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Functional Dissociation of Anoikis-like Cell Death and Activity of Stress Activated Protein Kinase

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Adhesion to the extracellular matrix is a crucial survival signal for epithelial and endothelial cells. Both cell types activate an endogenous death program termed "anoikis" when detached from the solid substratum. The signaling events culminating in anoikis are still unclear; recent studies have implicated Stress Activated Protein Kinase (SAPK), also known as Jun-N-Terminal kinase, as a potentially crucial signal transducer and mediator of anoikis. However, the generality and the causal role of SAPK in anoikis remain unclear and controversial. For these reasons we decided to examine the relationship between induction of anoikis and SAPK activation in three independent cell systems. We report here that in immortalized rat intestinal epithelial cells (IEC-18) and human umbilical vein endothelial cells (HUVEC), SAPK is activated weakly and transiently upon cell detachment while in canine kidney epithelial cells (MDCK) such induction is strong and protracted. However, cell types fail to commit to anoikis after remaining in three-dimensional culture for the time required for complete activation of SAPK. This suggests that there is no temporal correlation between SAPK activation and the onset of anoikis in any of the cell lines studied. We further examined the potential involvement of SAPK in the IEC-18 system by investigating a *ras* oncogene-transformed variant of IEC-18 cells (IEC-18 Ras 3) which are highly resistant to anoikis. Ras expression did not abrogate activation of SAPK, although these cells do exhibit altered kinetics of SAPK induction upon cell detachment. These results suggest that SAPK is not involved in anoikis regulation and that SAPK activation is likely a cell-type-specific epi-phenomenon.

It is now recognized that the relative failure of cancer cells to undergo programmed cell death (apoptosis) under a variety of circumstances can contribute to tumor development and disease progression, including metastasis, as well as resistance to anti-cancer drugs (1, 2). Consequently, elucidating the molecular and cellular factors which govern tumor cell survival mechanisms has assumed a dominant place in both basic and clinical studies of cancer. In this regard, one important manifestation of apoptosis is known as anoikis (3, 4). This refers to the phenomenon of detachment-induced cell death in which normal (or immortalized) epithelial or endothelial cells initiate a cellular suicide program when they become physically separated from the extracellular matrix (ECM) or basement membrane with which they are normally in contact (5, 6). Anoikis is thought to occur, at least in part, as a result of disruption of survival signals provided by components of the extracellular matrix through cell surface associated integrins (5, 6). In contrast to normal cells, transformed, tumorigenic epithelial cells (*i.e.*, carcinoma cells) appear readily capable of survival under similar detachment conditions (6). This relative resistance to anoikis may be an important factor in allowing detached tumor cells to survive as single cells, or small emboli, in the bloodstream, and hence, to establish distant metastases in foreign tissues (6). It also may help explain the well known property of cancer cells to survive and grow *in vitro* in an anchorage-independent fashion, *e.g.*, as small three dimensional colonies in soft agarose (6). Thus, there is considerable interest in uncovering the nature of the genetic changes and the signaling pathways associated with them which are responsible for the relative resistance of cancer cells to undergo anoikis.

We recently reported that cells from an immortalized but non-tumorigenic line of rat intestinal epithelium, called IEC-18, undergo a massive apoptotic cell death process when forced to grow as multicellular tumor spheroids in culture (7). In marked contrast, tumorigenic sublines of IEC-18, obtained by transfection with a mutant *H-ras* oncogene, survived and grew under similar growth conditions (7). The results suggested that mutant *ras* oncogenes may have an important

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anti-apoptotic/pro-survival function which is preferentially expressed under anchorage-independent multicellular growth conditions (7). Similar conclusions have been reported by others (8, 9).

Our results prompted us to initiate a series of investigations to uncover the molecular basis of activated *ras*-mediated survival mechanisms in epithelial cells growing anchorage independently. We found that oncogenic *ras* can induce a significant downregulation of the pro-apoptotic effector protein known as Bak (10), a member of the bcl-2 gene family of apoptosis regulators (11, 12). Moreover, this change appears necessary for *ras*-dependent resistance to anoikis, at least in the IEC-18 intestinal epithelial cells (10). However, *ras* transformed IEC cells, transfected with the *bak* gene failed to fully revert to the parental IEC-18 phenotype either in terms of survival in anchorage-independent conditions *in vitro* or tumorigenic growth *in vivo* (10), thus suggesting that additional changes are involved in the ability of *ras*-transformed IEC-18 cells to resist anoikis. Another important conclusion of this study was that the *ras*-induced downregulation of Bak requires PI3K activity. This conclusion is consistent with results published by several laboratories indicating that PI3K is a critical inhibitor of anoikis (9, 13). In our studies, however, we found that PI3K is *not* the *only* mediator of *ras*-induced resistance to anoikis, since treatment of IEC-ras cells with the PI3K inhibitor LY294002 only partially restored sensitivity to anoikis. In our search for other transducing molecules that could be involved in the regulation of anoikis we became particularly interested in SAPK since previous studies by others had implicated that the activation of this enzyme was involved in anoikis of MDCK epithelial cells (14). However, these results are controversial and could not be confirmed by others (15).

