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Cell Detachment Triggers p38 Mitogen-activated Protein Kinase-dependent Overexpression of Fas Ligand

A NOVEL MECHANISM OF ANOIKIS OF INTESTINAL EPITHELIAL CELLS*

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Many cell types undergo apoptosis when they are detached from the extracellular matrix (ECM). This phenomenon has been termed anoikis. Most epithelial cells, which are normally attached to a type of ECM called basement membrane, are particularly sensitive to anoikis. Conversely, carcinoma cells tend to be resistant to anoikis, and this resistance plays a critical role in tumor invasion and metastasis. We reported previously that detachment-induced down-regulation of the anti-apoptotic molecule Bcl-X_L makes a significant contribution to anoikis of intestinal epithelial cells. Here we demonstrate that exogenous Bcl-X_L, no matter how highly expressed in these cells, can significantly attenuate anoikis but cannot completely prevent it, suggesting that at least another pro-apoptotic event is activated by the loss of cell-ECM contacts. Indeed, in this study we identified a novel mechanism of anoikis in intestinal epithelial cells that involves detachment-induced overexpression of Fas ligand. We also demonstrated that this elevation in Fas ligand expression requires a detachment-induced increase of p38 mitogen-activated protein kinase activity. We conclude that the activation of at least two different pro-apoptotic events is required for anoikis of intestinal epithelial cells.

Survival of most normal epithelial cells requires adhesion to a type of extracellular matrix (ECM)[1] called basement membrane. Loss of cell-ECM contacts results in death of such cells by apoptosis, a phenomenon known as anoikis (1–3). Induction of anoikis is now thought to play a critical role in several physiological processes such as cavitation during vertebrate development and mammary gland regression after weaning (4–6). The process of cavitation transforms the solid embryonic ectoderm into a basement membrane-attached columnar epithelium surrounding a cavity. The cavity is generated by the apoptosis of the cells that are not attached to the basement membrane (7). In the mammary gland cessation of lactation induces the secretion of metalloproteases that destroy the basement membrane to which milk-producing mammary epithelial cells are attached. As a consequence such cells undergo apoptosis that leads to mammary gland regression (8, 9).

Anoikis is also thought to play an important role in the elimination of intestinal epithelial cells shed into the intestinal lumen. The surface of the intestine is covered by a single layer of epithelial cells that differentiate as they migrate upwards along the intestinal crypts and villi to be eventually shed into the lumen. As these cells reach the top of the crypt or villi they detach from the basement membrane and undergo apoptosis (10–12).

The interest in understanding the molecular mechanisms of anoikis has increased significantly during the last few years as it became evident that resistance to anoikis is a critical requirement for invasion and metastasis in cancers derived from epithelial cells (3, 13). A better understanding of anoikis could have an impact on the development of novel therapies for cancer, because it has been demonstrated that the reversion of anoikis resistance inhibits tumor progression (14, 15).

Despite the increasing interest in the study of anoikis, the basic mechanisms of this phenomenon are still poorly understood. Epithelial cells are known to attach to the ECM through specialized transmembrane receptors called integrins (5, 16). The loss of cell-ECM adhesion results in integrin disengagement as well as in rearrangement of the cell cytoskeleton. These events are known to lead to changes in the expression and/or activity of proteins that are directly involved in the control of apoptosis. As a consequence of these changes the apoptotic machinery becomes activated and anoikis is induced (6).

It is now believed that programmed cell death is regulated by two major pathways. One of them involves the release of cytochrome *c* from the mitochondria into the cytoplasm where this molecule participates in the activation of caspases (17, 18). Caspases are cysteine proteases that upon activation cleave a set of proteins critical for cell survival and thereby cause apoptosis (19, 20). The release of cytochrome *c* is both positively and negatively controlled by members of the Bc-2 family. Some members of this family such as Bcl-2 and Bcl-X_L are anti-apoptotic, and others are pro-apoptotic (*e.g.* Bak, Bax, and Bid) (21, 22). The second major pathway that triggers programmed cell death involves the activation of members of the death receptor family such as Fas, tumor necrosis factor receptor, DR-4, and DR-5 (23–26). In most cases these receptors are activated by their ligands, but ligand-independent mechanisms of activation have also been proposed (27, 28). The activation of the death receptors induces the formation of the so-called death-inducing signaling complex. In addition to the receptors,

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[1] The abbreviations used are: ECM, extracellular matrix; MAP, mitogen-activated protein; IEC, intestinal epithelial cells; FBS, fetal bovine serum; MEM, minimum Eagle's medium; ELISA, enzyme-linked immunosorbent assay; d.n., dominant negative; GFP, green fluorescent protein; IAPs, inhibitor of apoptosis proteins.

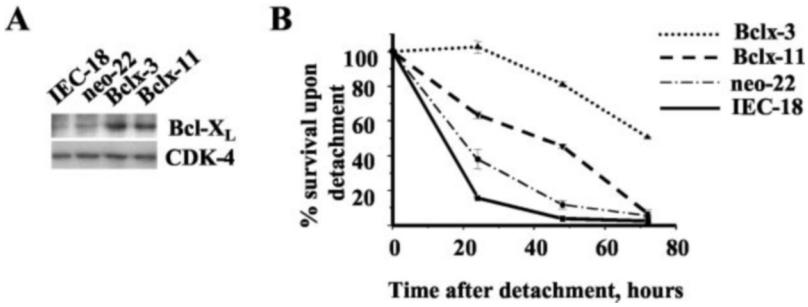

FIG. 1. **Ectopic Bcl-X$_L$ only partially inhibits anoikis of intestinal epithelial cells.** *A*, Western blot analysis of Bcl-X$_L$ expression. Cell lysates were prepared from the following cells grown in monolayer culture: IEC-18 cells (IEC-18), a control clone transfected with vector alone (neo-22), and two clones transfected with a Bcl-X$_L$ expression vector (Bclx-3 and Bclx-11). The membrane was re-probed with an anti-CDK-4 antibody as a loading control. *B*, effect of ectopic Bcl-X$_L$ on anoikis. IEC-18 cells as well as neo-22, Bclx-3, and Bclx-11 clones were plated in monolayer culture either immediately (0 h) or after being cultured in suspension for 24, 48, and 72 h. Cell colonies were allowed to form for 1 week and counted. % survival upon detachment was calculated as a ratio of the number of colonies formed after incubation in suspension culture for each of the indicated time periods to that at 0 h. Results represent the average of duplicates plus the S.D. This experiment was repeated twice with similar results.

this complex includes the adaptor FADD and caspases 8/10. Upon binding to death-inducing signaling complex these caspases are cleaved and activated and thus acquire the ability to trigger apoptosis (25, 29, 30).

