoptosis could be of benefit in designing new therapies for epithelial cell malignancies, which account for some 85% of human tumours.

In recent years, considerable advances have been made in understanding the signalling pathways acting downstream of the proteins encoded by these oncogenes, in particular the Ras proteins. Ras is now known to be able to interact directly with a number of families of target enzymes, or putative effectors (Marshall, 1996). The best characterized of these are the Raf proteins, which are proto-oncogene-encoded serine/threonine kinases that control the MAP kinase, or ERK, signalling pathway and are activated upon interaction with Ras (Marshall, 1994). The Raf/MAP kinase pathway is essential for fibroblast proliferation (Cowley *et al.*, 1994) and is likely to be important in the transformation of fibroblasts and other cell types by *ras* oncogenes. In addition to Raf, a number of other possible targets for Ras are known: the GTPase-activating proteins, p120^{GAP}, Gap1 and neurofibromin, all of which interact with Ras-GTP but not Ras-GDP, could possibly have some function downstream of Ras in addition to their negative regulatory function (Schweighoffer *et al.*, 1992; McGlade

et al., 1993). Ral-GDS, a family of exchange factors for the Ras-related Ral proteins, have been found to interact with activated Ras in the yeast two-hybrid system (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994). Activated Ras is able to regulate the activity of Ral-GDS and hence Ral (Urano *et al.*, 1996): the function of Ral is not well understood, but it has been implicated in the control of phospholipase D (Jiang *et al.*, 1995). Furthermore, phosphoinositide 3-OH kinase (PI 3-kinase) has been shown to interact with GTP-bound Ras, resulting in stimulation of the lipid kinase activity (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994, 1996). In addition to these Ras effectors, a number of other mammalian proteins have been described which interact with Ras in a GTP-dependent manner but for which further confirmation of their role in Ras signalling is not yet available.

In this report, we have set out to study the mechanism by which an activated *ras* oncogene protects the MDCK epithelial cell line from programmed cell death on removal from the extracellular matrix. We show that the Raf/MAP kinase pathway is not involved, but that the PI 3-kinase pathway is both necessary and sufficient for the Ras protection of epithelial cells from anoikis, and also from radiation-induced apoptosis. The serine/threonine kinase known as protein kinase B, Akt or Rac kinase (Bellacosa *et al.*, 1991; Coffey and Woodgett, 1991; Jones *et al.*, 1991), referred to here as PKB/Akt, but not the GTPase Rac or p70^{S6K}, is implicated downstream of PI 3-kinase in this survival pathway. In normal epithelial cells, detachment from matrix leads to a rapid decrease in the levels of the PI 3-kinase products and PKB/Akt activity, while reattachment causes rapid elevation of these lipids and kinase activity: a basal level of PI 3-kinase and PKB/Akt activity is normally maintained by matrix attachment which is essential for avoidance of programmed cell death. In Ras-transformed cells, the levels of the PI 3-kinase product lipids remain high even in suspension, and Akt/PKB is constitutively activated. A novel signalling pathway is therefore being activated by Ras, acting through PI 3-kinase and PKB/Akt to protect Ras-transformed epithelial cells from programmed cell death initiated by disengagement of integrins.

Results

Ras and PI 3-kinase protect MDCK cells from apoptosis in suspension culture

MDCK cells were transfected with constructs expressing activated forms of H-Ras (V12 Ras), Src (v-Src), Raf (Raf-CAAX) (Leever *et al.*, 1994), PI 3-kinase (p110*), Rac (V12 Rac) and PKB/Akt (gag-PKB) (Burgering and Coffey, 1995). A number of partial loss-of-function mutant Ras proteins were also expressed that distinguish between the various effector pathways downstream of Ras (Marshall, 1996): V12 S35 Ras (White *et al.*, 1995) and V12 E38 Ras (Krengel *et al.*, 1990) activate only the Raf pathway, V12 G37 Ras (White *et al.*, 1995) interacts only with Ral-GDS, V12 C40 Ras (Joneson *et al.*, 1996) activates only the PI 3-kinase pathway (Rodriguez-Viciana *et al.*, 1997), while V12 A38 Ras is totally inactive. The morphology of representative clones of these cells is shown in Figure 1. Wild-type MDCK cells had a typical epithelioid morphology, growing in islands of compact

cells. All the Ras effector site mutants showed wild-type morphology, with the exception of V12 C40 Ras, which had a dispersed spindly morphology very similar to V12 Ras- or v-Src-transformed MDCK cells. This morphology was also indistinguishable from that of cells expressing activated PI 3-kinase. Cells expressing activated Raf still grew in islands, but showed a slightly more spindly shape than wild-type cells. Cells expressing activated Rac appeared to be similar to activated PI 3-kinase- or Ras-expressing cells, while activated PKB/Akt-expressing cells had a morphology similar to wild-type (data not shown).

The ability of these cell lines to undergo apoptosis upon detachment from the extracellular matrix was measured. Cells were grown on plastic dishes in serum-containing medium before being removed from the dish, held in suspension in 10% fetal calf serum (FCS) for 8–12 h, and the extent of apoptosis determined by measuring characteristic DNA laddering (Figure 2A), the number of cells that are capable of surviving when replated (Figure 2B) or by enzyme-linked immunosorbent assay (ELISA) assay for cytoplasmic histone-associated DNA fragments (Figure 2C). Parental cells, and clones expressing inactive V12 A38 Ras, were highly susceptible to apoptosis following removal from the extracellular matrix, as were cells expressing activated Raf, and Ras mutants that stimulate only Raf (V12 E38 and V12 S35, data not shown) or Ral-GDS (V12 G37). All MDCK clones expressing V12 Ras or v-Src were almost completely protected from apoptosis following detachment. Clones expressing activated PI-3 kinase were also very highly protected, as were cells expressing V12 C40 Ras, which activates PI 3-kinase only. It therefore appears that the ability of Ras to protect these epithelial cells from anoikis is mediated through the PI 3-kinase, and not the Raf, pathway. In order to investigate which effector pathway downstream of PI 3-kinase is being used in the protection from anoikis, assays were performed on MDCK cell clones expressing V12 Rac or activated PKB/Akt. V12 Rac did not protect the cells from anoikis, but activated PKB/Akt gave comparable protection to activated PI 3-kinase or Ras. Furthermore, rapamycin (Price *et al.*, 1992), which indirectly inhibits p70^{S6K}, was unable to induce apoptosis of adherent MDCK cells, whereas the stable PI 3-kinase inhibitor LY294002 (Thelen *et al.*, 1994; Vlahos *et al.*, 1994) does (Figure 2C). Therefore PKB/Akt is likely to be involved in the protection from anoikis provided by PI 3-kinase, but Rac and p70^{S6K} are not important in this response.

