Activated *ras* Prevents Downregulation of Bcl-X$_L$ Triggered by Detachment from the Extracellular Matrix: A Mechanism of *ras*-induced Resistance to Anoikis in Intestinal Epithelial Cells

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Abstract. Detachment of epithelial cells from the extracellular matrix (ECM) results in a form of apoptosis often referred to as anoikis. Transformation of intestinal epithelial cells by oncogenic *ras* leads to resistance to anoikis, and this resistance is required for the full manifestation of the malignant phenotype. Previously, we demonstrated that *ras*-induced inhibition of anoikis in intestinal epithelial cells results, in part, from the *ras*-induced constitutive downregulation of Bak, a pro-apoptotic member of the Bcl-2 family. Since exogenous Bak could only partially restore susceptibility to anoikis in the *ras*-transformed cells, the existence of at least another component of the apoptotic machinery mediating the effect of activated *ras* on anoikis was suggested. Indeed, here we show that, in nonmalignant rat and human intestinal epithelial cells, detachment from the ECM or disruption of the cytoskeleton results in a significant downregulation of the antiapoptotic effector Bcl-X$_L$, and that activated H- or K-*ras* oncogenes completely abrogate this downregulation. In addition, we found that enforced downregulation of Bcl-X$_L$ in the *ras*-transformed cells promotes anoikis and significantly inhibits tumorigenicity, indicating that disruption of the adhesion-dependent regulation of Bcl-X$_L$ is an essential part of the molecular changes associated with transformation by *ras*. While the *ras*-induced downregulation of Bak could be reversed by pharmacological inhibition of phosphatidylinositol 3 kinase (PI 3-kinase), the effect of *ras* on Bcl-X$_L$ was PI 3-kinase– and mitogen-activated protein kinase (MAP kinase)–independent. We conclude that *ras*-induced resistance to anoikis in intestinal epithelial cells is mediated by at least two distinct mechanisms: one that triggers downregulation of Bak and another that stabilizes Bcl-X$_L$ expression in the absence of the ECM.

Key words: apoptosis • colorectal tumors • cytoskeleton • PI 3-kinase • MAP kinase

Introduction

Survival of normal epithelial cells is dependent on signals generated by the interaction of these cells with components of their basement membrane (Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997). The absence of such signals triggers a form of physiological cell death, which recently has been named anoikis or death of homelessness, as it is believed to affect cells outside of their proper tissue context (Frisch and Francis, 1994; Meredith and Schwartz, 1997). Anoikis is thought to play an important role in maintaining proper tissue architecture by precluding reattachment and growth of epithelial cells at ectopic locations (Frisch and Ruoslahti, 1997).

Several lines of evidence indicate that the acquisition of resistance to anoikis plays a central role in the progression of human carcinomas. First, solid tumors grow in vivo as multicellular masses in which the cells are forced to survive in the absence of attachment to a properly formed basement membrane. Second, most cell lines derived from such solid tumors are capable of growing in an anchorage-independent manner as colonies in soft agar or suspension culture (Schwartz, 1997). Third, nonmalignant epithelial cells, which were selected for the ability to resist anoikis in tissue culture, simultaneously acquire a tumor-forming capacity (Rak et al., 1999). Fourth, suppression of the resistance to anoikis in cultured transformed epithelial cells strongly inhibits their tumorigenicity (Rosen et al., 1998). Fifth, transfection of nonmalignant epithelial cells with various oncogenes commonly associated with epithelial malignancies, such as mutant H- or K-*ras*, induces resistance to anoikis (Frisch and Francis, 1994; Rak et al., 1995).

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Activating mutations of the *ras* proto-oncogene are among the most frequent oncogenic events in human cancer (Barbacid, 1987; Bos et al., 1987; Forrester et al., 1987; Almoguera et al., 1988). Ras is a small GTPase that acts as a molecular switch by regulating the passage of signals from growth factor receptors and other extracellular queues to signaling pathways that control expression of various effector genes (McCormick, 1993; Marshall, 1996). In this manner, *ras* exerts a regulatory effect on diverse cellular functions such as proliferation (Filmus et al., 1994), cytoskeletal organization (Hall, 1990; Rodriguez-Viciana et al., 1997), and survival (Downward, 1998). Oncogenic *ras*, which is locked in a constitutively active (GTP-bound) state, alters these cellular functions, and contributes in this way to the malignant transformation of various cell types including those from the intestinal epithelium (Bos et al., 1987; Forrester et al., 1987). One of the consequences of the disruptive effect of activated *ras* on normal cell physiology is the induction of resistance to anoikis (Frisch and Francis, 1994). We have recently demonstrated that this loss of susceptibility to anoikis is a critical component of the tumorigenic phenotype of *ras*-transformed intestinal epithelial cells (Rosen et al., 1998).

The molecular mechanisms governing the switch to the anoikis-resistant state associated with *ras*-induced transformation have just started to be uncovered. It is generally believed that programmed cell death can be triggered by a specific set of signals, which lead to the release of cytochrome *c* from the mitochondria into the cytoplasm (Nunez et al., 1998). Cytochrome *c* interacts with the regulatory protein Apaf-1, inducing the activation of caspases, which are serine proteases that cleave a set of critical cellular targets. At this point, the cell death program enters its irreversible stage (Green and Reed, 1998). The release of cytochrome *c* from the mitochondria is both positively and negatively regulated by members of the Bcl-2 protein family (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Kelekar and Thompson, 1998; Reed, 1998). Bcl-2, Bcl-X_L, and Bcl-w are some of the antiapoptotic members of this family, whereas Bak, Bax, and Bad are examples of the pro-apoptotic group (Adams and Cory, 1998). Caspase activity can also be directly inhibited by members of a separate gene family known as inhibitors of apoptosis (IAPs)[1] (LaCasse et al., 1998). In addition, the caspase cascade can be triggered by a specialized cell death pathway after engagement of members of the tumor necrosis factor receptor family (Nunez et al., 1998).