Several components of both the death receptor and mitochondrial apoptotic pathways have been shown to be regulated by cell attachment. The initial reports (31, 32) in this regard implicated the death receptor pathway in anoikis of some types of epithelial and endothelial cells. In addition to death receptor-mediated events, the mitochondria pathway has also been shown to participate in anoikis in that the pro-apoptotic molecule Bax changes its conformation and translocates to the mitochondria upon detachment of mammary epithelial cells (33). Another study implicating the mitochondrial pathway in anoikis reported that the pro-apoptotic Bcl-2 family member Bmf, which is normally bound to the cytoskeleton in attached cells, migrates to the mitochondria upon cell detachment (34).

Recently we have described another mechanism of anoikis. We found that detachment of intestinal epithelial cells results in a significant down-regulation of Bcl-X$_L$, and that ectopic expression of this anti-apoptotic molecule induces a significant inhibition of anoikis (14, 35). We have also shown that the detachment-induced down-regulation of Bcl-X$_L$ is not exclusive of the intestinal epithelium, because it is also observed in human ovarian epithelial cells (36).

The fact that the various studies on detachment-induced changes in components of the apoptotic machinery were performed in different cell types raises the question as to whether these changes are cell type-specific or whether more than one of these anoikis-triggering events occurs in the same cell type. We have recently started to investigate this question in intestinal epithelial cells, and we observed specific molecular events triggered by cell detachment which suggest that, in addition to the mitochondrial pathway, the death receptor pathway is also activated during anoikis (35). However, whether this pathway plays a causal role in this form of apoptosis in intestinal epithelial cells, which are the mechanisms of activation of the death receptor pathway in such cells are not known.

Here we report that Fas receptor signaling is required for efficient anoikis of intestinal epithelial cells. Moreover, we demonstrate that the activation of this pathway occurs because of the detachment-induced overexpression of Fas ligand, and that this increase in Fas ligand expression is mediated by an elevation in p38 MAP kinase activity.

EXPERIMENTAL PROCEDURES

Cell Culture—The IEC-18 cells were obtained from Dr. A. Quaroni (Cornell University) and were cultured in α-MEM containing 5% fetal bovine serum (FBS), 10 μg/ml insulin, and 0.5% glucose. DKS-8 cells were a gift of Dr. T. Sasazuki (Kyushu University). These cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS. Mouse colonocytes were derived from p53-deficient mice (37). These cells were cultured in α-MEM containing 5% FBS, 10 μg/ml insulin, and 0.5% glucose. For suspension cultures cells were plated above a layer of 1% sea plaque-agarose polymerized in α-MEM or Dulbecco's modified Eagle's medium. The IEC-18 clones transfected with a Bcl-X$_L$ expression vector were described previously (14).

Caspase-8 Activity Assay—A caspase-8 Colorimetric Assay kit from R&D Systems was used according to the manufacturer's instructions.

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% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 10 μg/ml leupeptin. After removing insoluble material, aliquots of supernatant containing 20–30 μg of protein were run under reducing conditions through a 12% polyacrylamide gel. Proteins were transferred to a nylon membrane that was subsequently incubated for 1 h at room temperature in a TBST buffer (125 mM Tris-HCl (pH 8.0), 625 mM NaCl, 0.5% Tween 20) containing 4% skim milk. The membrane was then incubated with one of the following antibodies: anti-Bcl-X$_L$, (Santa Cruz Biotechnology, to screen for exogenous Bcl-X$_L$, and BD Biosciences to screen for endogenous Bcl-X$_L$), anti-Bax (Upstate Biotechnology, Inc.), anti-Bmf (Alexis Biochemicals), anti-Fas ligand (Santa Cruz Biotechnology), anti-p38 (Cell Signaling), anti-phospho-p38 (New England Biolabs), anti-CDK-4 (Santa Cruz Biotechnology), and anti-β-actin (Sigma). Incubation with antibodies was performed in a TBST buffer containing 5% bovine serum albumin in case of anti-Bcl-X$_L$, 4% skim milk in case of anti-β-actin, and 2.5% skim milk in all other cases. Binding of the antibodies was detected with the enhanced chemiluminescence system (PerkinElmer Life Sciences).

Northern Blot Analysis—Northern blot analysis was performed on total RNA. A rat Fas ligand cDNA, kindly donated by Dr. S. Nagata (Osaka University Medical School), labeled with [^{32}P]dCTP by random priming was used as a probe.

Cell Death ELISA Assay—Cells growing in monolayer or in suspension culture were removed from the plates and assayed for the presence of nucleosomal fragments in the cytoplasm by a Cell Death Detection ELISA kit (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Assessment of Cell Survival by Colony Formation Assay—10^3 cells were placed in suspension culture for the indicated times and then plated in 100-mm tissue culture dishes. Cell colonies were allowed to form for 1 week and counted after crystal violet staining.

Assessment of Cell Survival by Morphological Changes—7.5×10^5 IEC-18 cells or a Bcl-X$_L$-transfected clone (Bclx-3) were plated in suspension for 17 h and subsequently visually assessed for apoptosis by light microscopy. Shrunk cells were scored as apoptotic.