Thus, we decided to investigate the contribution of SAPK in anoikis of IEC-18 cells and a *ras* transformed variant of IEC-18 called IEC-*ras*3. We also examined this relationship in MDCK cells and normal human umbilical vein endothelial cells (HUVECs), as additional control systems known to be susceptible to anoikis. We report here that activation of SAPK upon cell detachment and the induction of anoikis appear to be unrelated. Furthermore, *Ras* expression has a minimal effect on SAPK activation, and it appears that in three different anchorage dependent types of cells the magnitude and kinetics of SAPK activation is highly diverse and dissociable from the onset of anoikis.

MATERIALS AND METHODS

Cell lines and culture conditions. IEC-18 and IEC-18 *ras*-3 cells were obtained and cultured as described previously (7). Briefly, all cell lines were maintained in monolayer culture in growth medium composed of α-minimum essential medium (α-MEM) base supplemented with 5% fetal bovine serum (Gibco BRL, Gaithersburg, MD), 4 mmol/LL-glutamine, 20 mmol/L glucose, 10 μg/mL insulin (Sigma Chemical Co., St. Louis, MO). MDCK cells were obtained from the ATCC and maintained as monolayer cultures in medium composed of Dulbecco's modified Eagle's medium (DMEM) base supplemented with 10% fetal bovine serum (Gibco Canadian Life Technologies) precoated with 1% gelatin (Sigma) in medium composed of a MCDB131 base supplemented with 15% FBS (Gibco), 10 ng/mL epidermal growth factor (Upstate Technologies, Lake Placid, NY), 5 ng/mL basic fibroblast growth factor (R&D Systems Inc., Minneapolis, MN) and 10 units/mL heparin (Gibco). For spheroid culture over 2 hours in length cells were plated on 100-mm (Nunc) tissue culture dishes precoated with a thin layer of 1% Seaplaque agarose (FMC Corp., Rockland, ME). For spheroid culture under 2 hours in length cells were left in 14 mL Falcon polystyrene tubes (Becton Dickinson, Mississauga, Ont.) at 37°C and agitated every 30 minutes.

Anoikis assays. The characteristics of apoptotic death of IEC-18 cells in spheroid culture, and its abrogation in oncogenic ras transfectants IEC-18 *ras*-3 have been described in detail (7). For the purpose of the present study we have used the cell death detection Elisa Plus kit (Roche Molecular Biochemicals, Laval, Quebec) to estimate the rate of apoptotic cell death by quantitation of nucleosomes released into the cytoplasm by dying cells. Briefly, cells were allowed to become confluent prior to plating at 5×10^4 cells per 100-mm tissue culture dish (Nunc) in both monolayer culture and spheroid culture where the tissue culture dish (Nunc) was precoated with a thin layer of 1% Seaplaque agarose (FMC Corp.). At the time points indicated duplicate samples were collected from the dishes, lysed and incubated with a mixture of biotinylated antihistone antibody and peroxidase-conjugated anti-DNA antibody, both of which bind to histone DNA complexes and initiate color reaction in the presence of the ABST substrate. The OD reading at 405 nm was corrected to negative control and expressed as an enrichment factor, according to manufacturer's instructions.