Inhibition of PI 3-kinase or PKB/Akt reverses protection from anoikis

The effect of inhibition of PI 3-kinase activity on the ability of activated Ras, Src and PI 3-kinase to protect MDCK cells from apoptosis on detachment from matrix was studied. As shown in Figure 3A and B, LY294002 negated the protection from anoikis bestowed by V12 Ras, V12 C40 Ras, Src and PI 3-kinase, but did not reverse the protection due to activated Akt/PKB. PI 3-kinase activity is therefore required for activated Ras, Src and p110 to protect epithelial cells from anoikis, but activation of Akt/PKB can provide the entire survival signal generated by PI 3-kinase. PI 3-kinase inhibitory drugs could not completely reverse V12 Ras protection from anoikis: this is probably due to the inability of these

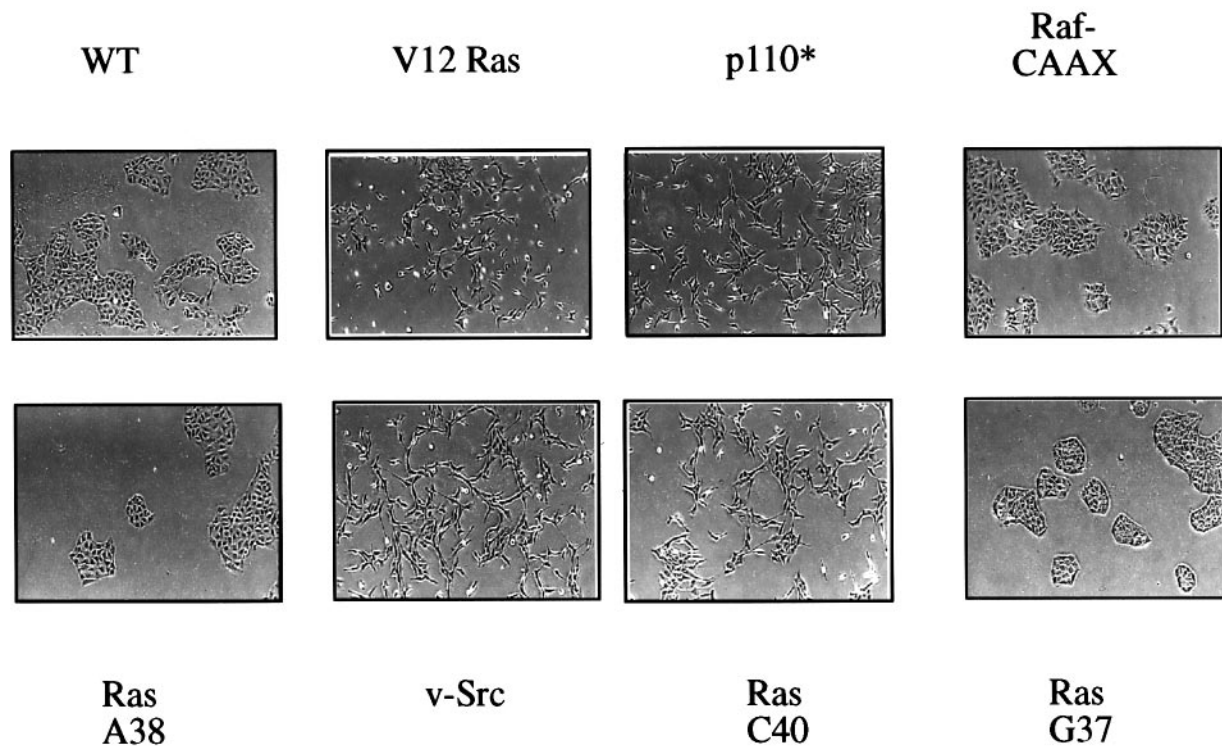


Fig. 1. Morphological appearance of wild-type MDCK and of stably transfected clones. These appearances were typical of at least four separate clones for each of the transfected lines.

drugs to completely reverse V12 Ras-induced elevation of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) levels in cells (Rodriguez-Viciana *et al.*, 1997) and PKB/Akt stimulation (data not shown), possibly due to Ras interaction with drug-resistant PI 3-kinase isoforms (Virbasius *et al.*, 1996). To assess whether PKB/Akt function was required for normal survival of epithelial cells on matrix, a deletion mutant (Datta *et al.*, 1995) of PKB/Akt (AH-Akt, retaining only residues 1–147, the regulatory domain of PKB/Akt) which has dominant-negative function was transiently expressed in normal MDCK cells. As shown in Figure 3C, expression of this construct induced programmed cell death in adherent cells growing normally in serum. The ability of this AH-Akt construct to act as a dominant-negative in terms of the regulation of wild-type PKB/Akt was tested by co-transfecting it along with tagged wild-type PKB/Akt into MDCK cells under the same conditions as in Figure 3C. Tagged PKB/Akt was immunoprecipitated from the transfected cells and assayed for kinase activity towards histone 2B as described in Materials and methods. Four μ g of AH-Akt vector completely abolished the ability of hepatocyte growth factor/scatter factor to induce activation of tagged PKB/Akt (1 μ g of vector), which was ~2-fold in the absence of the dominant negative. The unstimulated basal kinase activity of PKB/Akt (corrected for expression level) was reduced by >50% by AH-Akt under these conditions.

Activated PI 3-kinase blocks DNA damage-induced apoptosis

In order to investigate whether activation of PI 3-kinase was able to suppress other forms of apoptosis in MDCK cells, the various MDCK cell lines were tested for their sensitivity to UV irradiation-induced apoptosis when

growing in serum-containing medium as an adherent monolayer. As shown in Figure 4, V12 Ras strongly protects from UV radiation-induced apoptosis, as do activated PI 3-kinase, v-Src and V12 C40 Ras, all to a slightly lesser extent. V12 G37 Ras fails to protect, while Raf-CAAX shows only a low, but statistically significant, level of protection. PI 3-kinase is thus important in protecting cells from more than one type of programmed cell death, although somewhat different mechanisms may be involved in each case. The weak protection by Raf-CAAX from UV irradiation-induced apoptosis might possibly be mediated by the autocrine growth factors known to be produced by cells expressing activated Raf (McCarthy *et al.*, 1995), which are capable of activating PI 3-kinase and PKB/Akt. As shown in Figure 6D, growth factors have a greatly reduced ability to activate PKB/Akt via PI 3-kinase in cells in suspension (as in the anoikis assay) compared with on the dish (as in the UV-induced apoptosis assay); conditioned medium from activated Ras- or Raf-expressing cells, and indeed added growth factors or serum, will not protect normal MDCK cells from anoikis (data not shown).