As a result of our initial attempt to investigate the effect of the *ras* oncogene on the apoptotic machinery of a nonmalignant intestinal epithelial cell line (IEC-18), we have reported that activated *ras* induces constitutive downregulation of Bak (Rosen et al., 1998). Interestingly, downregulation of Bak has been found in a large proportion of human colorectal carcinomas, indicating that our finding has clinical implications (Krajewska et al., 1996). At the functional level, we have shown that ectopic expression of Bak in *ras*-transformed rat intestinal epithelial cells markedly diminishes *ras*-induced resistance to anoikis, and significantly reduces tumorigenicity of these cells in nude mice. Overall, our results indicated that the ability of activated *ras* to downregulate Bak, and the consequent resistance to anoikis, is essential for the malignant transformation of intestinal epithelial cells induced by this oncogene. At the mechanistic level, we noted that the impact of activated *ras* on Bak expression could be partially prevented by pharmacological inhibition of phosphatidylinositol 3 kinase (PI 3-kinase), an immediate downstream target of *ras* (Rodriguez-Viciana et al., 1994). This observation is consistent with a previous report implicating this enzyme in the induction of resistance to anoikis in a *ras*-transformed epithelial cell line derived from the kidney (Khwaja et al., 1997). These data are also compatible with a general perception that PI 3-kinase is a mediator of cell survival signals under a variety of circumstances acting through the activation of protein kinase B (PKB; Franke et al., 1997; Marte and Downward, 1997). Our study also suggested that effectors other than Bak must be involved in *ras*-induced resistance to anoikis. This conclusion was based on the fact that expression of exogenous Bak in the *ras*-transformed cells at levels similar to or even higher than those of the parental IEC-18 cells caused only partial restoration of the susceptibility to anoikis. Therefore, we decided to investigate whether other components of the apoptotic machinery act as effectors of the *ras*-induced resistance to anoikis in intestinal epithelial cells.

Bcl-X_L, an antiapoptotic member of the Bcl-2 family, is upregulated in ~50% of cancers derived from intestinal epithelium (Krajewska et al., 1996). Here, we report that Bcl-X_L is an important mediator of the effect of *ras* on anoikis in intestinal epithelial cells. Our results show that detachment of such nonmalignant cells from the ECM results in a strong downregulation of Bcl-X_L expression, and that this downregulation is blocked by transformation with activated H- and K-*ras* oncogenes. In addition, we show that ectopic expression of Bcl-X_L in nontransformed intestinal epithelial cells strongly inhibits anoikis, whereas enforced downregulation of Bcl-X_L in the *ras*-transformed cells has an opposite effect with a parallel decrease in tumorigenicity of such cells.

Materials and Methods

Cell Culture

The IEC-18 cells were obtained from Dr. A. Quaroni (Cornell University, Ithaca, NY). The generation of the IEC clones expressing activated H-*ras* constitutively or under the control of the inducible metallothionein promoter (MT-*ras*) has been previously described (Filmus et al., 1992, 1993). All IEC clones were cultured in α-MEM containing 5% FBS, 10 μg/ml insulin, and 0.5% glucose. H-*ras* expression in the MT-*ras* clone was induced by adding 100 μM $ZnCl_2$ and 2 μM $CdCl_2$ to cells 48 h before the experiment. The DLD-1, DKO-3, and DKS-8 colorectal tumor cell lines were provided by T. Sasazuki (Kyushu University, Fukuoka, Japan; Shirasawa et al., 1993). These cells were cultured in DME containing 10% FBS. The generation of the IEC-18 variant, which is resistant to anoikis (AR 1.10), has been described elsewhere (Rak et al., 1995). For suspension cultures, 10^6 cells were plated above a layer of 1% sea plaque agarose polymerized in α-MEM or DME.

Vector Construction and Transfection

To generate the sense and antisense Bcl-X_L expression vectors, the human Bcl-X_L cDNA was inserted into the EcoRI site of pcDNA3 (Invitrogen Corp.) in the sense and antisense orientations. To generate IEC-18 cells

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[1] *Abbreviations used in this paper:* AR, anoikis resistant; CD, cytochalasin D; ECM, extracellular matrix; IAP, inhibitor of apoptosis; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B.

stably expressing exogenous Bcl-X_L, 10^6 IEC-18 cells were transfected with 10 μg of the sense Bcl-X_L expression vector by using lipofectin. Transfected cells were selected in 400 μg/ml of G418. Selected clones were expanded, and Bcl-X_L expression was assessed by Western blotting. To generate IEC-*ras* cells stably expressing antisense Bcl-X_L, 2.5×10^5 IEC-*ras*-3 cells were cotransfected by using lipofectin with 10 μg of the expression vector, carrying the human Bcl-X_L cDNA in an antisense orientation, and 1 μg of pZeoSV vector carrying a zeocin resistance gene. Transfected cells were selected in 250 μg/ml of zeocin. Surviving clones were expanded, and Bcl-X_L expression was assessed by Western blotting.

Transfection with Antisense Oligonucleotides

5×10^4 IEC-*ras*-3 cells were plated on a 60-mm dish. The next day, cells were incubated with the oligonucleotides (300 nM) in the presence of 7.5 μg/ml of lipofectin in 1 ml of OPTI MEM for 4 h. The transfection mixture was replaced by α-MEM with standard ingredients (see above). Cells were grown overnight and processed for Western blotting or assayed for anoikis as described below. Oligonucleotides were obtained from ISIS Pharmaceuticals (Taylor et al., 1999). The sequence of the antisense Bcl-X_L oligonucleotide (ISIS 16009) was CTACGCTTTCCACGCACAGT, and the sequence of the control-scrambled oligonucleotide (ISIS 20574) was CTCCGATGTCCCCTCAAAGT. All internucleotide bonds were phosphorothioated. Underlined residues indicate 2′-O-methoxyethyl modification.

Western Blot Analysis

Cells were lysed for 30 min on ice in a buffer containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 100 mM NaF, 0.5% NP-40, 1 mM PMSF, 50 μg/ml aprotinin, and 10 μg/ml leupeptin. After removing the insoluble material, aliquots of supernatant containing 20–30 μg of protein were run through a 10% polyacrylamide gel under reducing conditions. Proteins were transferred to a nylon membrane that was subsequently incubated for 1 h at room temperature in TBST buffer (125 mM Tris-HCl, pH 8.0, 625 mM NaCl, and 0.5% Tween 20) containing 4% skim milk. The membrane was incubated with one of the following antibodies: anti-Bcl-X_L, anti-Bad (Transduction Laboratories) or, in case of the IEC-18–derived clones transfected with human Bcl-X_L and in the experiments with cell lines derived from human colorectal carcinomas, anti-Bcl-$X_{S/L}$ (Santa Cruz Biotechnology), anti-rat Akt (UBI), anti–phospho-Akt (Ser 473), anti–phospho-MAPK, anti-MAPK (New England Biolabs), or anti-Bax, anti-CDK4 (Santa Cruz Biotechnology). Incubation with antibodies was performed in a TBST buffer containing 5% BSA for 1–2 h. Binding of the antibodies was detected with the enhanced chemiluminescence system (New England Nuclear).