Dominant Negative FADD Vector Construction and Assay of Apoptosis by Transient Transfection—Plasmid DNA PAS2–1 carrying the cDNA fragment corresponding to amino acids 79–205 of mouse FADD in the *Eco*RI-*Sal*I site was kindly provided by Dr. W. C. Yeh (Ontario Cancer Institute). This FADD cDNA fragment was cloned into the *Hpa*I site of pDON-AI expression vector (Takara) to generate pDON-AI-d.n.FADD. 3.5 μg of the pEGFP-C1 expression vector (Clontech) alone or in combination with either 17.5 μg pDON-AI or 17.5 μg pDON-AI-d.n.FADD were incubated in 350 μl of serum-free α-MEM for 5 min at

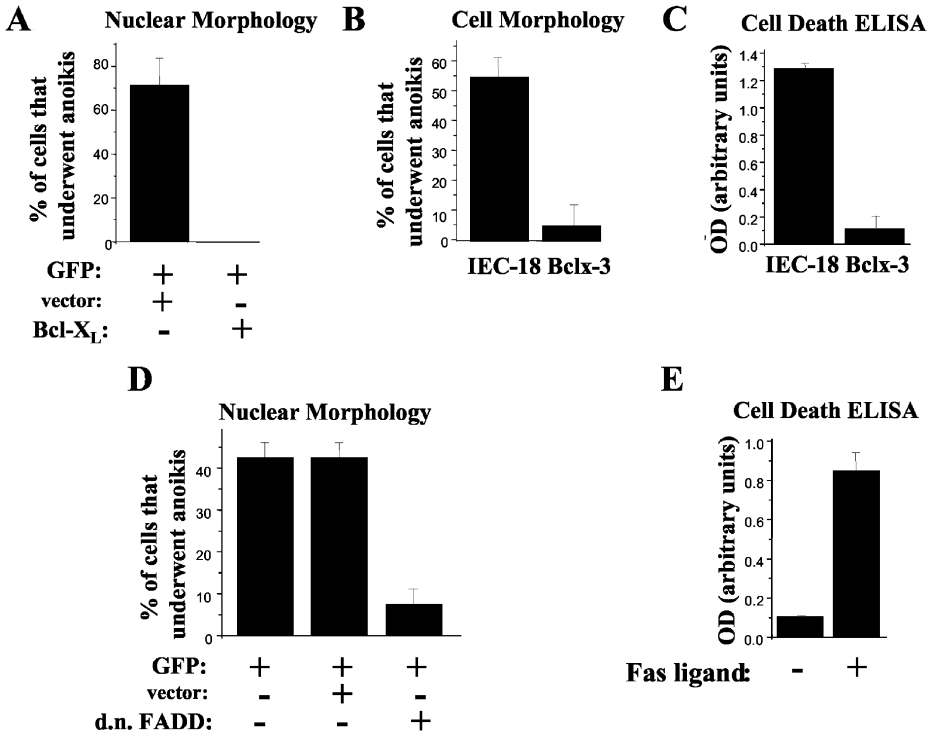

FIG. 2. **Death receptor signaling is required for anoikis of intestinal epithelial cells.** *A*, transient transfection of Bcl-X_L inhibits detachment-induced morphological changes of the nuclei associated with apoptosis. IEC-18 cells were transfected with a green fluorescent protein expression vector (*GFP*) in combination with either vector control (*vector*) or a Bcl-X_L expression vector. Cells were then cultured in monolayer or in suspension for 17 h. Cell nuclei were subsequently stained with 4′,6-diamino-2-phenylindole dihydrochloride hydrate, and the morphology of the nuclei of GFP-positive cells was assessed by fluorescence microscopy. Cells with fragmented or shrunk nuclei were scored as apoptotic. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. Cell death observed in monolayer culture was subtracted from that observed for suspension cells as background. *B*, exogenous Bcl-X_L blocks detachment-induced changes in cell morphology associated with apoptosis. IEC-18 cells or an IEC-18 clone expressing exogenous Bcl-X_L (Bclx-3) were plated in suspension for 17 h and subsequently visually assessed for apoptosis by light microscopy. Shrunk cells were scored as apoptotic. Results represent the average of duplicates plus the S.D. This experiment was repeated three times with similar results. *C*, exogenous Bcl-X_L blocks detachment-induced release of oligonucleosomes into the cytoplasm. IEC-18 cells or an IEC-18 clone of these cells expressing exogenous Bcl-X_L (Bclx-3) were plated in suspension for 17 h and subsequently assessed for apoptosis by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. *D*, FADD is required for anoikis of intestinal epithelial cells. IEC-18 cells were transfected with a green fluorescent protein expression vector (*GFP*) alone or in combination with either vector control (*vector*) or a dominant negative FADD expression vector (*d.n. FADD*). Cells were then cultured in monolayer or in suspension for 17 h, and nuclei were subsequently assayed for apoptosis-associated features as in *A*. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. Cell death observed in monolayer culture was subtracted from that observed for suspension cells as background. *E*, exogenous Fas ligand causes apoptosis of intestinal epithelial cells. IEC-18 cells were cultured in monolayer for 17 h in the absence (−) or in the presence (+) of recombinant His-tagged Fas ligand and anti-His antibody. Apoptosis was measured by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate.

room temperature. These plasmids were then mixed with 30 μg/ml superfect (Qiagen) in 2.5 ml of culture medium for 10 min at room temperature and added to 7.5×10^5 IEC-18 cells plated into 100-mm tissue culture dish. Cells were incubated with the transfection mixture for 3 h at 37 °C. The medium was then replaced with 10 ml of the regular IEC-18 medium. Cells were grown for another 24 h, collected, and placed either in suspension or monolayer culture overnight. Apoptosis was then assessed according to a procedure published previously (38, 39). In brief, cells were trypsinized, washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 30 min at room temperature, washed again with phosphate-buffered saline, and stained with 1 μg/ml of 4′,6-diamino-2-phenylindole dihydrochloride hydrate in phosphate-buffered saline for 30 min at room temperature. 4′,6-Diamino-2-phenylindole dihydrochloride hydrate-positive (blue) nuclei of GFP-positive (green) cells were visualized by fluorescent microscopy using the respective light filters.