Attachment of cells to matrix induces elevation of 3' phosphorylated phosphoinositides

The data from Figures 2 and 3 suggest that attachment of MDCK cells to the extracellular matrix may stimulate the activity of PI 3-kinases leading to the production of elevated levels of PIP₃ and phosphatidylinositol 3,4-diphosphate [PI(3,4)P₂] which then act to protect the cells from apoptosis by stimulating PKB/Akt, possibly by direct binding to its pleckstrin homology domain (Franke *et al.*, 1995; James *et al.*, 1996). In this model, removal of cells from matrix would be expected to result in a decrease in

PIP₃ levels, as would treatment of adherent cells with PI 3-kinase inhibitors, both of which trigger apoptosis. In order to test this hypothesis, the levels of PIP₃ and PI(3,4)P₂ were compared in cells in suspension versus adherent cells. As shown in Figure 5A and B, parental MDCK cells growing on dishes in serum-free medium have levels of PIP₃ and PI(3,4)P₂ that are 3- to 5-fold higher than those in cells which have been in suspension for 2 h. Upon replating cells on collagen, there is a rapid increase in PIP₃ and PI(3,4)P₂ to levels similar to those of the original adherent cells. Cells growing in the continuous

presence of 10% FCS show very similar changes in PIP₃ and PI(3,4)P₂ levels following detachment and reattachment. Cycling cells in the presence of serum do not show significantly elevated levels of 3' phosphorylated phosphoinositides relative to quiescent cells in the absence of serum. Although acute treatment with serum does cause moderate elevation of these lipids, the effect is very transient, rapidly returning to basal levels (data not shown).

These effects on PIP₃ and PI(3,4)P₂ levels are not due to global changes in phospholipid metabolism since in this cell type the levels of PI(4,5)P₂ are not altered on detachment (Figure 5C). The fact that PI 3-kinase lipid product levels are elevated by matrix attachment alone in the absence of serum factors indicates that attachment of epithelial cells to extracellular matrix is sufficient for activation of PI 3-kinase. In the case of p110-CAAX- and V12 Ras-expressing cells, the level of PIP₃ in adherent cells in serum-free medium was markedly higher than in parental cells (Figure 5D). Upon suspension, the levels of PIP₃ and PI(3,4)P₂ drop only slightly in p110-CAAX- and V12 Ras-expressing cells, remaining well above those in adherent parental MDCK cells. v-Src- and V12 C40 Ras-expressing cells in suspension also maintain levels of PIP₃ that are above that of adherent wild-type cells. The ability of cells to resist anoikis thus correlates with their ability to maintain levels of PI 3-kinase activity in suspension that are at least as great as in normal adherent cells.

Attachment induces activation of PKB/Akt

In addition to studying lipid levels, the activation state of the known downstream protein kinase targets of PI 3-kinase, p70^{S6K} and PKB/Akt, was also measured (Burgering and Coffey, 1995; Franke *et al.*, 1995; Weng *et al.*, 1995). p70^{S6K} shows elevated activity in V12 Ras-, V12 C40 Ras-, v-Src- or p110-CAAX-expressing cells in suspension, while normal MDCK cells and those expressing Raf-CAAX or V12 G37 Ras show no p70^{S6K} activity under these conditions (Figure 6A). Endogenous Ras function previously has been reported not to be necessary for activation of p70^{S6K} by growth factors (Ming *et al.*, 1994): these data show that expression of V12 Ras is, however, sufficient to stimulate this kinase, both in adherent and suspended cells. By contrast, only V12 Ras- and Raf-CAAX-expressing cells show high Erk2 MAP kinase activity in suspension. In addition, attachment of normal MDCK cells to collagen-coated plates is found to

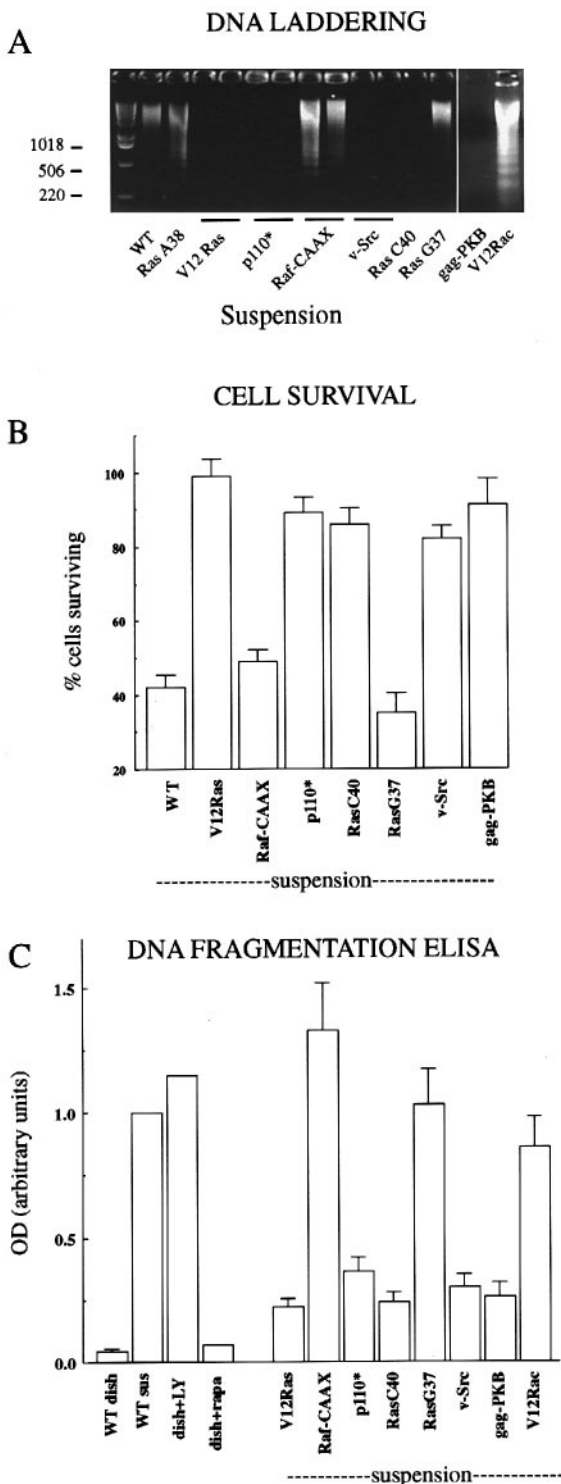


Fig. 2. Effect of detachment from matrix on apoptosis of wild-type and transfected MDCK cells. (A) MDCK cells were maintained in suspension for 8 h, low molecular weight DNA extracted, run on a 1.5% agarose gel and visualized with ethidium bromide. The left hand lane shows a 1 kb ladder for comparison. Samples from two separate clones are shown for V12 Ras, activated p110, activated Raf and v-Src. Similar results were seen for all constructs tested in at least four distinct clones. (B) Cell survival measured by the MTS method after maintenance in suspension for 12 h. Points were assayed in quadruplicate in each experiment and the data (mean ± SEM) are from six to nine experiments employing at least three different clones for each cell type. (C) DNA fragmentation ELISA. Comparison is made with wild-type cells maintained on the tissue culture dish. Treatment of attached cells with the PI 3-kinase inhibitor LY294002 (20 μM) but not rapamycin (5 ng/ml) causes apoptosis (mean of two estimations). DNA fragmentation was measured in wild-type and transfected cells kept in suspension for 8 h. Data are mean ± SEM from five to ten separate estimations with at least three distinct clones for each cell type.