Northern Blot Analysis

Northern blot analysis was performed on total RNA. A human Bcl-X_L cDNA labeled with [^{32}P]dCTP by random priming was used as a probe.

Apoptosis Assay

5×10^4 cells were plated on a 60-mm or a 100-mm dish in a monolayer or in suspension. At the indicated time points, cells were removed from the plates, washed once with PBS, and assayed for the presence of nucleosomal fragments in the cytoplasm by a cell death detection ELISA kit (Boehringer Mannheim) according to the manufacturer's instructions.

Soft Agar Colony Formation Assay

5,000 cells were suspended in 2 ml of IEC medium containing 0.3% of melted bacto-agar. The resulting suspension was added to a 60-mm plate covered with a 2-ml layer of solidified 0.5% bacto-agar in α-MEM. Cell colonies (>50 cells) were allowed to form for 7–10 d and counted. Each experiment was performed in triplicate.

Tumorigenicity Assay

1.6×10^5 cells were suspended in 0.2 ml of PBS and injected subcutaneously into an 8–12-wk-old female nude athymic BALB/C mice. The tumors were measured at the indicated time points by using a Vernier's calliper, and tumor volume was calculated by using the standard formula:

$$\frac{a^2 \times b}{2}, \tag{1}$$

where *a* is width and *b* is length of the ellipsoid tumor perimeter.

Results

Activated ras Inhibits Downregulation of Bcl-X_L Triggered by Detachment from the ECM

The search for effectors that mediate the *ras*-induced resistance to anoikis led us to compare the levels of Bcl-X_L in the nonmalignant rat intestinal epithelial cell line IEC-18 and in the previously characterized IEC-*ras*–transformed clone IEC-*ras-3* (Filmus et al., 1992) that was cultured in a monolayer and in suspension. Detachment of IEC-18 cells from the ECM resulted in a strong downregulation of Bcl-X_L expression. On the other hand, this downregulation was completely abrogated in case of the *ras*-transformed clone (Fig. 1 A). Similar changes were observed at the mRNA level (Fig. 1 B). The *ras*-induced stabilization of Bcl-X_L levels in the absence of ECM was also observed in two other previously characterized IEC-18–derived clones constitutively expressing the activated H-*ras* oncogene (*ras*-4 and *ras*-7; Fig. 1 C; Filmus et al., 1992, 1994). To confirm that this stabilization of Bcl-X_L expression in detached cells is a direct consequence of the action of oncogenic *ras*, we used an IEC-18–derived clone (MT-*ras*) in which exogenous activated H-*ras* is expressed under the control of a metallothionein promoter, which is inducible by Zn^{2+} and Cd^{2+} (Filmus et al., 1994). Similar to what was observed in clones expressing activated *ras* constitutively, the induction of activated H-*ras* expression in the MT-*ras* cells significantly inhibited downregulation of Bcl-X_L upon cell detachment (Fig. 1 D).

To validate this observation in a different cellular system, we employed the highly tumorigenic human colorectal cancer cell line DLD-1, which was harboring a single copy of the activated K-*ras* oncogene, and its two variants DKS-8 and DKO-3 in which the mutant *ras* allele has been disrupted by homologous recombination (Shirasawa et al., 1993). It already has been reported that the ablation of activated *ras* from DLD-1 cells strongly inhibits their anchorage-independent growth and tumorigenicity (Shirasawa et al., 1993). Fig. 2 A shows that the removal of the oncogene from DLD-1 cells also induces sensitivity to anoikis, since when DKS-8 and DKO-3 cells were placed in suspension, they displayed significantly higher levels of death compared with the K-*ras*–expressing DLD-1 cells. In agreement with what was found in IEC cells, deletion of activated K-*ras* from the DLD-1 cells restored the adhesion-dependent regulation of Bcl-X_L expression in DKS-8 and DKO-3 cells (Fig. 2 B).

Transduction of many signals generated by cell–ECM interactions requires the maintenance of an intact actin cytoskeleton (Clark and Brugge, 1995). Therefore, we decided to verify whether treatment with drugs that disrupt actin assembly, such as cytochalasin D (CD), has the same effect on Bcl-X_L expression as culturing cells in suspension. As shown on Fig. 3 A, incubation of IEC-18 and IEC-*ras*-3 cells with CD strongly inhibited spreading of these cells on the tissue culture dish. Similar to what was observed after detachment from the ECM, CD treatment induced a sig-