To test the effect of Bcl-X_L on anoikis we followed a protocol similar to that described for dominant negative FADD. The Bcl-X_L expression vector was described previously (14).

Inhibition of Anoikis with the Anti-Fas Ligand Antibody—2×10^4 cells were plated in suspension culture in 1 ml of growth medium above 0.5 ml of sea plaque-agarose in the absence or in the presence of 5 μg/ml of the anti-Fas ligand monoclonal antibody NOK-2 (Pharmingen) for 8 h. Cells were then assayed for apoptosis by the Cell Death ELISA.

Induction of Apoptosis with Recombinant Fas Ligand—10^5 cells were incubated with 4 μg/ml recombinant histidine-tagged Fas ligand (R&D Systems) supplemented with 10 μg/ml anti-histidine antibody (R&D Systems) for the indicated times. Cells were then assayed for apoptosis by the Cell Death ELISA.

RESULTS

Inhibition of Detachment-induced Down-regulation of Bcl-X_L Results in a Delayed Cell Death Rather Than a Complete Suppression of Anoikis—We have recently reported (35) that detachment of intestinal epithelial cells induces molecular events that are consistent with the activation of the Fas receptor pathway, but we have not yet investigated whether this activation plays a role in anoikis. On the other hand, we have already established that detachment-induced down-regulation of Bcl-X_L plays a causal role in anoikis of intestinal epithelial cells (14). Thus, before investigating whether the Fas receptor pathway plays a role in anoikis of these cells, we decided to find out whether Bcl-X_L-independent events may contribute to anoikis. To this end, we directly compared the long term sensitivity to anoikis of the non-malignant rat intestinal epithelial cells (IEC-18) with that of the two previously published IEC-18-derived clones transfected with Bcl-X_L (14). It is important to note that these two Bcl-X_L-transfected clones express significantly higher levels of this anti-apoptotic protein than the parental cells both in monolayer (Fig. 1*A*) and in suspension

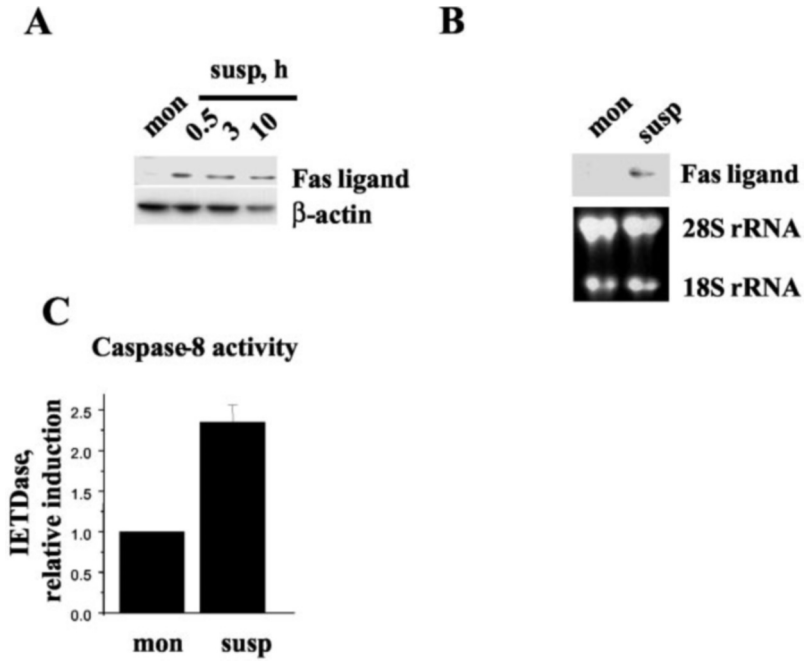

FIG. 3. **Detachment of IEC-18 cells results in overexpression of Fas ligand.** *A*, cell detachment induces up-regulation of Fas ligand. IEC-18 cells were cultured in monolayer (*mon*) or in suspension (*susp*) for the indicated times and assayed for Fas ligand expression by Western blot. The membrane was reprobed with an anti-β-actin antibody as a loading control. *B*, cell detachment induces Fas ligand mRNA. IEC-18 cells were cultured in monolayer or in suspension for 3 h and assayed for Fas ligand expression by Northern blot. 18 S and 28 S ribosomal RNA were used as loading controls. *C*, cell detachment induces caspase-8 activity. IEC-18 cells were cultured in monolayer or in suspension for 3 h, and the cleavage of IETD-pNA tetrapeptide, a known substrate of caspase-8, was measured in the respective cell lysates by a colorimetric assay. Results represent the average of two independent experiments plus the S.D.

culture (14). As shown in Fig. 1*B*, overexpression of Bcl-X_L in IEC-18 cells causes a significant delay of anoikis but not a complete inhibition of this process. These data indicate that at least one Bcl-X_L-independent molecular event contributes to the induction of anoikis in intestinal epithelial cells.

Death Receptor Signaling Contributes to Anoikis of Intestinal Epithelial Cells—In an effort to investigate a possible involvement of the death receptor pathway in anoikis of intestinal epithelial cells, we decided to find out whether transfection of dominant negative FADD, a truncated mutant of this adaptor molecule that prevents endogenous FADD from transmitting death receptor-induced apoptotic signals, is capable of blocking anoikis of IEC-18 cells (40). To this end, we used an assay based on monitoring transiently transfected cells for nuclear fragmentation and shrinkage, which are characteristic features of apoptosis (41, 51). To validate this assay in our model system, we first decided to verify that transient transfection of IEC-18 cells with Bcl-X_L is capable of blocking detachment-induced apoptotic changes in nuclear morphology. Indeed, transient transfection with a Bcl-X_L expression vector resulted in a significant inhibition of apoptosis-specific nuclear alterations caused by detachment of IEC-18 cells for 17 h (Fig. 2*A*). This result correlated well with the ability of exogenous Bcl-X_L to block anoikis measured by changes in the cell morphology or the release of oligonucleosomes into the cytoplasm (Fig. 2, *B* and *C*). Likewise, as shown in Fig. 2*D*, transient transfection of IEC-18 cells with dominant negative FADD strongly suppressed detachment-induced apoptosis. Next we asked whether signals triggered by the activation of death receptors in attached intestinal epithelial cells, for example in response to treatment with exogenous Fas ligand, are capable of inducing apoptosis. To this end, we treated IEC-18 cells cultured as monolayer with recombinant Fas ligand. We found that this treatment strongly induces apoptosis (Fig. 2*E*). We conclude, therefore, that death receptor-mediated signaling plays a causal role in anoikis of intestinal epithelial cells.