cause activation of Erk2 and p70^{S6K}, even in the absence of serum factors (Figure 6B). The Erk2 activation seen here is considerably stronger than the very weak activation reported by Frisch *et al.* (1996): the reason for this is not clear. The activation of p70^{S6K} found here is sensitive to inhibition by the PI 3-kinase inhibitor wortmannin, and also by LY294002 (data not shown). The activation state of PKB/Akt was also studied: on detachment, cellular PKB/Akt activity drops by 3- to 5-fold, and on reattach-

ment to collagen it increases by 10-fold (Figure 6C and D). PKB/Akt is constitutively very active in V12 Ras-, p110-CAAX- or V12 C40 Ras-expressing cells, both when adherent (Figure 6C) and in suspension (data not shown) but not in Raf-CAAX- or V12 G37 Ras-expressing cells. Thus PKB/Akt activity, and also that of p70^{S6K}, closely reflects the levels of PIP₃ and PI(3,4)P₂, with matrix attachment alone providing a significant level of PI 3-kinase, PKB/Akt and p70^{S6K} activity. Serum factors are unable to maintain high levels of any of these three activities in the absence of integrin engagement. In response to specific growth factors, saturating concentrations of epidermal growth factor (EGF) will give a weak acute stimulation of PKB/Akt in cells in suspension, to a similar extent to the chronic stimulation seen due to adhesion alone in the absence of growth factors (Figure 6D). Presumably, adhesion in the absence of growth factors can protect from apoptosis because it causes long lasting activation of PKB/Akt, whereas the stimulation of this kinase by growth factor in the absence of adhesion is only transient. However, adhesion and EGF together give a strongly synergistic activation of PKB/Akt. V12 Ras or p110-CAAX keep both 3' phosphorylated phosphoinositide lipid levels and p70^{S6K} and PKB/Akt activity elevated even in the absence of attachment.

In common with parental MDCK cells, V12 Ras- and p110-CAAX-expressing cells show no tyrosine phosphorylation of p125^{FAK} when in suspension (data not shown), suggesting that the protection from apoptosis of these clones is not mediated by constitutive activation of p125^{FAK}. However, in MDCK cells growing on the dish, or adhering to collagen, complexes of p125^{FAK} with p85, the regulatory subunit of PI 3-kinase, can be detected; these disappear on placing the cells in suspension (data not shown). Similar complexes have been observed in other cell types (Chen and Guan, 1994). It is possible that adhesion-induced activation of PI 3-kinase activity is at least in part mediated by this interaction, and possibly also by Ras which is activated on integrin engagement (Kapron-Bras *et al.*, 1993; Clark and Hynes, 1996).

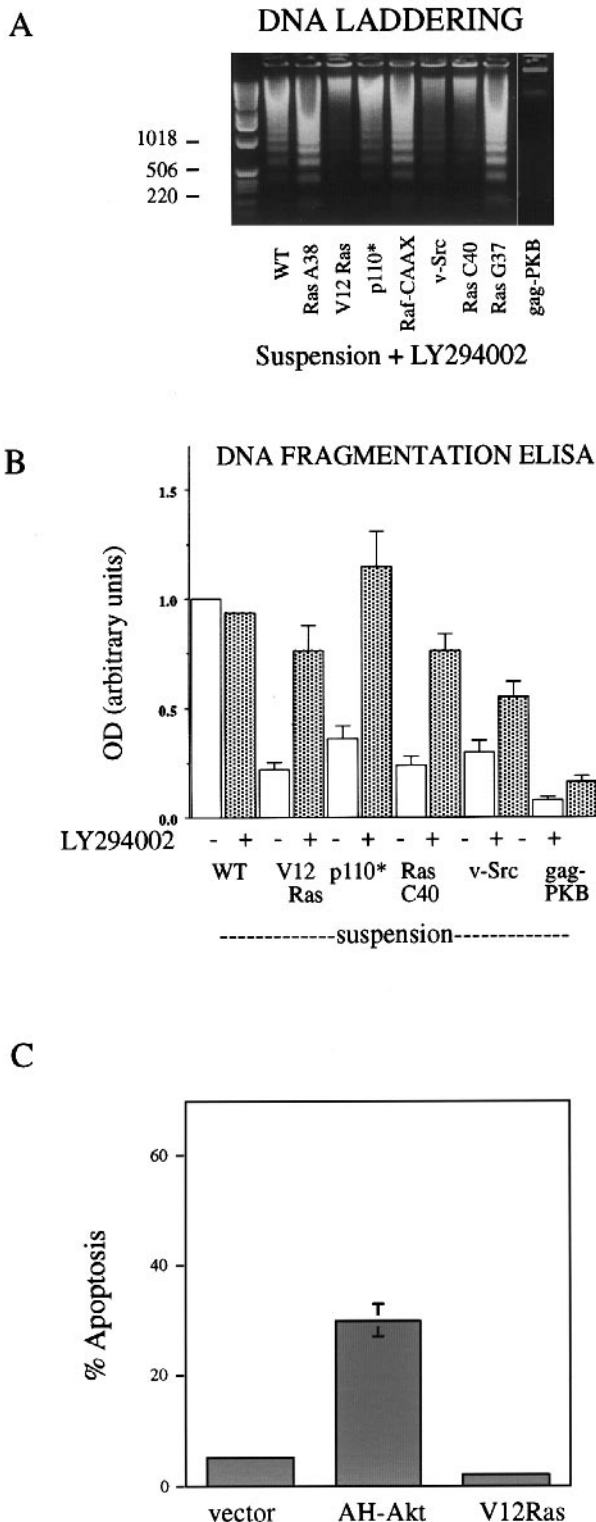


Fig. 3. Effect of the PI 3-kinase inhibitor LY294002 and dominant-negative PKB/Akt on apoptosis. (A) MDCK cells were maintained in suspension for 8 h in the presence of 20 μ M LY294002, low molecular weight DNA extracted, run on a 1.5% agarose gel and visualized with ethidium bromide. The left hand lane shows a 1 kb ladder for comparison. Samples from two separate clones are shown for V12 Ras, activated p110, activated Raf and v-Src. Similar results were seen for all constructs tested in at least three distinct clones. (B) LY294002 reverses Ras and PI 3-kinase but not activated PKB protection from anoikis. DNA fragmentation was quantified by ELISA after cells were kept in suspension for 8 h in the presence of 20 μ M LY294002 (50 μ M V12 Ras). Comparison is made with wild-type cells maintained in suspension without LY294002. Data are mean \pm SEM from three to five separate estimations with at least two distinct clones for each cell type. (C) Dominant-negative PKB/Akt induces apoptosis in MDCK cells. MDCK were co-transfected with pCMV β gal and pSG5 AH-Akt (dominant-negative Akt), pSG5 V12Ras or empty vector. At 24 h after transfection, cells were fixed and stained for β -galactosidase activity. Positively stained cells were scored as normal or apoptotic on the basis of cellular morphology. Data are mean \pm SE of three separate estimations. For comparison, treatment of MDCK with the PI 3-kinase inhibitor LY294002 for a similar period results in the apoptosis of 60% of cells.

