Oncogenic Ras Inhibits Anoikis of Intestinal Epithelial Cells by Preventing the Release of a Mitochondrial Pro-apoptotic Protein Omi/HtrA2 into the Cytoplasm

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Resistance of cancer cells to anoikis, apoptosis induced by cell detachment from the extracellular matrix, is thought to represent a critical feature of the malignant phenotype. Mechanisms that control anoikis of normal and cancer cells are understood only in part. Previously we found that anoikis of non-malignant intestinal epithelial cells is driven by detachment-induced down-regulation of Bcl-XL, a protein that blocks apoptosis through preventing the release of death-promoting factors from the mitochondria. Mitochondrial proteins the release of which causes anoikis are presently unknown. Similar to what was previously observed by others for keratinocytes and fibroblasts, we show here that anoikis of intestinal epithelial cells does not involve caspase-9, a target of a mitochondrial protein cytochrome c. Furthermore, Smac/Diablo, another mitochondrial pro-apoptotic factor, does not appear to play a role in detachment-dependent anoikis of these cells either. Instead, anoikis of intestinal epithelial cells is triggered by the release of a mitochondrial protein Omi/HtrA2, an event driven by detachment-induced down-regulation of Bcl-XL. Moreover, we established that oncogenic ras inhibits anoikis by preventing the release of Omi/HtrA2. This effect of ras required ras-induced down-regulation of a pro-apoptotic protein Bak and could be blocked by an inhibitor of phosphoinositide 3-kinase, a target of Ras that was previously implicated by us in the down-regulation of Bak and blockade of anoikis. We conclude that Omi/HtrA2 is an inducer of anoikis and an important regulator of ras-induced transformation.

Normal epithelial cells typically grow in vivo in contact with a form of extracellular matrix (ECM) called basement membrane. Detachment of non-malignant epithelial cells from the ECM triggers their apoptosis (1, 2). This type of programmed cell death has been observed in various types of cells and is often referred to as anoikis (3–5). Unlike normal epithelial cells, carcinomas (cancers of epithelial origin) grow, invade, and metastasize as disorganized three-dimensional cellular aggregates in which cells are deprived of adhesion to the basement membrane but remain anoikis-resistant. This resistance is now thought to be critical for the progression of the disease and could therefore serve as a carcinoma-specific novel therapeutic target (4, 6–8).

Some of the molecular events that are involved in the inhibition of anoikis in tumor cells have been identified but the knowledge about the mechanisms by which these cells escape anoikis is far from complete. It is now known that activated ras, one of the most frequently occurring oncogenes, acts as a strong inhibitor of anoikis of various types of cells, including those of the intestinal epithelium (1, 3, 10, 11). Ras is a small GTPase that can be activated by numerous mitogenic stimuli and, once activated can, in turn, trigger various signaling pathways that alter diverse biological cellular characteristics (12). The oncogenic form of Ras is locked in a GTP-bound state as a result of point mutations and thus acquires the ability to activate its downstream targets in a constitutive manner. So far oncogenic Ras was shown to block anoikis by multiple mechanisms that alter the expression of several direct regulators of apoptosis (10, 13).

Two pathways are now thought two play a major role in apoptosis of mammalian cells. One of them is driven by the release of factors such as cytochrome c, Smac/DIABLO, Omi/HTRA2, and Arts from the mitochondria into the cytoplasm (14–21), and subsequent activation of caspases, proteases that cleave vital targets, and thus trigger death (22). Upon induction by cytochrome c and another pro-apoptotic molecule APAF-1, initiator caspase-9 activates the executioner caspses, such as caspases-3 and -7 (23). Activity of caspses can be blocked by members of the IAP family (cIAP1, -2, XIAP etc.) (24). On the other hand, mitochondrial factors, such as Smac/Diablo and Omi/HtrA2 are capable of sequestering the IAPs and thus promoting caspase activation (15, 17). In addition, Omi/HtrA2 possesses a serine protease activity that contributes to the ability of Omi to cause caspase-independent apoptosis by cleaving targets that remain to be fully characterized (17, 18, 25, 26). AlF and endonuclease G are two other mitochondrial factors that upon release from the mitochondria trigger programmed cell death by inducing fragmentation of chromosomal DNA (27, 28). The release of the death-promoting proteins from the mitochondria into the cytoplasm can be stimulated or blocked by pro-apoptotic (Bak, Bim, etc.) and anti-apoptotic (Bcl-2, Bcl-XL, etc.) proteins of the Bcl-2 family, respectively (20, 21, 29, 30). The second major apoptotic pathway is typically triggered by members of the death receptor family, such as Fas, after activation by their ligands, such as Fas ligand. Once engaged, death receptors induce initiator caspses-8 and -10 through the adaptor molecule FADD (31, 32).
We and other have shown that detachment of epithelial cells from the ECM results in changes in the expression or activity of various components of apoptotic machinery (13, 33–41). For example, according to our data, anokis of non-malignant intestinal epithelial cells is driven by two simultaneously occurring death-promoting events. The first event involves detachment-dependent inhibition of expression of the anti-apoptotic factor Bcl-XL (42, 43). The second mechanism is mediated by detachment-triggered induction of p38 MAP kinase and subsequent p38 MAP kinase-dependent overexpression of Fas ligand (36).