Cell Detachment Triggers Overexpression of Fas Ligand—To determine the mechanism by which the death receptor pathway is activated by detachment of intestinal epithelial cells, we investigated the effect of such detachment on Fas ligand expression. We found that suspended IEC-18 cells display a significant increase of Fas ligand both at the protein and mRNA levels (Fig. 3, *A* and *B*).

We have reported that anoikis of IEC-18 cells is not detectable until 4 h after detachment (14). Here we show that Fas ligand expression is induced as early as 30 min after loss of cell-ECM adhesion (Fig. 3*A*). This suggests that overexpression of this pro-apoptotic molecule could be one of the causes of anoikis.

In a previous study (35), we found that detachment of IEC-18 cells triggers a relatively strong activation of caspase-10 and a relatively weak activation of caspase-8. However, whereas in that study caspase-10 activation was measured as early as 30 min after detachment, caspase-8 activity was assessed at later time points (the earliest time point was 5 h). We therefore decided to investigate caspase-8 activation at an earlier time point, before the onset of anoikis. As shown in Fig. 3*C*, caspase-8 is strongly activated 3 h after detachment of IEC-18 cells. We conclude that, similarly to what was observed for caspase-10, activation of caspase-8 precedes the onset of anoikis.

In addition to being directly triggered by the engagement of death receptors, caspase-8 and caspase-10 can be activated by the release of cytochrome *c* from the mitochondria. Such release is known to lead to the sequential activation of caspase-9, caspase-3, caspase-6, and eventually to caspase-8 and caspase-10 (42). However, we showed previously (35) that caspase-3 is not activated until 10 h after detachment of IEC-18 cells, suggesting that the induction of caspase-8 and caspase-10 is not a post-mitochondrial event but rather represents a direct consequence of the activation of Fas.

In order to confirm that the induction of Fas ligand by the loss of cell-ECM interaction is not a unique property of IEC-18 cells, we studied the effect of cell detachment on the expression levels of this molecule in non-malignant human intestinal epithelial DKS-8 cells. These cells were derived from the colorectal carcinoma cell line DLD-1 by targeted ablation of the activated K-*ras* allele (43). As a result of the loss of oncogenic Ras, DKS-8 cells became non-tumorigenic *in vivo* and acquired significant susceptibility to anoikis (14, 43) (Fig. 4*A*). Fig. 4*B* shows that in DKS-8 cells, similarly to what was observed in IEC-18 cells, Fas ligand is strongly induced by detachment.