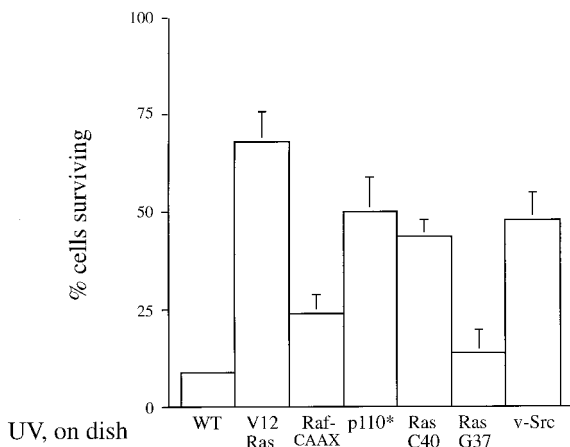


Fig. 4. Effect of UV irradiation on cell survival in wild-type and stably transfected MDCK cells. Cells were plated in 96-well plates at 5×10^3 /well and treated with UVC irradiation at $10\,000 \mu\text{J}/\text{cm}^2$ 8 h later. Cell survival compared with unirradiated control cells was measured 12 h later by the MTS method. Points were assayed in quadruplicate in each experiment and the data (mean \pm SEM) are from six to nine experiments employing at least two different clones for each cell type.

Discussion

The data presented here support the following model of integrin signalling to the apoptotic machinery in untransformed epithelial cells. Upon engagement of integrins by extracellular matrix proteins, PI 3-kinase becomes constitutively activated to a significant level, independently of signals from serum factors. The PI 3-kinase lipid products provide a protective signal acting through PKB/Akt which blocks entry into apoptosis; PI 3-kinase, although not PKB/Akt, previously has been reported to be required for cell survival in other systems (Yao and Cooper, 1995, 1996). When epithelial cells are detached from the matrix, PI 3-kinase and PKB/Akt become inactive, even in the presence of serum factors, and an apoptosis pathway is engaged. Commitment to apoptosis is blocked if PI 3-kinase activity is stimulated in these suspended cells by expression of p110-CAAX or activated Ras, which directly binds and stimulates p110 (Kotani *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994, 1996; Klinghofer *et al.*, 1996), or if activated PKB/Akt is expressed. Thus PI 3-kinase and PKB/Akt play a critical role in sensing the adhesion status of the cell and promoting its survival. The ability of Ras in cells in suspension to activate PI 3-kinase, and hence PKB/Akt, explains the survival of *ras* oncogene-expressing cells in the absence of adhesion to the extracellular matrix, one of the principal hallmarks of malignant transformation.

PI 3-kinase previously has been implicated in growth factor protection from apoptosis in PC12 cells (Yao and Cooper, 1995) and in fibroblasts (Yao and Cooper, 1996). The PI 3-kinase inhibitors wortmannin and LY294002 induce apoptosis in PC12 cells, and the ability of the platelet-derived growth factor (PDGF) receptor to protect these cells from apoptosis relies on the presence of Tyr740, which when phosphorylated is a binding site for the SH2 domains of the p85 regulatory subunit of PI 3-kinase. In addition, the use of these inhibitors has implicated PI 3-kinase in protecting some haemopoietic cells from apoptosis (Scheid *et al.*, 1995; Minshall *et al.*, 1996). The

present report shows for the first time that activation of PI 3-kinase is sufficient to protect cells from apoptotic death.

Activated Ras has variable effects on apoptosis in different cell types, although most commonly it suppresses programmed cell death induced by a variety of agents (Lin *et al.*, 1995). In MDCK cells, activated Ras uses a PI 3-kinase-mediated mechanism to protect from at least two forms of apoptosis, induced either by detachment from matrix or by UV radiation-induced DNA damage. Activated Ras probably protects by directly stimulating PI 3-kinase: it is unlikely that the PI 3-kinase involvement downstream of Ras is through a mechanism involving autocrine production of growth factors since the presence of 10% serum in the medium in which cells are suspended is unable to rescue them from programmed cell death. Conversely, adherent MDCK cells are able to survive in the complete absence of serum for considerably longer than the period of the apoptosis assays performed here (data not shown). Since Raf does not provide any protection, factors produced in response to MAP kinase cascade activation cannot be protective.

GTP-bound Ras interacts directly with the catalytic subunit of PI 3-kinase and activates it (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994, 1996): activated mutants of Ras acting alone can stimulate PI 3-kinase activity in this way, and endogenous normal Ras can contribute to PI 3-kinase activation in synergy with other pathways, particularly those involving the interaction of phosphoproteins with the SH2 domains of p85 (Klinghofer *et al.*, 1996; Rodriguez-Viciana *et al.*, 1996). The various partial loss-of-function effector mutants of Ras used in this study have been described in detail elsewhere (Rodriguez-Viciana *et al.*, 1997): they define a number of effector pathways that Ras can activate. Although the various mutants are 3- to 10-fold less efficient than normal activated Ras in switching on their targets, under conditions of modest overexpression such as here they are able to give close to maximal activation of the downstream pathways that they engage. While we cannot rule out the possibility that effectors other than PI 3-kinase contribute slightly to the Ras protection from anoikis, our data would indicate that Raf and Ral-GDS play no significant part in this pathway.