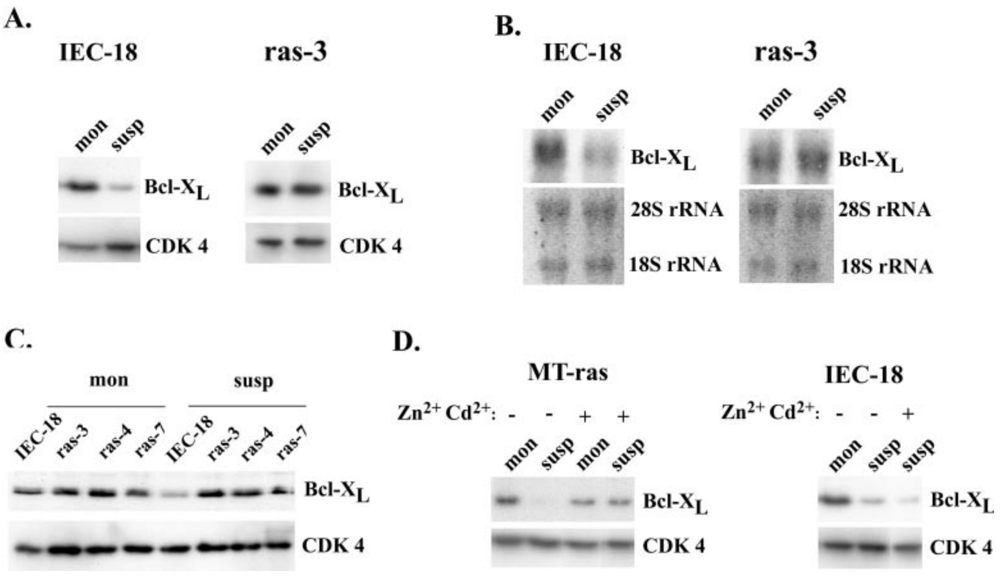

Figure 1. Expression of activated H-*ras* in intestinal epithelial cells prevents downregulation of Bcl-X_L, which is caused by cell detachment. (A) Western blot analysis of Bcl-X_L in IEC-18 cells and an H-*ras*–transformed clone IEC-ras-3 that were cultured in monolayer (mon) or in suspension (susp) overnight. (B) Northern blot analysis of Bcl-X_L in IEC-18 cells and clone ras-3 that were cultured in monolayer (mon) or in suspension (susp) overnight. (C) Western blot analysis of Bcl-X_L in IEC-18 cells and three independently derived H-*ras*–transformed clones (ras-3, ras-4, and ras-7) cultured in monolayer (mon) or in suspension (susp) overnight. (D) Western blot analysis of Bcl-X_L in MT-*ras* and IEC-18 cells that were cultured in monolayer (mon) or in suspension (susp) for 4.5 h in the absence (−) and in the presence (+) of Zn^{2+} and Cd^{2+}. CDK 4 in A, C, and D, and ribosomal RNAs in B were used as loading controls.

nificant downregulation of Bcl-X_L in IEC-18 cells, but did not affect the expression of this antiapoptotic molecule in the *ras*-transformed clones (Fig. 3 B). As might be expected, treatment with CD had a severe impact on the survival of the parental IEC-18 cells, whereas in case of the *ras*-transfectants CD-induced apoptosis was strongly inhibited (Fig. 3 C). These results are consistent with those obtained with the cells cultured in suspension, and indicate that the integrity of the actin cytoskeleton represents an important requirement for the maintenance of the constitutive Bcl-X_L expression and survival in case of IEC-18 but not IEC-*ras* cells.

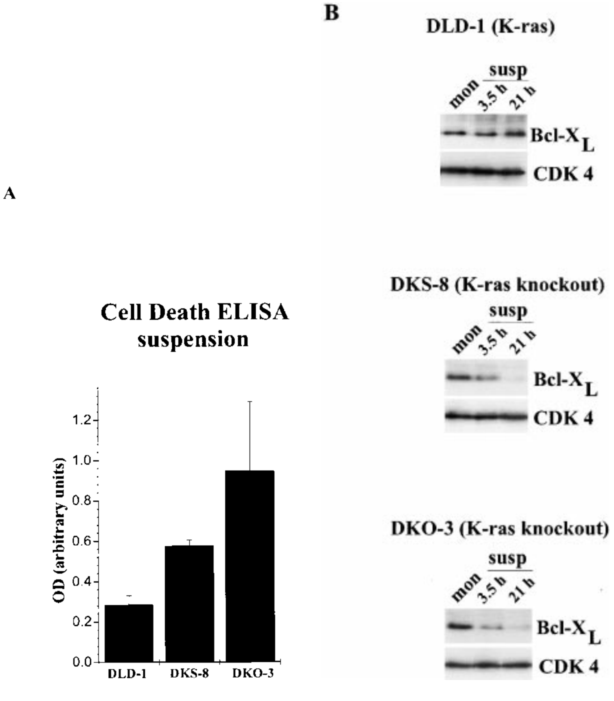

Figure 2. Targeted disruption of the activated K-*ras* allele in cells derived from human colorectal carcinoma restores sensitivity to anoikis, and adhesion-dependent regulation of Bcl-X_L expression. (A) Analysis of apoptosis by cell death ELISA in DLD-1, DKS-8, and DKO-3 cells that were cultured in suspension overnight. Results represent the average of two independent experiments plus the SD. (B) Western blot analysis of Bcl-X_L was performed in cells that were cultured in monolayer (mon) or in suspension (susp) for the indicated time periods. Membranes were reprobed with an anti-CDK 4 antibody as a loading control.

Downregulation of Bcl-X_L in Response to Cell Detachment Contributes to Anoikis

To verify whether the downregulation of Bcl-X_L expression in detached cells plays a role in anoikis, it was important to establish that such downregulation occurs before the detachment-induced cell death. As shown in Fig. 4 A, Bcl-X_L expression was inhibited as early as 0.5 h after placing the IEC-18 cells in suspension, whereas no significant apoptosis was observed even after 1 h of suspension culture (Fig. 4 B), suggesting that the loss of Bcl-X_L plays a causal role in this process. To confirm this more definitively, we transfected IEC-18 cells with a vector in which Bcl-X_L expression was driven by a heterologous promoter. Four independent clones (Bcl-X 3, 11, 27, and 41) expressing exogenous Bcl-X_L at levels significantly higher than in the IEC-18 cells (Fig. 5 A) were generated and tested for survival in the suspension culture. In all of the Bcl-X_L–transfected clones, a strong protection from apoptosis was consistently observed (Fig. 5 B). It is important to note that, although three of the Bcl-X_L–transfected clones displayed significantly higher levels of Bcl-X_L than the IEC-*ras*-3 cells when placed in suspension, the protection from apoptosis in clone 41, which expressed Bcl-X_L at levels similar to those in the IEC-*ras*-3 cells, was considerable. Interestingly, expression of Bcl-X_L in IEC-18 cells at levels even higher than in the IEC-*ras*-3 clone did not provide the degree of protection against anoikis that was observed in the case of *ras*-transformed cells. These data suggest that *ras* may inhibit anoikis through both Bcl-X_L–depen-