Kinase assays. The effect of cell detachment on SAPK activation was measured in IEC-18, IEC-18 *ras*-3, HUVEC and MDCK cells. Cells were plated at 3.8×10^6 and detached 24 hours later after a brief incubation with 10% trypsin. Trypsin was neutralized with one wash with media containing FBS (Gibco BRL); the cells were then resuspended in media with no FBS in 14 mL Falcon polystyrene tubes (Becton Dickinson) and incubated at 37°C with agitation every 30 minutes for the period of time indicated. Positive controls were provided by cells treated with 250 μg/ml anisomycin for 30 minutes at 37°C. Cells were then rapidly pelleted and lysed in lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 1% IEGPAL, 1 mM EDTA, 20 μg/ml aprotinin/leupeptin, 1 mM PMSF and 1 mM Na_3VO_4 for 30 minutes on ice. Lysates were centrifuged at 12 000 g for 10 minutes at 4°C, equalized for protein (determined by Bio-Rad protein assays), and the supernatant immunoprecipitated with rabbit polyclonal JNK antibody sc-474 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4°C. Samples were further incubated in the presence of protein A Sepharose beads (Sigma Chem. Co., St. Louis, MO) for 1 hour at 4°C. Pellets were washed twice in wash buffer (50 mM Hepes (pH 7.4), 150 mM NaCl, 1% IEGPAL, 1 mM EDTA) and once in kinase buffer wash (10 mM $MgCl_2$, 50 mM Tris-Cl, pH 7.5 and 1 mM EDTA, pH 7.5). The pellet was resuspended in kinase buffer and 100 μM [γ-^{32}P]ATP (Amersham Intl., Arlington Heights, IL); and c-Jun sc-4113 (Santa Cruz). The reaction was stopped with sample buffer after 30 minutes of incubation at 37°C. After boiling for 5 minutes and SDS-PAGE, the phosphorylation of c-Jun protein was quantified by using a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Survival Properties of Epithelial and Endothelial Cell Lines under Three-Dimensional Growth Conditions

Since it has been reported that SAPK is involved in the *ras*-induced resistance to anoikis in MDCK cells, we decided to use those cells as our positive controls for

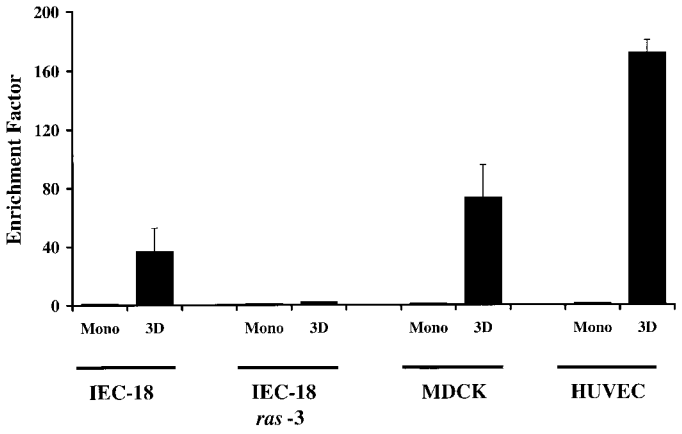

FIG. 1. Apoptosis of MDCK, IEC-18, IEC-18 *ras*-3 and HUVEC in suspension culture. Following detachment from the plates and 24 hours in either monolayer or three-dimensional culture conditions the apoptosis of IEC-18, IEC-18 *ras*-3, MDCK and HUVEC cells was quantitated by ELISA. The enrichment of mono- and oligonucleosomes in the cytoplasm was detected by biotin-labeled anti-histone and peroxide conjugated anti-DNA antibodies (see Methods). The data represents the average of two independent experiments, each one was done in duplicate.

our studies of IEC-18 cells. In addition, we have also included in our studies HUVECs, as endothelial cells are also known to be highly sensitive to anoikis (5). Previous studies have reported that cell-cell contact sensitizes MDCK cells to anoikis, with maximal apoptosis being detectable when cells were confluent prior to suspension (14). Accordingly, all cells were grown until confluent prior to plating for the anoikis assay; cells were then placed in either monolayer or three-dimensional culture conditions for 24 hours before being assayed for apoptosis. Apoptosis, although virtually undetectable in cells placed in monolayer conditions, was detected in all the cell lines or populations tested, except the *ras* transformed IEC-18 variant, after incubation in three-dimensional culture (Fig. 1). Interestingly, although all three anoikis sensitive cell lines exhibited marked apoptosis after substratum detachment, differences were found between cell types; HUVECs being the most sensitive, whereas immortalized MDCK and IEC-18 cells were found to be somewhat less sensitive.