The mechanism whereby integrin engagement leads to PI 3-kinase activation in normal epithelial cells is not known. At present we do not know whether endogenous normal Ras plays a role in this pathway: it is unknown whether Ras is activated in response to integrin engagement in epithelial cells, although it may well be since integrin engagement alone causes activation of Erk2 in these cells (Figure 6). In other cell types, dominant-negative Ras has been reported to block integrin-induced Erk2 activation in fibroblasts (Clark and Hynes, 1996) and integrin engagement has been shown to increase Ras-GTP levels in lymphocytes (Kapron-Bras *et al.*, 1993). Another possible component of this pathway is p125^{FAK}; this focal adhesion kinase has been found to associate with the SH2 domains of p85 (Chen and Guan, 1994), so it may contribute to the constitutive activation of PI 3-kinase during adherent growth when p125^{FAK} is continuously tyrosine phosphorylated and capable of binding p85. Since serum factors are not required, it is likely that this interaction by itself is enough to maintain a level of

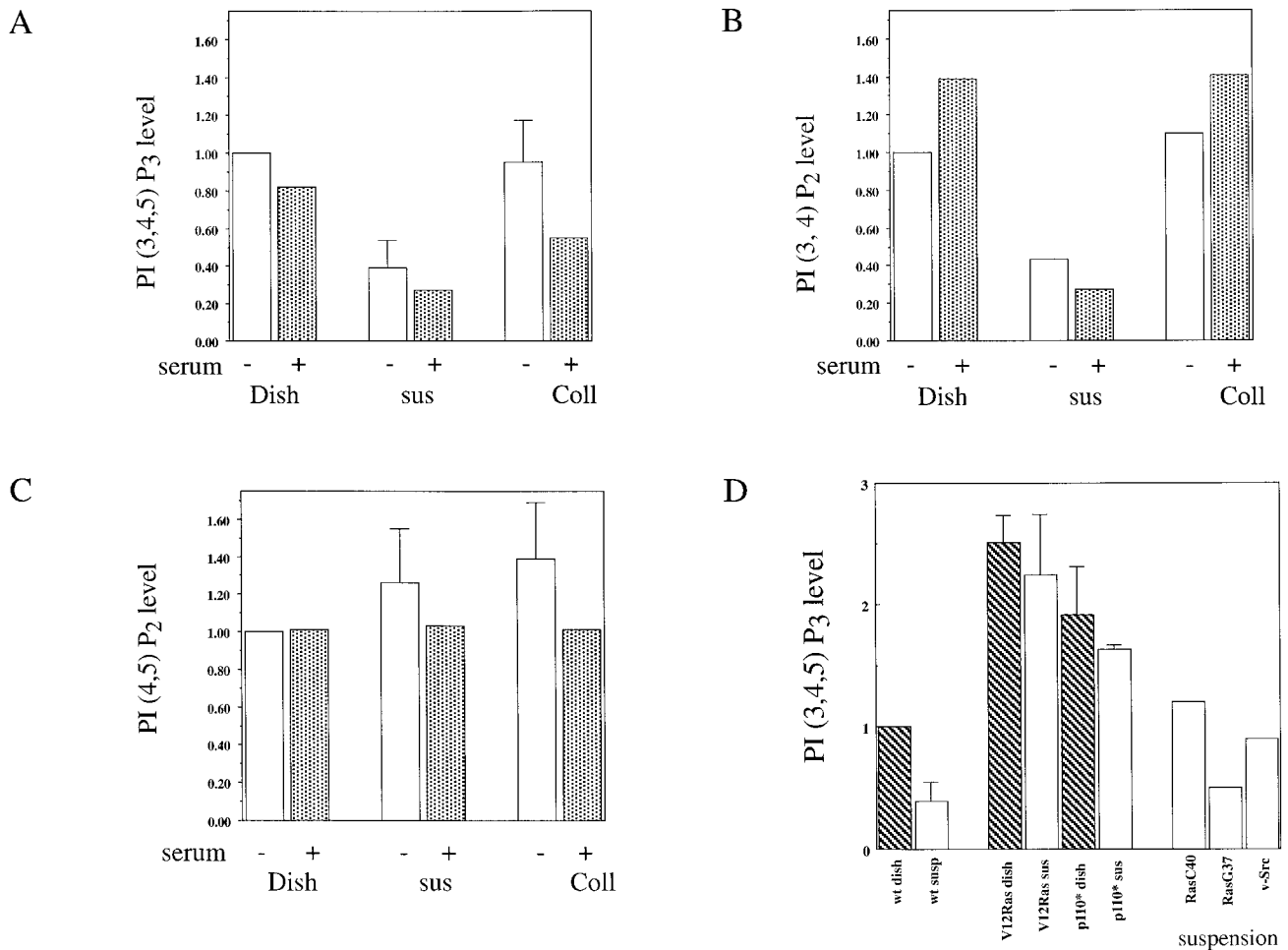


Fig. 5. Phosphoinositide levels in wild-type and stably transfected MDCK cells. Wild-type MDCK cells were labelled with [³²P]orthophosphate and lipids extracted from attached cells (dish), cells maintained in suspension for 2 h (susp) or cells maintained in suspension for 2 h and then replated onto collagen-coated plates for 60 min (coll). Assays were done either in the absence of serum (–) or in the continuous presence of 10% FCS throughout the experiment (+). Cellular phosphoinositides were analysed by HPLC. Data are presented after correction of PI(3,4,5)P₃ (A), PI(3,4)P₂ (B) and PI(4,5)P₂ (C) levels for the number of total input c.p.m. and are normalized to the counts in the wild-type attached cells assayed in the absence of serum. As a percentage of total input c.p.m., the mean value for PI(3,4,5)P₃ in attached cells in the absence of serum was 0.022, for PI(3,4)P₂ 0.016 and for PI(4,5)P₂ 0.139 (results are the means of three separate experiments without serum and two with serum). (D) Comparison of PI(3,4,5)P₃ levels in wild-type MDCK and stably transfected lines. Data are presented after correction of PI(3,4,5)P₃ levels for the number of total input c.p.m. and are normalized to the counts in the wild-type attached cells. PI(3,4,5)P₃ levels were measured in three separate experiments for wild-type, V12 Ras and activated p110 in attached cells and cells kept in suspension for 2 h, and in a single experiment for V12 C40 Ras-, V12 G37 Ras- and v-Src-expressing cells in suspension.

PI 3-kinase activity sufficient to protect epithelial cells from anoikis. The decrease in PIP₃ and PI(3,4)P₂ levels on detachment may be due either to loss of p125^{FAK} complexes with p85, loss of Ras activity or a combination of these and perhaps other factors. Decreases in PI(4,5)P₂ levels due to loss of PI 5-kinase activity occur in detached fibroblasts (McNamee *et al.*, 1993; Chong *et al.*, 1994), but not in MDCK cells, so loss of substrate lipid cannot account for the decreases in PI 3-kinase activity.

Since we have shown here that PI 3-kinase is involved in allowing Ras-transformed epithelial cells to survive in the absence of contact with extracellular matrix, inhibitors of this enzyme may have some potential in the treatment of carcinoma. However, the ability of PI 3-kinase inhibitors to induce apoptosis in untransformed cells under normal culture conditions is likely to severely limit any such usage. This may partly explain the considerable toxicity of these agents in whole animals (Gunther *et al.*, 1989).