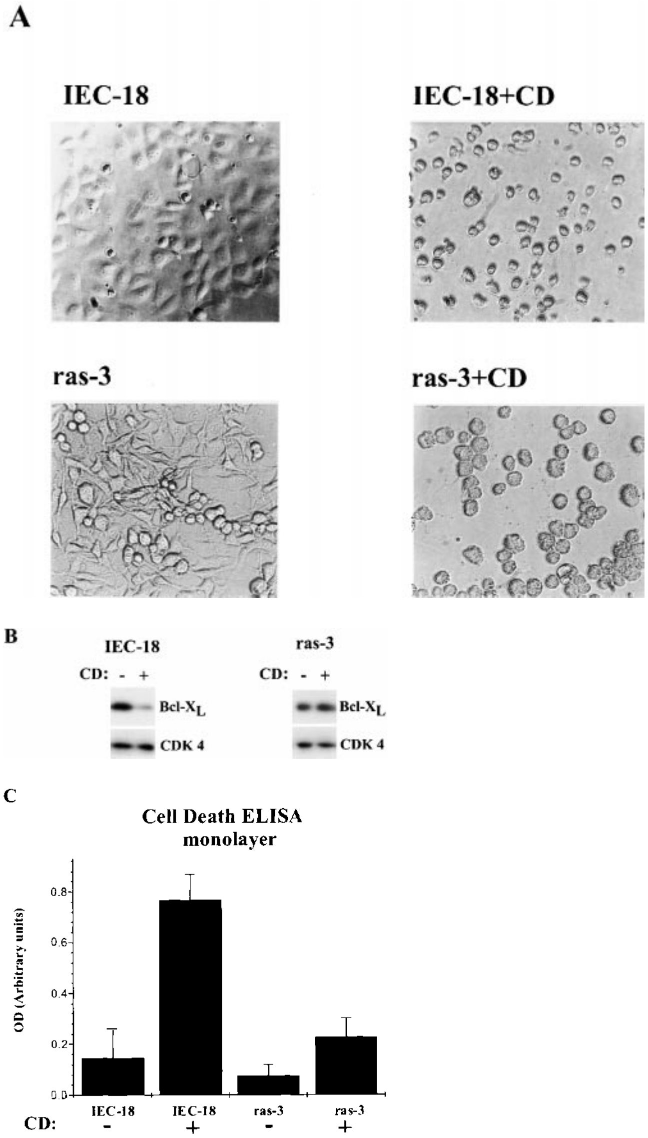

Figure 3. Activated *ras* inhibits downregulation of Bcl-X$_L$ and apoptosis in IEC-18 cells treated with cytochalasin D (CD). (A) Microphotographs of IEC-18 and IEC-*ras*-3 cells that were grown in monolayer with and without treatment with 10 μg/ml of CD for 16 h. (B) Western blot analysis of Bcl-X$_L$ in IEC-18 and IEC-*ras*-3 cells that were grown in monolayer with (+) and without (−) treatment with CD for 16 h. Membranes were reprobed with anti-CDK 4 antibody as a loading control. (C) Analysis of apoptosis by cell death ELISA in IEC-18 and IEC-*ras*-3 cells grown in monolayer with (+) and without (−) treatment with 10 μg/ml of CD for 16 h. A similar volume of DMSO (vehicle) was added to the untreated cells. Results represent the average of three independent experiments plus the SEM.

dent and –independent mechanisms, which is consistent with our previous finding indicating that a part of the effect of *ras* is exerted through a constitutive downregulation of Bak (Rosen et al., 1998).

Further evidence that is consistent with a causal role of Bcl-X$_L$ in the inhibition of anoikis came from the analysis of an anoikis-resistant (AR) IEC-18 variant which was obtained by serial passage of these cells in intermittent suspension culture, as we described earlier (Rak et al., 1995). This variant is significantly anoikis resistant (Fig. 6 A), and, unlike the parental IEC-18 cells, is tumorigenic in nude mice (Rak et al., 1999). Interestingly, Bcl-X$_L$ levels in these cells were not reduced by lack of attachment. On the contrary, the expression of Bcl-X$_L$ was dramatically increased upon placing the AR variant in the suspension culture (Fig. 6 B).

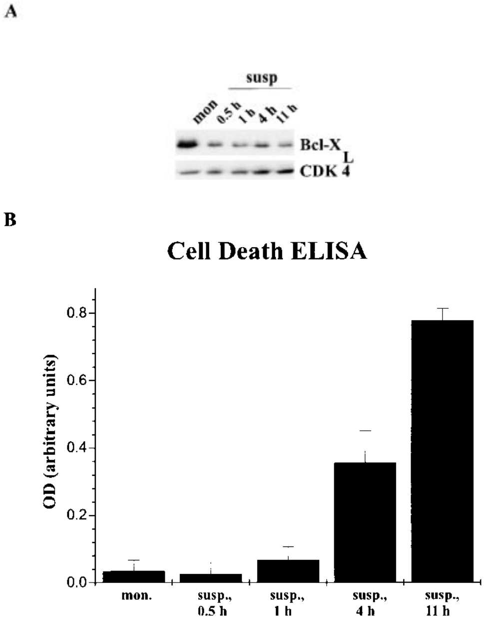

Figure 4. Inhibition of Bcl-X$_L$ expression caused by cell detachment precedes the onset of anoikis. (A) Western blot analysis of Bcl-X$_L$ in IEC-18 cells that were cultured in monolayer (mon) or in suspension (susp) for the indicated time periods. The membrane was reprobed with anti-CDK 4 antibody as a loading control. (B) Analysis of apoptosis by cell death ELISA in IEC-18 cells that were cultured in monolayer (mon) or in suspension (susp) for the indicated time periods. Results represent the average of two independent experiments plus the SD.

ras-induced Stabilization of Bcl-X$_L$ Expression Contributes to the Anoikis Resistance and Tumorigenicity of *ras*-transformed Cells

To assess the role of Bcl-X$_L$ in *ras*-induced resistance to anoikis, IEC-*ras*-3 cells were transiently transfected with an antisense Bcl-X$_L$ oligodeoxyribonucleotide, which was previously demonstrated to induce downregulation of Bcl-X$_L$ expression in a specific manner (Taylor et al., 1999). As shown in Fig. 7 A, such transfection resulted in a significant downregulation of Bcl-X$_L$ compared with the mock-transfected cells or cells transfected with a control (scrambled) oligonucleotide. Transfected cells were cultured in monolayer or in suspension and apoptosis was measured. We observed that transfection of IEC-*ras*-3 cells with the antisense oligonucleotide resulted in a noticeable increase of anoikis of these cells compared with the controls (Fig. 7 B).

To be able to study the effect of Bcl-X$_L$ downregulation in long-term assays, IEC-*ras*-3 cells were transfected with an antisense Bcl-X$_L$ expression vector to generate permanent cell lines. Two clones (designated as Bcl-X 65 and 66)

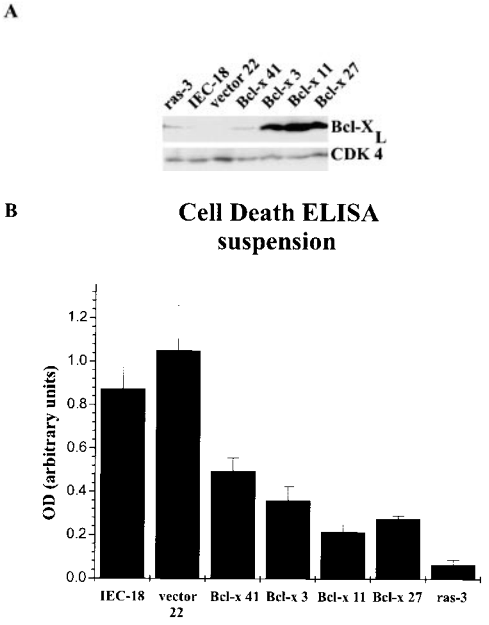

Figure 5. Anoikis of IEC-18 cells can be inhibited by expression of exogenous Bcl-X$_L$. (A) Western blot analysis of Bcl-X$_L$ in the following clones placed in suspension: IEC-*ras*-3, parental IEC-18 cells, and IEC-18 clones transfected with a Bcl-X$_L$ expression vector (Bcl-X 3, 11, 27, and 41), or vector alone (vector 22). The membrane was reprobed with anti-CDK 4 antibody as a loading control. (B) Analysis of apoptosis by cell death ELISA in IEC-18 and the Bcl-X$_L$–transfected clones cultured in suspension overnight. Results represent the average plus the SEM of three independent experiments.