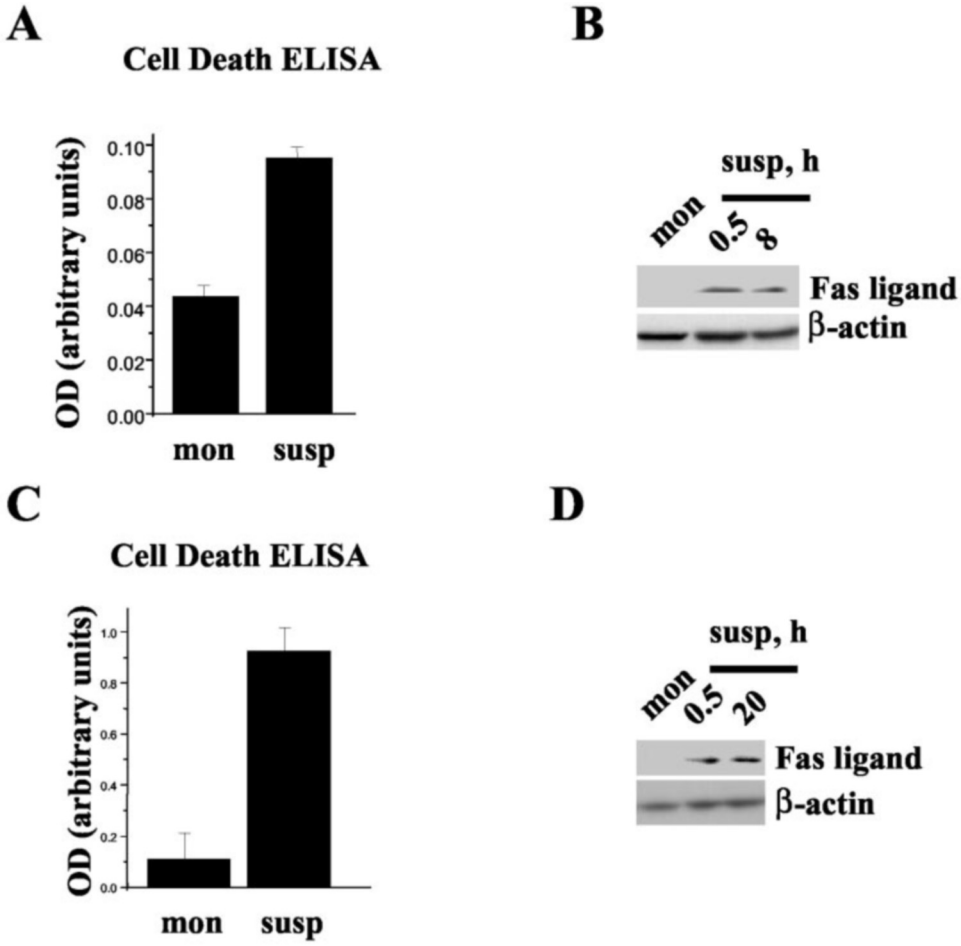

FIG. 4. **Detachment of human and mouse colonic cells results in overexpression of Fas ligand.** *A*, detachment of non-malignant DKS-8 human intestinal epithelial cells results in anoikis. DKS-8 cells were cultured in monolayer (*mon*) or in suspension (*susp*) for 21 h and assayed for apoptosis by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was performed in duplicate. *B*, detachment of DKS-8 cells induces Fas ligand expression. DKS-8 cells were cultured in monolayer or in suspension for the indicated times and assayed for Fas ligand expression by Western blot. The membrane was re-probed with an anti-β-actin antibody as a loading control. *C*, detachment of non-malignant mouse colonocytes results in anoikis. Colonocytes derived from p53-deficient mice were cultured in monolayer or in suspension for 21 h and assayed for apoptosis by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. *D*, detachment of colonocytes derived from p53-deficient mice induces Fas ligand expression. Colonocytes were cultured in monolayer or in suspension for the indicated times and assayed for Fas ligand expression by Western blot. The membrane was re-probed with an anti-β-actin antibody as a loading control.

Overexpression of Fas ligand in suspended cells was also observed in non-malignant colonocytes derived from p53-deficient mice that are highly susceptible to anoikis (Fig. 4, *C* and *D*). It can be concluded, therefore, that detachment-induced overexpression of Fas ligand is a general feature of intestinal epithelial cells.

Fas Ligand Overexpression Is Required for Anoikis of Intestinal Epithelial Cells—In order to find out whether detachment-induced Fas ligand is required for anoikis, we cultured suspended DKS-8 human intestinal epithelial cells in the presence of the anti-human Fas ligand monoclonal antibody NOK-2. This antibody has a well documented ability to block the function of human Fas ligand (44–46). As shown in Fig. 5, NOK-2 significantly inhibited anoikis. This result indicates that detachment-induced overexpression of Fas ligand contributes, at least in part, to anoikis of intestinal epithelial cells.

Detachment-induced Increase in the Activity of p38 MAP Kinase Is Required for the Induction of Fas Ligand Expression—Next, we investigated the molecular mechanism involved in the detachment-induced overexpression of Fas ligand. Several recent reports have implicated the activation of p38 MAP kinase as the cause of induction of Fas ligand expression. A p38 kinase-dependent overexpression of Fas ligand has been observed in pheochromocytoma cells PC12 in response to treatment with corticotropin-releasing hormone (47), in T cells during activation-induced cell death (48), as well as in transformed

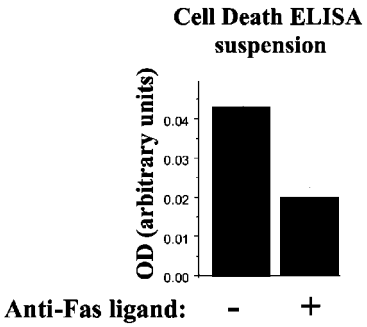

FIG. 5. **Detachment-induced overexpression of Fas ligand is required for anoikis of intestinal epithelial cells.** DKS-8 cells were cultured in suspension for 8 h in the absence (−) or in the presence (+) of 5 μg/ml of the function blocking monoclonal anti-Fas ligand antibody (NOK-2) and assayed for apoptosis by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. Cell death in monolayer culture was also measured and subtracted from that observed for suspended cells as background.

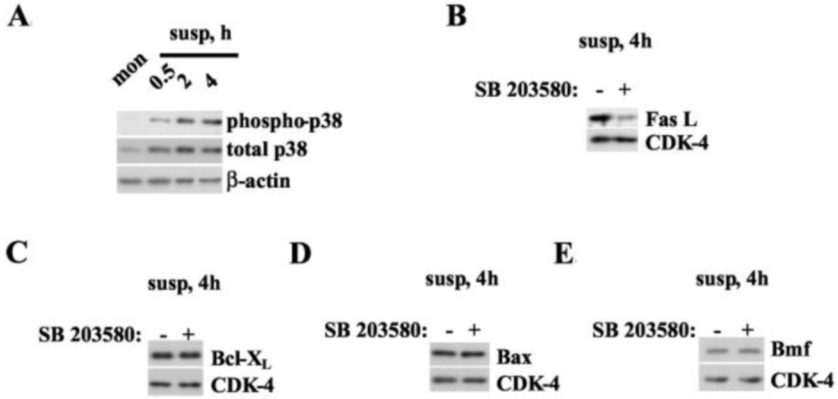

FIG. 6. **Detachment-induced overexpression of Fas ligand occurs in a p38 MAP kinase-dependent manner.** *A*, detachment induces p38 MAP kinase activity. IEC-18 cells were cultured in monolayer (*mon*) or in suspension (*susp*) for the indicated times and assayed for p38 kinase phosphorylation (*phospho-p38*) and p38 kinase expression (*total p38*) by Western blot. The membrane was re-probed with an anti-β-actin antibody as a loading control. *B*, Fas ligand induction triggered by cell detachment is p38 kinase-dependent. IEC-18 cells were cultured in suspension for 4h in the absence (−) or in the presence (+) of 20 μM SB 203580 and assayed for Fas ligand expression by Western blot. The membrane was re-probed with an anti CDK-4 antibody as a loading control. Me_2SO (vehicle) was added to the untreated cells. *C*, inhibition of p38 kinase has no effect on Bcl-X_L expression. IEC-18 cells were cultured in suspension for 4 h in the absence (−) or in the presence (+) of 20 μM SB 203580 and assayed for Bcl-X_L expression by Western blot. The membrane was re-probed with an anti CDK-4 antibody as a loading control. Me_2SO (vehicle) was added to the untreated cells. *D*, inhibition of p38 kinase has no effect on Bax expression. IEC-18 cells were cultured in suspension for 4 h in the absence (−) or in the presence (+) of 20 μM SB 203580 and assayed for Bax expression by Western blot. The membrane was re-probed with an anti-CDK-4 antibody as a loading control. Me_2SO (vehicle) was added to the untreated cells. *E*, inhibition of p38 kinase has no effect on Bmf expression. IEC-18 cells were cultured in suspension for 4 h in the absence (−) or in the presence (+) of 20 μM SB 203580 and assayed for Bmf expression by Western blot. The membrane was re-probed with an anti CDK-4 antibody as a loading control. Me_2SO (vehicle) was added to the untreated cells.

primary embryonal kidney 293T cells upon anisomycin treatment (49).