From indirect (see Figure 3) and direct (Rodriguez-Viciana *et al.*, 1997; A.Khwaja, P.Rodriguez-Viciana and J.Downward, unpublished data) observations, there are indications that some of the PI 3-kinase isoforms activated by V12 Ras might be less sensitive than p110 α to inhibitors such as LY294002 and wortmannin. A novel PI 3-kinase isoform that is ~50-fold less sensitive than p110 α to wortmannin has been identified recently (Virbasius *et al.*, 1996). It is possible that oncogenic Ras might activate a set of PI 3-kinase isoforms distinct from those activated by adhesion: if this is so, then specific inhibitors might possibly be developed that could selectively target the Ras-regulated enzymes and target Ras-transformed as opposed to normal cells.

Several direct and indirect downstream targets for PI 3-kinase have been identified: these include p70^{S6K} (Chung *et al.*, 1994), novel and atypical protein kinase C isoforms (Nakanishi *et al.*, 1993; Toker *et al.*, 1994), the Rho family

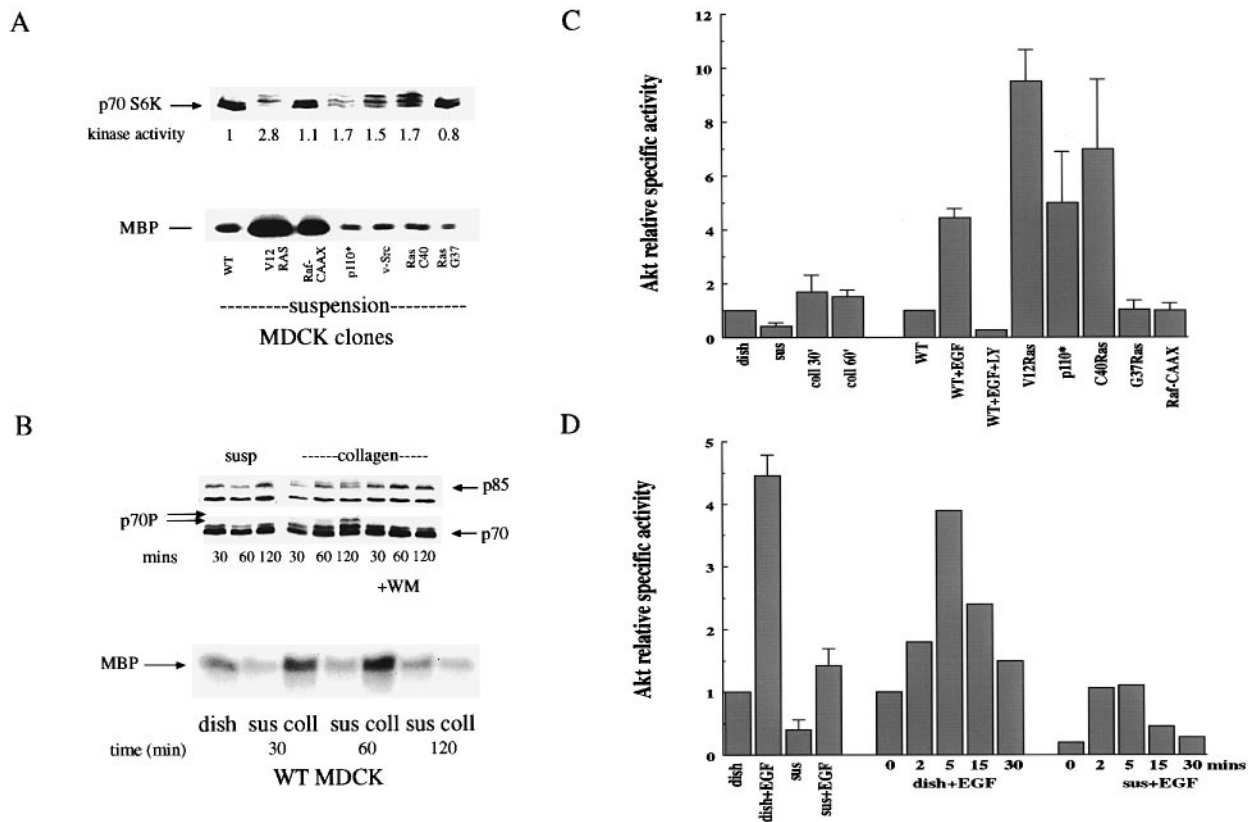


Fig. 6. Activation of PKB/Akt, p70^{S6K} and MAP kinase in MDCK clones and in wild-type MDCK cells replated onto collagen. (A) Wild-type MDCK or the indicated transfected clones were detached and kept in suspension for 2 h in serum-free medium before analysis. p70^{S6K} mobility shift was detected by immunoblotting and kinase activity by phosphorylation of peptide substrate in S6 kinase immunoprecipitates. For comparison, EGF stimulation in these cells results in a 1.6-fold increase in S6 kinase activity. MAP kinase activity was measured by immunoprecipitation of Erk2 and *in vitro* phosphorylation of myelin basic protein. Similar results were obtained for at least two different clones for each cell type. (B) Wild-type MDCK cells were detached, kept in suspension for 2 h and then replated onto collagen-coated plates. The effect of the PI 3-kinase inhibitor wortmannin (100 nM) on p70^{S6K} activation was also examined. (C) MDCK cells expressing epitope-tagged PKB/Akt were detached and replated as in (B), and PKB/Akt activity measured in HA epitope tag immunoprecipitates. The activity of PKB/Akt in the indicated MDCK clones was measured using adherent cells. EGF treatment was 50 ng/ml for 5 min, LY294002 pre-treatment was 20 μ M for 60 min. Data are mean \pm SE of four to six separate experiments. (D) Effect of EGF (50 ng/ml) on PKB/Akt activation in adherent and suspended MDCK cells. Data are shown as the mean \pm SE of four separate experiments with EGF for 5 min and also of a single time course.

small GTP-binding protein Rac (Hawkins *et al.*, 1995) and the serine/threonine kinase PKB/Akt (Burgering and Coffey, 1995; Franke *et al.*, 1995). Since rapamycin fails to induce apoptosis in MDCK cells (Figure 2D) and in several other cell types (Yao and Cooper, 1996), it seems unlikely that p70^{S6K} is involved in the PI 3-kinase protection from anoikis. From our data, activated Rac does not give significant protection from anoikis, thus ruling out this pathway downstream of PI 3-kinase. In other cell types, protein kinase C isoforms have been implicated in both protection from, and induction of, apoptosis; however, in MDCK cells, phorbol esters promote apoptosis (Flach *et al.*, 1995), making it unlikely that conventional or novel protein kinase C isoforms are able to mediate the protective effect. By contrast, activated PKB/Akt is able to protect MDCK cells substantially from anoikis, so this is likely to be the pathway whereby PI 3-kinase influences cell survival. MDCK cells expressing activated PKB/Akt are highly resistant to the apoptosis-inducing effects of PI 3-kinase inhibitors (Figure 3B), so PKB/Akt probably represents the major pathway by which PI 3-kinase protects cells from apoptosis. Dominant-negative mutants of PKB/Akt are able to induce apoptosis when expressed in

adherent MDCK cells, indicating that a constitutive level of activity of PKB/Akt is required for epithelial cell survival. PKB/Akt was identified as a retroviral oncogene (Bellacosa *et al.*, 1991), as well as by its similarity to protein kinases A and C (Coffey and Woodgett, 1991; Jones *et al.*, 1991), and the close relative Akt 2 recently has been shown to be frequently overexpressed in human ovarian and pancreatic cancers (Bellacosa *et al.*, 1995; Cheng *et al.*, 1996). PKB/Akt may therefore be able to promote the survival of transformed cells under physiological conditions and may influence the pathology of many human tumours.