Oncogenic Ras is now known to block anokis by activating several pathways. For example, we found that in the case of intestinal epithelial cells, the inhibitory effect of Ras on anokis is mediated by Ras-dependent activation of phosphoinositide 3-kinase (PI 3-kinase) and subsequent PI 3-kinase driven down-regulation of the pro-apoptotic protein Bak (10). We also established that Ras prevents anokis of these cells by blocking detachment-induced down-regulation of Bcl-XL (13). In addition, data obtained by others suggest that this oncogene suppresses detachment-dependent apoptosis of epithelial cells from breast, intestine, and other organs by down-regulating a death-inducing Bcl-2 family member Bim (39).

Given that oncogenic Ras blocks detachment-dependent apoptosis by more than one mechanism, therapeutic targeting of anokis resistance of ras-transformed cells based on the simultaneous suppression of these mechanisms could obviously be practically difficult. In this regard, one common feature of the mediators of the anti-anokis effect of ras, such as Bak, Bcl-XL, and Bim, is that they ultimately control the release of various mitochondrial factors into the cellular cytoplasm. Thus, delivery of such factor(s) into the cytoplasm of those tumor cells that carry the ras oncogene may represent an efficient strategy for overcoming their anokis resistance. However, mitochondrial mediators of apoptosis that are involved in the control of anokis of normal and cancer cells are presently unknown. According to recent studies, caspase-9, a target of cytochrome c, does not appear to be involved in the execution of detachment-induced apoptosis (44, 45). This conclusion is based on the fact that embryonic fibroblasts derived from caspase-9- and Apaf-1 knock-out mice are as susceptible to anokis as their wild type counterparts (45). Furthermore, a caspase-9 inhibitor, Z-LEHD-fmk (46), was demonstrated to be unable to block anokis of human keratinocytes (44).

We show in this study that anokis of intestinal epithelial cells is caspase-9- and Smac/Diablo-independent but requires the activity of Omi/HtrA2. We demonstrate that the release of Omi into the cytoplasm of these cells is driven by detachment-induced down-regulation of Bcl-XL. We also show that the ras oncogene prevents the release of Omi and subsequent anokis by blocking the expression of Bak.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The generation of the IEC clones expressing activated H-ras and Bcl-XL of mouse colonicocytes as well as of ras knock-out derivatives of the HCT-116 cells. HhK-2 has been described elsewhere (1, 47–49). IEC-18 cells and their derivatives as well as mouse colonicocytes were cultured in α-minimal essential medium containing 5% fetal bovine serum, 10 μg/ml insulin, and 0.5% glucose. HCT-116 and HhK-2 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. To culture cells in the absence of adhesion to the ECM, cells were plated in suspension culture above a layer of 1% sea plaque-agarose polymerized in α-minimal essential medium or Dulbecco’s modified Eagle’s medium.

**Vectors**—pcDNA-3-encoded Omi-GFP fusion construct was provided by Dr. J. Downward, Imperial Cancer Research Fund. pEGFP-C1 expression vector was from Clontech.

**Western Blot Analysis**—Western blot analysis was performed as described elsewhere (13). The following antibodies were used in this study: anti-cytochrome c (Santa Cruz Biotechnology), anti-Smac (R & D Systems), anti-Omi (R & D Systems), anti-β-actin (Sigma), anti-Bak (Upstate), and anti-caspase-9 (Cell Signaling).

**Preparation of Cytosolic Fraction**—Preparation of cytosolic fraction was performed as described by others (2). Cells were first washed and then re-suspended in 25 μl of a buffer containing 70 mM Tris base, 0.25 M sucrose, and 1 mM EDTA, pH 7.4. Subsequently an equal volume of digitonin dissolved at 0.2 mg/ml in a buffer containing 19.8 mM EGTA, 19.8 mM EDTA, 0.25 M mannitol, and 19.8 mM MES, pH 7.4, was added to the samples for 5 min on ice. This mixture was then subjected to centrifugation at 900 × g for 2 min. The resulting supernatant was further centrifuged for 5 min at 20,000 × g. The supernatant obtained after this centrifugation was subsequently analyzed for the presence of cytochrome c, Smac, or Omi by Western blot.

**Caspase-9 Activity Assay**—A caspase-9 Colorimetric Assay kit from R & D Systems was used according to the manufacturer’s instructions.