We reasoned that if p38 MAP kinase plays a role in the detachment-induced overexpression of Fas ligand in intestinal epithelial cells, such cells should display higher levels of p38 kinase activity in suspension than in monolayer culture. As shown in Fig. 6*A*, we found that cell detachment induces a strong increase in the amount of phospho-p38. Interestingly, we also found that the loss of cell-ECM contact results in a noticeable overexpression of this enzyme (Fig. 6*A*).

We further investigated whether the increase in active p38 kinase caused by cell detachment mediates the concomitant accumulation of Fas ligand. To this end we treated IEC-18 cells grown in suspension culture with SB 203580, a specific inhibitor of p38 kinase (50). We found that this treatment results in a strong inhibition of Fas ligand expression (Fig. 6*B*). On the other hand, at least in our experimental conditions, SB 203580 had no significant effect on the levels of Bcl-X_L (Fig. 6*C*), Bax (Fig. 6*D*), or Bmf (Fig. 6*E*), which have been implicated previously (14, 34, 33) in the regulation of anoikis.

If p38 kinase mediates the detachment-induced overexpression of Fas ligand in intestinal epithelial cells, and this pro-apoptotic molecule plays a role in the induction of anoikis, treatment of detached intestinal cells with the p38 kinase inhibitor SB 203580 would be expected to reduce this form of cell death. Indeed, when suspended IEC-18 cells were treated with the p38 kinase inhibitor, a noticeable suppression of anoikis was observed (Fig. 7*A*). This effect was relatively SB 203580-specific as inhibitors of other signaling molecules such as MEK (PD 98059) (51) and NF-κB (52) stimulated anoikis (Fig. 7, *B* and *C*).

In order to find out whether p38 kinase causes anoikis in a Fas ligand-dependent manner, we brought IEC-18 cells in suspension and treated them either with SB 203580 alone or in combination with recombinant Fas ligand. As shown in Fig. 7*A*, exogenous Fas ligand significantly inhibited the anti-anoikis effect of SB 203580. This result confirms that anoikis of intestinal epithelial cells occurs, at least in part, due to detachment-induced p38 kinase-dependent overexpression of Fas ligand.

Bcl-X_L Blocks Fas Ligand-induced Apoptosis in Intestinal Epithelial Cells—Depending on the cell type, Fas ligand-induced cell death may or may not require the activation of the mitochondrial pathway (53). In those cells whose apoptosis requires the activation of this pathway, cell death induced by Fas ligand can be partially inhibited by anti-apoptotic members of the Bcl-2 family. Because we have shown here that Fas ligand plays a causal role in anoikis, and we have previously demonstrated that this form of cell death can be inhibited by exogenous Bcl-X_L (14), it is reasonable to propose that in intestinal epithelial cells Fas ligand-induced cell death can be regulated by Bcl-X_L. To test this hypothesis we investigated whether ectopic Bcl-X_L is capable of inhibiting Fas ligand-induced apoptosis of IEC-18 cells. To this end, Bcl-X_L-transfected IEC-18 clones, parental untransfected IEC-18 cells, and vector-transfected control cells were treated with recombinant Fas ligand in monolayer culture. We found that Bcl-X_L confers significant protection from Fas ligand-induced apoptosis (Fig. 8), confirming that in intestinal epithelial cells the mitochondrial pathway makes an essential contribution to cell death triggered by the activation of the death receptor. It can be proposed, therefore, that one of the mechanisms by which detachment-induced down-regulation of Bcl-X_L contributes to anoikis is by facilitating Fas receptor-induced cell death.

DISCUSSION

In this study we have demonstrated that anoikis of intestinal epithelial cells requires detachment-induced increase in p38 MAP kinase activity, and subsequent p38 kinase-dependent overexpression of Fas ligand.

Activation of the death receptor pathway during anoikis has been shown by others previously (31, 32, 54) and appears to occur via distinct cell type-specific mechanisms. For example, anoikis of kidney and skin epithelial cells can be blocked by dominant negative FADD but not by decoy receptors that inhibit Fas ligand or TRAIL activity (31). These data suggest that detachment-induced apoptosis of these types of cells is either triggered by one of the respective death receptors in a ligand-independent manner or that it is induced by death receptor(s)