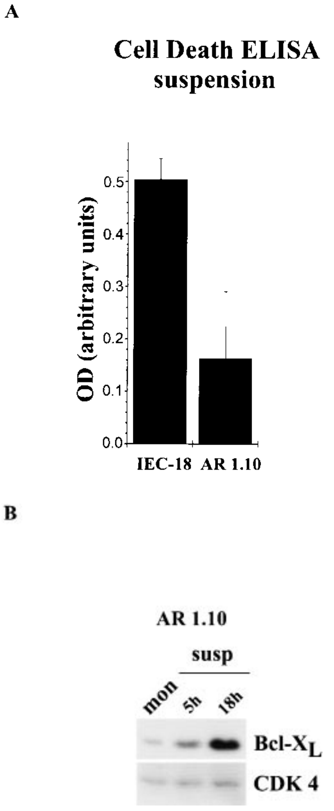

Figure 6. Bcl-X$_L$ is not downregulated in suspension in the IEC-18 variant AR 1.10 that spontaneously acquired resistance to anoikis. (A) Analysis of apoptosis by cell death ELISA in IEC-18 and AR 1.10 cells that were cultured in suspension. Results represent the average of two independent experiments plus the SD. (B) Western blot analysis of Bcl-X$_L$ in AR 1.10 cells that were cultured in monolayer (mon) or in suspension (susp) for the indicated time periods. The membrane was reprobed with an anti-CDK4 antibody as a loading control.

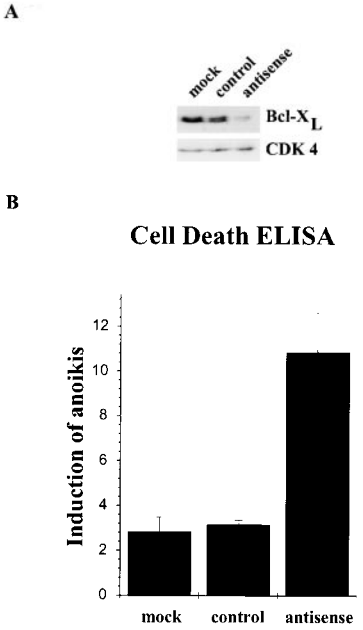

Figure 7. Enforced downregulation of Bcl-X$_L$ in the *ras*-transformed cells with the antisense oligodeoxyribonucleotide results in the inhibition of *ras*-induced resistance to anoikis. (A) Western blot analysis of Bcl-X$_L$ in mock-transfected IEC-*ras*-3 cells, IEC-*ras*-3 cells transfected with a control, or antisense Bcl-X$_L$ oligonucleotides. The membrane was reprobed with anti-CDK 4 antibody as a loading control. (B) Analysis of apoptosis by cell death ELISA in mock-transfected IEC-*ras*-3 cells, IEC-*ras*-3 cells transfected with a control, or antisense Bcl-X$_L$ oligonucleotides that were cultured in monolayer or suspension overnight. Results represent the average of duplicates plus the SD. Induction of anoikis was calculated as the ratio of the apoptotic signal observed in suspension versus the monolayer. This experiment was performed three times with similar results.

were found to express significantly less Bcl-X$_L$ than the parental IEC-*ras*-3 cells or a clone transfected with vector alone (designated vector 75; Fig. 8 A). When cultured in suspension, the antisense Bcl-X clones displayed levels of Bcl-X$_L$ comparable to those in IEC-18 cells (Fig. 8 B). The levels of other Bcl-2 family members such as Bak, Bax, and Bad were not changed in response to the antisense vector (Fig. 8 A), and no obvious differences in morphology and growth rates in monolayer culture were observed between any of these cell lines. Unlike the controls, cells expressing antisense Bcl-X$_L$ displayed a significant increase in anoikis (Fig. 8 C). Consistent with these data, the ability of the antisense Bcl-X$_L$ clones to grow in soft agar was strongly inhibited (Fig. 8 D). Furthermore, in good agreement with the tissue culture studies, cells expressing low levels of Bcl-X$_L$ were markedly less tumorigenic in vivo than the respective controls (Fig. 8 E). Taken together, these results indicate that the *ras*-induced stabilization of Bcl-X$_L$ levels under anchorage-independent conditions is required for full manifestation of anoikis resistance and the tumorigenic phenotype caused by this oncogene. However, it is important to note that although the effects of Bcl-X$_L$ downregulation on anoikis and tumor growth were significant, they were incomplete. This was expected since, as discussed previously, part of the effect of activated *ras*