SAPK Is Differentially Activated after Detachment of MDCK, IEC-18, and HUVECs

After trypsinization and suspension, SAPK was assayed at various times points in the anoikis sensitive cell lines (MDCK, IEC-18 and HUVEC). All cells were confluently plated prior to assay in an effort to ensure maximal cell-cell interactions. MDCK cells showed a strong and rapid induction of SAPK activity upon detachment (Fig. 2A), which was sustained for up to 4 hours (data not shown). This temporal order was previously interpreted as an indicator of a linkage between SAPK activation and commitment to undergo cell death in three-dimensional culture (14). In contrast, IEC-18 cells exhibit a markedly different pattern of SAPK induction. SAPK activity was weak and transient after the detachment of IEC-18 cells. Maximal activity was attained approximately 15 minutes after detachment and decreased rapidly to below the basal level after 1 hour (Fig. 2B). This was not due to inability of IEC-18 cells to activate SAPK. A known inducer of the stress pathway, anisomycin was capable of a strong and sustained activation of SAPK in these cells (Fig. 2B) (16). These results prompted us to further examine the kinetics of SAPK activation in HUVEC, another anoikis-sensitive cell line, in order to determine if the kinetics of SAPK activation is similar to either IEC-18 cells or MDCK cells. Our results show that HUVECs exhibit a strikingly similar pattern of SAPK activation to that of IEC-18 cells; maximal activity is, again, reached within 15 minutes of detachment and activity declines to almost the basal level after 1 hour in suspension (Fig. 2C). Thus, in two out of three anoikis-sensitive cell lines we observed discordance between detachment from the substratum and an induction of SAPK activity.

Activated ras Alters the Kinetics of SAPK Activation in IEC-18 Cells

A number of reports have indicated that *Ras* can signal through a variety of pathways to activate SAPK (17–21). The involvement of SAPK activation in triggering apoptosis seems somewhat paradoxical in view of the survival abilities imparted by *Ras* expression in anchorage-independent growth conditions. We decided then to examine the degree and kinetics of SAPK induction in cells overexpressing oncogenic *Ras* when placed in three-dimensional culture conditions. Not surprisingly, a different pattern of SAPK activation upon cell detachment was detected. Similar to previous reports, in MDCK cells (15), *Ras* does not abrogate SAPK activity in IEC-18 cells placed in suspension. The maximal activity of SAPK occurs between 45 and 60 minutes after detachment of IEC-18 *ras-3* cells (Fig. 3) contrasting sharply with the transient SAPK activation expressed after 15 minutes by IEC-18 parental cells upon detachment.

Maximal Activation of SAPK in Spheroid Culture Does Not Commit Cells to Undergo Anoikis

To investigate whether SAPK activity is sufficient to induce anoikis, we designed a rescue experiment to test cell viability after the maximal SAPK activation in suspension culture has been reached. The various cell lines, in which we had previously established the pattern of SAPK induction, were plated and allowed to