Materials and methods

Expression vectors

The Ras expression vectors were generated as described in Marte *et al.* (1996). Activated PI 3-kinase constructs were made by the addition of a carboxy-terminal farnesylation signal from H-Ras to p110 to cause its membrane localization (myc-tagged p110-CAAX in pcDNA3) (S.Wennström and J.Downward, in preparation). Raf-CAAX and myc-tagged Erk2 cDNA were provided by R.Marais and C.J.Marshall (ICR, London). Gag-PKB cDNA was provided by B.Burgering and P.Coffey (Utrecht). AH-Akt cDNA was provided by Thomas Franke.

Cell lines

Early passage MDCK cells were provided by J.Taylor-Papadimitriou (ICRF) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. To generate stably transfected lines, wild-type cells were transfected with the various constructs by lipofection (Lipofectamine, GibcoBRL). Cells were plated at 10^5 /well of a 6-well plate and transfected the following day with 4 μ g of DNA (plus 0.4 μ g of a hygromycin resistance plasmid for those vectors not encoding a neomycin resistance gene) and 10 μ l of Lipofectamine/well. After 48 h, cells were replated into a 10 cm dish and selected with 250 μ g/ml hygromycin or 500 μ g/ml G418, as appropriate. Control cells were transfected with empty vector alone or were untransfected. After selection in antibiotic for 10–14 days, individual resistant colonies were picked and expanded. At least 24 clones were picked for each transfection and expression of protein assessed by Western blotting of cell lysates using appropriate antibodies. Expression of V12 H-Ras and the Ras mutant proteins (C40, G37 and A38) varied between 5- and 10-fold excess over endogenous Ras levels.

Anoikis assays

These were performed as described in Frisch and Francis (1994). For DNA laddering, 5×10^6 cells were maintained in suspension for 8 h. Cells were then washed once in phosphate-buffered saline (PBS) and low molecular weight DNA was extracted. The cell pellet was lysed using 0.5 ml of extraction buffer (0.5% Triton X-100, 5 mM Tris pH 7.5, EDTA 20 mM) for 20 min on ice. The low molecular weight DNA-containing soluble fraction was isolated by centrifugation at 15 000 g for 10 min, phenol/chloroform extracted three times, ethanol precipitated, resuspended in Tris/EDTA, pH 8.0, containing 20 μ g/ml RNase A and incubated at 37°C for 2 h. DNA was run on a 1.5% agarose gel and visualized by ethidium bromide staining. To examine the effects of the PI 3-kinase inhibitor LY294002, this was added (20 μ M) for the duration of the assay.

For the survival assay, cells were detached and equal numbers either replated immediately or maintained in suspension as described above for 12 h and then replated into 96-well plates. Four hours after replating, cell survival was measured by a colorimetric method based on the conversion of MTS tetrazolium to formazan (CellTiter AQueous Kit, Promega) used according to the manufacturer's instructions. DNA fragmentation ELISA was done using the Cell Death Detection ELISA kit (Boehringer Mannheim). Samples were assayed in duplicate according to the manufacturer's instructions. Lysates from 10^3 cells/point were assayed.

Apoptosis quantitation in transient transfections

A total of 10^5 MDCK cells in 6-well plates were co-transfected with 0.5 μ g of pCMV β gal and 4 μ g of pCMV6 AH-Akt (dominant-negative Akt), pSG5V12Ras or empty vector using Lipofectamine. At 24 h after transfection, cells were fixed and stained for β -galactosidase activity. Positively stained cells were scored as normal or apoptotic on the basis of cellular morphology (membrane blebbing, cytoplasmic and nuclear condensation). At least 500 positively stained cells were scored per assay.

UV irradiation

Cells were plated in 96-well plates at 5×10^3 /well and treated with UVC irradiation at 10 000 μ J/cm² 8 h later. Cell survival compared with unirradiated cells was measured 12 h later (MTS method).

Measurement of phosphoinositides

Subconfluent cells were incubated overnight in 250 μ Ci of [³²P]orthophosphate (Amersham) in phosphate-free DMEM supplemented with 0.2% dialysed FCS (10 cm dish). For measurement of phosphoinositide levels in suspension, cells were detached with trypsin/EDTA, washed once in phosphate-free DMEM/2% FCS, resuspended in their original [³²P]orthophosphate-containing medium and maintained in suspension on polyHEMA-coated plates for 2 h. In experiments to examine the effect of reattachment of cells on phosphoinositide levels, suspension cells were replated onto 10 cm plates pre-coated with 50 μ g/ml collagen (Vitrogen 100, Collagen Corporation, Palo Alto) for 60 min. Parallel experiments were carried out with the same protocol but in the presence of 10% FCS. Lipid extraction from cells attached to the dish or from the cell pellet obtained after centrifugation of suspension cells and HPLC analysis was carried out as described (Rodríguez-Viciana *et al.*, 1996). Phosphoinositide levels were corrected for the number of total c.p.m. loaded.

Measurement of MAP kinase, PKB/Akt and S6 kinase activation

Cells were serum starved overnight in DMEM/0.2% FCS and analysed, or detached with trypsin/EDTA, washed once with DMEM/10% FCS and resuspended in serum-free DMEM. Cells were maintained in suspension for 120 min and analysed or then replated onto collagen-coated dishes for varying times. p70^{S6K} activation was assessed by retardation of electrophoretic mobility due to phosphorylation and by phosphorylation of substrate peptide (KRRRLASLAA) in immunoprecipitates. MAP kinase activation was measured by immunoprecipitation of Erk2 and phosphorylation of myelin basic protein as described (Leevers and Marshall, 1992). PKB/Akt activity was measured in HA tag immunoprecipitates using H2B as a substrate as described (Franke *et al.*, 1995). Immunoprecipitations and Western blotting protocols were as described (Khawaja *et al.*, 1996).

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