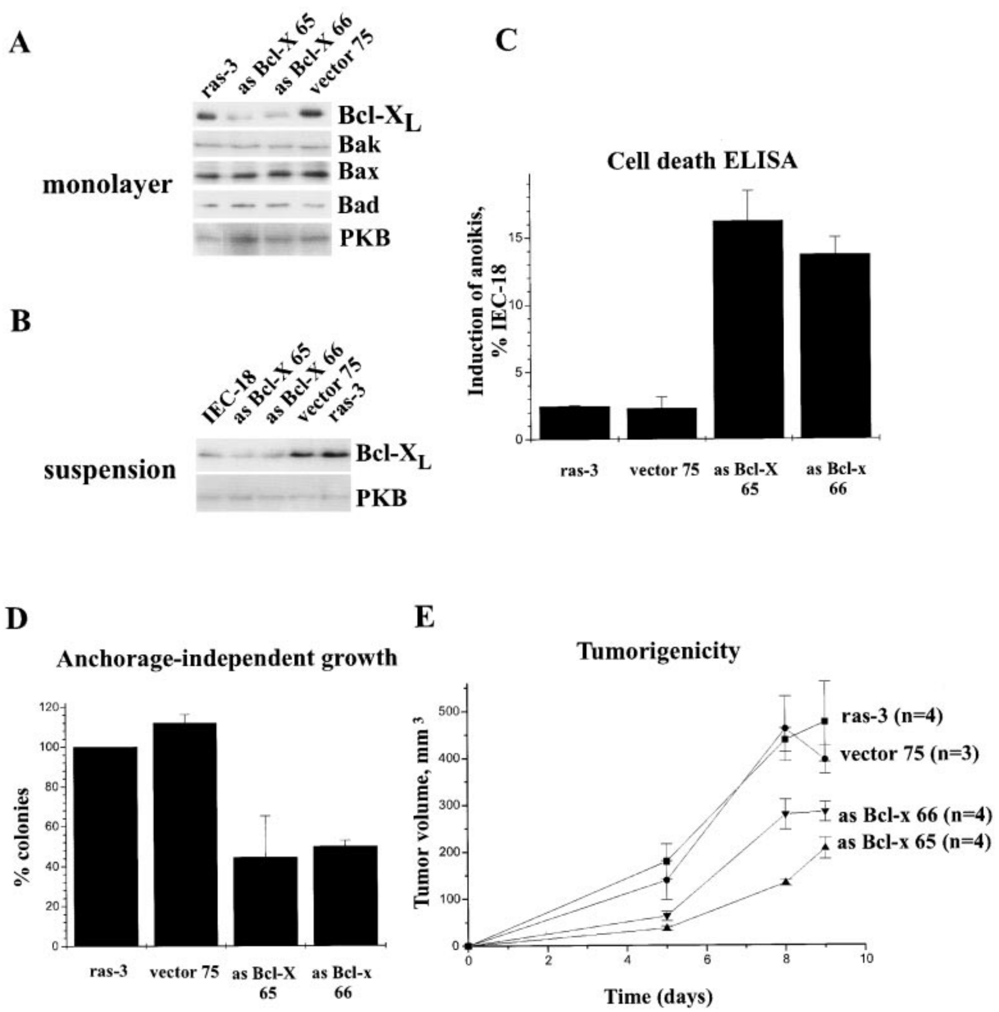

Figure 8. Enforced downregulation of Bcl-X$_L$ in the *ras*-transformed cells by transfection with the full-length antisense Bcl-X$_L$ cDNA results in the inhibition of *ras*-induced resistance to anoikis, anchorage-independent growth, and tumorigenicity. (A) Western blot analysis of Bcl-X$_L$ in IEC-*ras*-3 cells, IEC-*ras*-3 clones transfected with an antisense Bcl-X$_L$ expression vector (as Bcl-X 65 and 66), or vector alone (vector 75) that were cultured in monolayer. The membrane was probed with anti-PKB antibody as a loading control. Cells were also probed for Bak, Bax, and Bad. (B) Western blot analysis of Bcl-X$_L$ in IEC-18, IEC-*ras*-3 cells, and IEC-*ras*-3 clones transfected with an antisense Bcl-X$_L$ expression vector (as Bcl-X 65 and 66), or vector alone (vector 75) cultured in suspension overnight. The membrane was probed with anti-PKB antibody as a loading control. (C) Analysis of apoptosis by cell death ELISA in IEC-*ras*-3 and antisense Bcl-X$_L$–transfected clones cultured in monolayer or suspension overnight. Results represent the average of two independent experiments plus the SD. Induction of anoikis was calculated as the ratio of the apoptotic signal observed in suspension versus monolayer, and the data are expressed as a percentage of the ratio measured for IEC-18 cells. (D) Growth of IEC-*ras*-3 and antisense Bcl-X$_L$–transfected clones in anchorage-independent conditions. Cells were plated in soft agar in triplicates, and colonies were counted after 10 d. Results are expressed as a percentage of the number of colonies obtained with IEC-*ras*-3 cells, and represent the average plus the SD of two independent experiments. (E) Transfection of antisense Bcl-X$_L$ reduces the tumorigenicity of IEC-*ras*-3 cells. The indicated cell lines were injected subcutaneously into nude mice, and tumor volumes were measured at the indicated time points. The number of mice (*n*) used in each case is indicated. Bars represent the SEM. This experiment was repeated twice with similar results.

on the induction of anoikis resistance stems from the downregulation of Bak (Rosen et al., 1998).

ras-induced Stabilization of Bcl-X$_L$ Expression in Suspension Culture Does Not Require PI 3- and MAP Kinases

ras is known to activate several signaling pathways through effector molecules such as PI 3-kinase, Raf, Ral GDS, MEKK, and AF-6 (Marshall, 1996; Katz and McCormick, 1997; Khosravi-Far et al., 1998). Two of these pathways, the PI 3-kinase/PKB and Raf/MEK/ERK signaling cascades, have been shown to be involved in the inhibition of apoptosis in several cellular systems (Downward, 1998; Scheid et al., 1999). Therefore, we decided to investigate whether the *ras*-induced stabilization of Bcl-X$_L$ in IEC-18 cells requires activation of any of these two pathways. To investigate the involvement of the PI 3-kinase/PKB pathway, IEC-*ras*-3 cells were brought into suspension and treated with the PI 3-kinase inhibitor LY 294002 at a concentration that we previously found to efficiently suppress the activity of the enzyme in this particular cellular system (Rosen et al., 1998). As expected, treatment with LY 294002 strongly suppressed the phosphorylation of PKB (Fig. 9 A) and, as previously reported (Rosen et al., 1998), caused a significant increase in expression of Bak (Fig. 9 A). The levels of Bcl-X$_L$, on the other hand, were not affected (Fig. 8 A). This result is consistent with the fact that activated PKB, a downstream mediator of the antiapoptotic effect of PI 3-kinase, does not change the expression of Bcl-X$_L$ in fibroblasts (Kennedy et al., 1997). Previously, we found that ectopic expression of Bak in IEC-*ras* cells at levels similar to, or even higher than, those in the parental IEC-18 cells causes only a partial reversal of *ras*-induced resis-