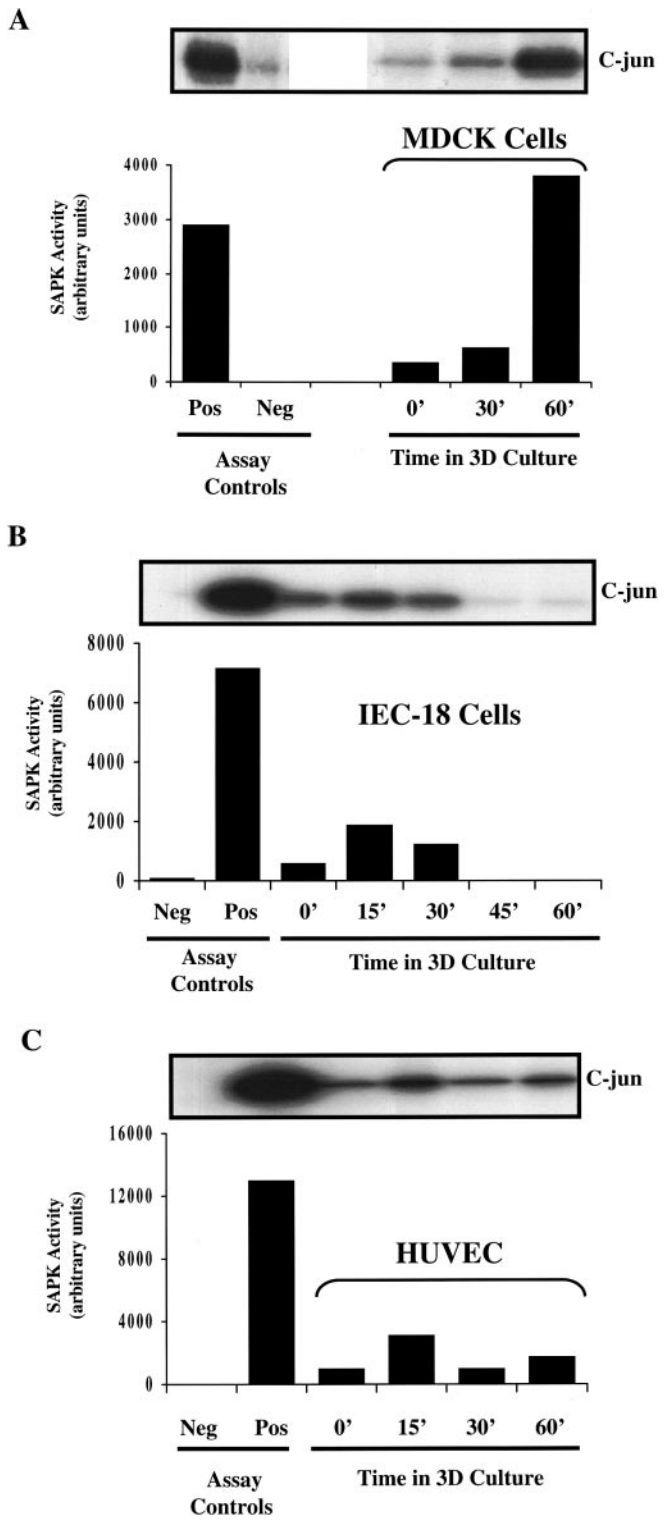

FIG. 2. Differential activation of stress protein kinase in three anoikis sensitive cell lines. SAPK immunoprecipitates from MDCK (A), IEC-18 (B), and HUVEC (C) cell line lysates were assayed for associated c-jun phosphorylating activity. Radioactivity in c-jun bands is shown in the inset and quantitated (in arbitrary units) in the graphs shown below. For positive assay controls IEC-18 cells treated with 250 μg/mL of anisomycin were used while negative controls are IEC-18 cell lysates with no c-jun substrate added.

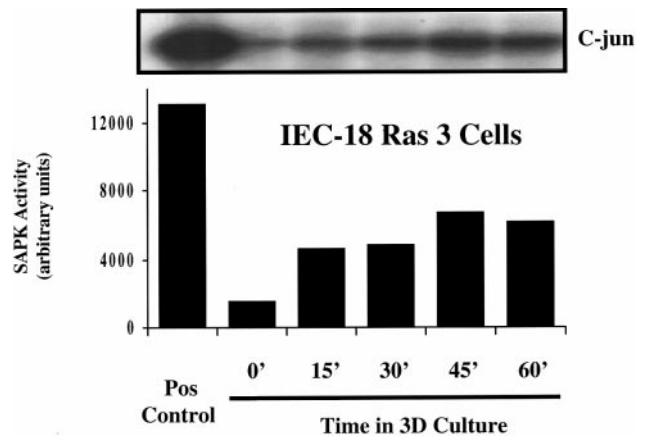

FIG. 3. Delayed activation of stress activated protein kinase in anoikis resistant IEC-18 Ras 3 cells. SAPK immunoprecipitates from IEC-18 Ras 3 cell lysates were assayed for associated c-jun kinase activity. Radioactivity in c-jun bands is shown in the inset and quantitated in the graph shown below. Positive control is IEC-18 Ras cells treated with 250 μg/mL of anisomycin.

become confluent. After 24 hours, cells were trypsinized and then either replated in monolayer or in three-dimensional culture conditions for 2 hours. This time point was selected to ensure that all cells lines would attain the maximal activation of SAPK induced by substratum detachment. After such 2 hour incubation in suspension culture the cells were replated in monolayer culture for a further 22 hours. Apoptosis was determined 24 hours after the initial cell plating by a cell death ELISA (Roche). Interestingly, none of the cells pre-incubated in suspension showed detectable levels of apoptosis (Fig. 4) thus, confirming the hypothesis that SAPK activity induced by cell detachment is not sufficient to commit cells to apoptosis.

DISCUSSION

Two main findings are presented in this paper. First, the magnitude of SAPK activation upon cell detachment from the extracellular matrix appears to be a cell-type specific phenomenon, and, second, SAPK activation is dissociable not only from the onset of anoikis in three anoikis-sensitive cell lines, but also, from the lack of anoikis in the *ras* transformed variants of one of these lines. The first finding is of particular interest, as there are few available reports specifically focused on the role of SAPK in anoikis. Moreover, the causal role of SAPK has been examined only in the MDCK cell system and even this result is controversial and not yet confirmed (15). Our results shed some light on the role of SAPK in the broader phenomenon of anoikis as a result of investigating a number of different cell lines. Furthermore, this work is consistent with the growing number of reports indicating that SAPK activation is highly dependent on both the nature of the inducing stimulus and the cellular context (22–24).