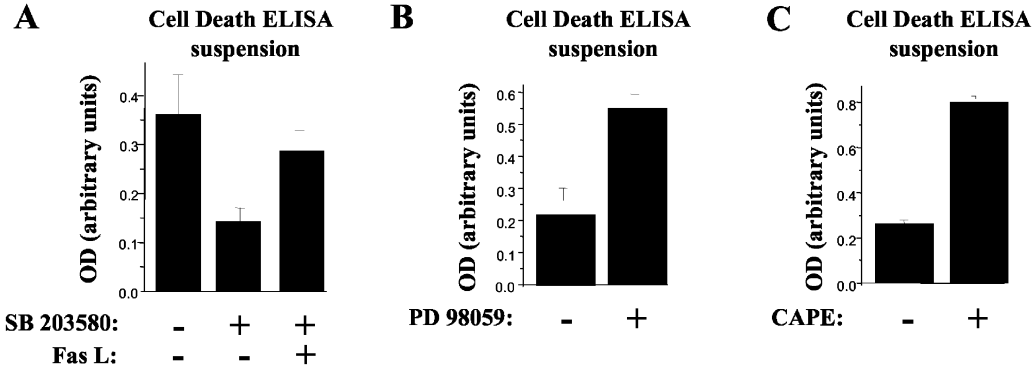

FIG. 7. **p38 MAP kinase activity is required for anoikis, and Fas ligand induces apoptosis in suspended cells treated with SB 203580.** *A*, IEC-18 cells were cultured in suspension for 4 h with recombinant His-tagged Fas ligand and anti-His antibody in the absence (−) or in the presence (+) of 20 μM SB 203580. Apoptosis was measured by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. Me_2SO (vehicle) was added to the untreated cells. The level of apoptosis of cells cultured in monolayer was subtracted from that observed for suspension cells as background. *B*, IEC-18 cells were cultured in suspension for 4 h in the absence (−) or in the presence (+) of 25 μM PD 98059. Apoptosis was measured by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. Me_2SO (vehicle) was added to the untreated cells. The level of apoptosis of cells cultured in monolayer was subtracted from that observed for suspension cells as background. *C*, IEC-18 cells were cultured in suspension for 4 h in the absence (−) or in the presence (+) of 1 μg/ml CAPE. Apoptosis was measured by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. Me_2SO (vehicle) was added to the untreated cells. The level of apoptosis of cells cultured in monolayer was subtracted from that observed for suspension cells as background.

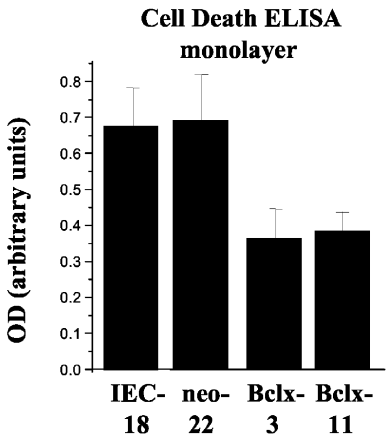

FIG. 8. **Fas ligand-induced apoptosis of intestinal epithelial cells can be blocked by Bcl-X_L.** IEC-18 cells as well as neo-22, Bclx-3, and Bclx-11 clones were cultured in monolayer in the absence or in the presence of recombinant His-tagged Fas ligand supplemented with the anti-His antibody for 12 h. Apoptosis was assayed by the Cell Death ELISA. Results represent the average of two independent experiments plus the S.D. Each experiment was done in duplicate. Cell death observed in case of untreated cells was subtracted from that of Fas ligand-treated cells as background.

ligand(s) other than Fas ligand and TRAIL. In endothelial cells it has been found that anoikis requires the interaction between Fas ligand and its receptor (54). However, in these cells the main molecular events involved in the activation of the Fas receptor pathway in anoikis are the detachment-induced overexpression of Fas receptor, and the detachment-induced down-regulation of c-Flip, an inhibitor of apoptosis that blocks the interaction between FADD and caspases-8 and 10 (54). It can be concluded, therefore, that the activation of the Fas pathway plays an important role in anoikis but that the specific mechanism of activation of this receptor (and possibly other members of the death receptor family) depends on the particular cell type.

One important discovery of this study is the involvement of p38 MAP kinase in anoikis. According to our data, detachment-induced elevation in p38 kinase activity is accompanied by an increase in p38 kinase expression. Normally this enzyme is known to be activated by MAP kinases such as MKK3 and/or MKK6 (55). Whether elevated expression of p38 *per se* is sufficient for the increased p38 enzymatic activity observed during anoikis, or if other upstream MAP kinases need to be induced to achieve such activation, remains to be investigated.

To our knowledge, activation of the p38 MAP kinase pathway has not been associated previously with anoikis. However, c-Jun N-terminal kinase, another stress-associated enzyme, has been suggested to play a causal role in anoikis of Madin-Darby canine kidney epithelial cells (56), although this finding is controversial (57). We have reported previously (58) that c-Jun N-terminal kinase activation is not involved in anoikis of intestinal epithelial cells.

Our finding that p38 kinase plays a role in the induction of Fas ligand expression during anoikis is consistent with previous reports implicating this kinase in the induction of Fas ligand expression in T lymphocytes, PC12, and 293 kidney cells (47–49). Moreover, in one of these reports (49) it has been demonstrated that p38 kinase can activate expression driven by the Fas ligand promoter.

From the results obtained in this study and in previous ones (14, 15), we conclude that anoikis of intestinal epithelial cells is triggered by at least two molecular events that are induced by cell detachment and which activate the apoptotic machinery: overexpression of Fas ligand, and down-regulation of Bcl-X_L. Certainly, at the present time we cannot exclude the possibility that other pro-apoptotic molecular events are triggered by detachment of intestinal epithelial cells, because such events have been described in other cell types (33, 34).

Based on our results, Fas ligand-deficient mice would be expected to contain a higher proportion of viable cells in the gut lumen than their wild type counterparts. Specific technical approaches aimed at measuring apoptosis of cells in the lumen will have to be developed to study this problem.

Another interesting finding of this study is that Bcl-X_L can modulate Fas-induced signaling in intestinal epithelial cells. It is reasonable to propose, therefore, that this modulation plays a significant role in the induction of anoikis. In several cell types members of the Bcl-2 family have been shown to be able to protect from death receptor-induced apoptosis (53, 59). It is now understood that in such cells the Fas pathway is unable to activate threshold amounts of caspases unless the mitochondrial pathway contributes to caspase activation by releasing pro-apoptotic molecules such as cytochrome *c* and Smac/Diablo (59–61). The latter is known to induce apoptosis by blocking

the activity of the IAPs, which are potent inhibitors of caspases (62). The pro-apoptotic role of Smac/Diablo is critical for the induction of apoptosis in cells that express high levels of IAPs (60). Interestingly, it has been reported recently (63) that in intestinal epithelial cells susceptibility to apoptosis by Fas ligand can be regulated by IAPs. It is therefore possible that in such cells the ratio between Smac/Diablo and IAPs is regulated by Bcl-X_L and thereby plays a critical role in anoikis.

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