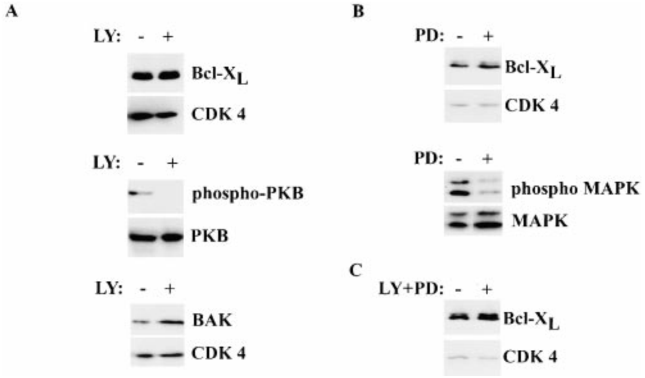

Figure 9. The stabilizing effect of activated *ras* on Bcl-X$_L$ expression is not suppressed by inhibition of PI 3-kinase, and Mek. (A) Western blot analysis of Bcl-X$_L$, phospho-PKB, PKB, and Bak in IEC-*ras*-3 cells cultured in suspension overnight in the presence (+) or absence (−) of 40 μM LY 294002 (LY). A similar volume of DMSO (vehicle) was added to the untreated cells. The membranes were reprobed with an anti-CDK 4 antibody as a loading control. (B) Western blot analysis of Bcl-X$_L$ and phospho-MAP kinase in IEC-*ras*-3 cells cultured in suspension overnight in the presence (+) or absence (−) of 50 μM PD 98059 (PD). A similar volume of DMSO (vehicle) was added to the untreated cells. The membranes were reprobed with an anti-CDK 4 and MAP kinase antibodies as loading controls. (C) Western blot analysis of Bcl-X$_L$ in IEC-*ras*-3 cells cultured in suspension overnight in the presence (+) or absence (−) of 40 μM LY 294002 and 50 μM PD 98059 (LY + PD). A similar volume of DMSO (vehicle) was added to the untreated cells. The membrane was reprobed with an anti-CDK 4 antibody as a loading control.

tance to anoikis (Rosen et al., 1998). Consistent with these results, we found that the degree of death induced by LY 294002 in suspended IEC-*ras* cells constitutes only a fraction of what is observed in untreated IEC-18 cells (data not shown).

To investigate the potential involvement of the Raf/MEK/ERK signaling IEC-*ras*-3 cells were placed in suspension and treated with PD 98059, an inhibitor of MEK. The treatment strongly suppressed phosphorylation of ERK but did not change Bcl-X$_L$ expression (Fig. 9 B). Simultaneous treatment of suspended IEC-*ras*-3 cells with LY 294002 and PD 98059 had no effect either (Fig. 9 C). Overall, these results indicate that the resistance to anoikis caused in intestinal epithelial cells by oncogenic *ras* is executed by at least two major contributing pathways: one that regulates Bcl-X$_L$ expression and, at least in our experimental conditions, is independent of PI 3-kinase and MEK, and another that downregulates Bak and requires PI 3-kinase activity.

Discussion

We have shown here that in nonmalignant rat and human intestinal epithelial cells, detachment from the ECM results in a significant downregulation of the antiapoptotic protein Bcl-X$_L$, and that activated *ras* completely abrogates such downregulation. The functional significance of this finding was made evident by the demonstration that ectopic expression of Bcl-X$_L$ in IEC-18 cells protected them from anoikis, whereas enforced inhibition of Bcl-X$_L$ expression in the *ras*-transformed cells promoted anoikis and reduced tumorigenicity.

Activated *ras* is known to trigger multiple downstream targets (Marshall, 1996; Katz and McCormick, 1997; Khosravi-Far et al., 1998). We found that two of such targets, PI 3-kinase and MEK, are not involved in the effect of Ras on Bcl-X$_L$ expression. The potential role of other *ras* effectors in this phenomenon is the subject of our ongoing research.

About 50% of human colorectal tumors display overexpression of Bcl-X$_L$ when compared with the adjacent normal intestinal mucosa (Krajewska et al., 1996). Thus, based on the findings reported here, it is tempting to speculate that Bcl-X$_L$ overexpression may play a role in conferring anoikis resistance to colorectal tumor cells, which are forced to survive in the absence of contact with a properly formed basement membrane during invasion and metastasis.

The overall conclusion that can be drawn from this study as well as the previous one (Rosen et al., 1998) is that the inhibition of anoikis in *ras*-transformed cells is, at least in part, the result of the ability of this oncogene to regulate the expression of two members of the Bcl-2 family: Bcl-X$_L$, an inhibitor of cell survival, and Bak, a well established inducer of apoptosis. Interestingly, the regulation of the expression of these molecules represents two fundamentally different patterns of cell survival control in the face of malignant transformation. Bak expression in the nonmalignant IEC-18 cells is constitutively high, relatively adhesion-independent, and a threshold level of expression of this protein is presumably required for the induction of apoptosis upon cell detachment. Oncogenic activation of *ras* leads to a dramatic downregulation of Bak below the threshold levels that are required for apoptosis of detached cells. In contrast, expression of Bcl-X$_L$ is highly adhesion-dependent, and activated *ras* prevents Bcl-X$_L$ downregulation, thereby providing the transformed cells with an additional survival advantage under three-dimensional growth conditions. Dissimilar modes of regulation of both Bak and Bcl-X$_L$ are, understandably, associated with differences in signaling events governing expression of these molecules. While suppression of Bak in *ras*-transformed IEC-18 cells can be reversed by the PI 3-kinase inhibitor LY294002, such treatment is ineffective in abrogating the stabilizing effect of activated *ras* on Bcl-X$_L$.

Even though our data identify Bcl-X$_L$ and Bak as two important mediators of the inhibitory effect of activated *ras* on anoikis, the involvement of other components of the apoptotic machinery in this effect cannot be excluded. For example, in various cellular systems, the PI 3-kinase signaling pathway is known to regulate the activity of Bad (Datta et al., 1997; del Peso et al., 1997; Scheid and Duronio, 1998), and Fas (Peli et al., 1999), two molecules with well established roles in apoptosis. Among other candidate proteins that may act as mediators of the anoikis-inhibitory effect of activated *ras* in intestinal epithelial cells are members of the IAP family. In this respect, it is important to note that the IAP survivin is deregulated in several human tumors including colorectal carcinomas (Ambrosini et al., 1997).

In summary, our study indicates that *ras*-induced resistance to anoikis in intestinal epithelial cells is the result of the ability of activated *ras* to alter the expression of at least two components of the apoptotic machinery. This suggests that therapeutic treatment aimed at the restoration of sensitivity to anoikis of tumors carrying activated *ras*, while expected to have therapeutic effect, may require targeting the *ras* oncogene directly instead of its downstream effector molecules involved in the control of apoptosis.

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