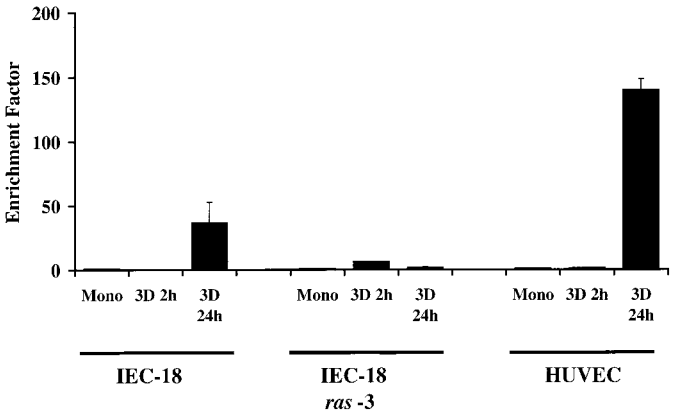

FIG. 4. Activation of SAPK in suspension culture is insufficient to cause anoikis. Apoptosis of IEC-18, IEC-18 Ras 3 and HUVEC cells was assessed after the cells remained in three-dimensional culture for the time exceeding the requirement for activation of SAPK (2 hours). The enrichment of mono- and oligonucleosomes in the cytoplasm was detected by cell death ELISA assay. The data represents the average of two independent experiments, each one was done in duplicate.

The second finding, namely the complete lack of correlation between SAPK activity and the induction of anoikis in any of the cell lines examined, is not surprising in light of the first finding because two out of three cell lines exhibited only a weak and transient activation of SAPK upon substratum detachment, although all cells displayed comparable levels of apoptosis in three-dimensional culture. In addition, *ras* transformed IEC-18 cells are completely resistant to anoikis but, nevertheless, show SAPK activity when detached, albeit with different kinetics from the parental cells. These results support the findings of Khwaja *et al.* who reported that although SAPK is strongly activated under the conditions that initiate anoikis in MDCK cells, it is neither necessary nor sufficient for the induction of anoikis in this particular cell line (15).

What factors, then, are responsible for induction of anoikis, and development of resistance to this form of apoptosis in normal epithelial and transformed epithelial cells, respectively? The answer, to some extent, may depend upon the nature and origin of the cell population. For example, as summarized in the Introduction, we have previously reported that pro-apoptotic effector molecule known as Bak plays an important role in the induction of anoikis of IEC-18 intestinal epithelial cells (10). In this regard it is interesting to note that Bak has been previously implicated as a critical endogenous promoter of apoptosis in the intestinal epithelium (25). For example, Arber *et al.* (26) found that a *K-ras* oncogene can increase resistance to sulindac-induced apoptosis in IEC-18 cells and that this may be the result of downregulation of Bak. Similarly, Houghton *et al.* found that apoptosis was inhibited after 'thymineless stress' by oncogenic *K-ras* in thymidylate synthase deficient human colon cancer cells, and that this may be due to altered levels of Bak and Bcl-X_L, *i.e.*, upregulation of Bcl-X_L and downregulation of Bak, by oncogenic *K-ras* (27). Thus, Bak seems to be an especially important regulator of programmed cell death, including anoikis, in intestinal epithelial cells, and moreover, mutant *ras* may function as an important survival factor in colon cancer through its ability to modulate (downregulate) Bak expression. This *ras* oncogene-Bak inter-relationship appears to be mediated, at least in part by PI3 kinase (9, 10, 13). However, as discussed in the Introduction, additional factors appear to be involved in *ras*-mediated resistance to anoikis, the nature of which are currently under investigation.

In summary, the data reported here does not dispute that SAPK is activated by the loss of cell-ECM interactions (14, 15), but serves to provide further support for the lack of causative effects this activation has in the overall phenomenon of anoikis. SAPK has been implicated in a variety of cellular functions, such as cell cycle regulation (28) or the apoptosis induced by such divergent stimuli as heat stress or chemotherapeutic drugs (22, 28). However, SAPK activation appears to be functionally unimportant in the direct signaling that commits cells to anoikis. It would appear that anoikis regulation is a complex process regulated by numerous and possibly redundant signaling pathways.

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