**RNA Interference**—All transfections with siRNAs were performed by using Lipofectamine 2000 (Invitrogen). To block caspase-9 expression in IEC-18 cells by caspase-9 siRNA1 and caspase-9 siRNA2 or Omi expression by Omi siRNA2 and Omi siRNA3, 5 × 105 cells were transfected with 100 nM of either previously published nonspecific control siRNA (50) or each of the respective caspase-9- or Omi-specific siRNAs for 48 h. In the case of Omi siRNA1 efficient inhibition of Omi expression could only be achieved after 5 × 105 IEC-18 cells were subjected to 2 consecutive 48-h rounds of transfection with 250 nM Omi-siRNA1. In this case the control cells were also subjected to 2 consecutive 48-h rounds of transfection with 250 nM of the control siRNA. In the case of the experiments involving simultaneous transfection of cells with Omi siRNA3 and caspase-9 siRNA2 we found that these RNAs when co-transfected at concentrations higher than 25 nM each, reduced the ability of each other to down-regulate their respective targets, possibly because of the fact that at these concentrations they competed for the same components of the cellular RNA interference machinery. When cells were co-transfected with Omi- and caspase-9-specific siRNAs at 25 nM each, these RNAs were capable of efficiently blocking the expression of their respective targets. Thus, 5 × 105 cells were co-transfected with 25 nM Omi siRNA3 and 25 nM caspase-9 siRNA2 for 48 h. To ensure that in this series of experiments concentration of the total transfected RNA remained identical in all samples, the expression of Omi alone was blocked by co-transfecting cells with 25 nM Omi siRNA3 and 25 nM of the control RNA. The sequences of the sense strands of the RNAs used in this study were as follows: control RNA, UGUUGUUGUGAGGGGACCGTT; Omi siRNA1, GGGGAGUGUGUGUGUGCCATT; Omi siRNA2, CGAUGCAGCUAUGACUUUU; Omi siRNA3, CAUUAGGAAUUCAAAACAAUAU; caspase-9 siRNA1, CAAUGGGACUAAUCAAAU; and caspase-9 siRNA2, CGAAGACAUUGACAGUAUGU. All siRNAs were synthesized by Dharmacon.

**Survival Assay after Omi RNA Interference**—After transfection with the control or Omi-specific siRNAs, 1 × 103 IEC-18 cells were plated in monolayer either immediately or after being cultured in suspension for various times. Cells were then cultured for 7 days, colonies were counted, and then visualized by Crystal Violet staining and counted.
Omi-induced Apoptosis Assay—5 × 10^5 of IEC-ras or IEC-BclX cells grown in 60-mm dishes were transfected for 4 h with either 2 μg of pEGFP-C1 or 2 μg of Omi-GFP expression vector in the presence of 2 μl of Lipofectamine 2000 (Invitrogen). Cells were further grown for 48 h under standard tissue culture conditions and plated in monolayer and suspension culture for 17 h. Cells were then trypsinized, washed with

**FIGURE 1. Caspase-9 antagonists do not block anoikis of intestinal epithelial cells.** A, IEC-18 cells were cultured being attached (att) or detached (det) from the ECM in suspension culture for the indicated times, cytosolic fractions were obtained from these cells and analyzed for the presence of cytochrome c by Western blot. α-Tubulin was used as a loading control. B, IEC-18 cells were cultured being attached (att) or detached (det) from the ECM for the indicated times, and the cleavage of the LEHD-pNA tetrapeptide, a known substrate of caspase-9, was measured in the respective cell lysates by a colorimetric assay. Results represent the average of two independent experiments plus the S.D. C, IEC-18 cells were transfected with a control nonspecific RNA (cont RNA) or caspase-9-specific siRNA1 (casp-9 siRNA1) and assayed for caspase-9 expression by Western blot. β-Actin was used as a loading control. Cells were subsequently placed in monolayer either immediately or after 24 h of suspension in culture. Colonies formed by the cells that survived after being cultured in suspension were counted 7 days later. Results are expressed as the percentage of the number of colonies obtained after culturing cells in monolayer. D, IEC-18 cells were transfected with a control nonspecific RNA (cont RNA) or caspase-9-specific siRNA2 (casp-9 siRNA2) and assayed for caspase activity in the respective cell lysates by a colorimetric assay. Levels of cleaved caspase-9 were determined by Western blotting. Results represent the average of three independent experiments plus the S.D. E, IEC-18 cells were cultured being attached (att) or detached (det) from the ECM for 24 h and assayed for survival as in C. Cells were subsequently transfected with a control nonspecific RNA (cont RNA) or caspase-9-specific siRNA2 (casp-9 siRNA2) and assayed for caspase activity as in B. Results in F and G represent the average of two independent experiments plus the S.D. H, mouse colonocytes were cultured being attached (att) or detached (det) from the ECM for 17 h in the presence of vehicle (DMSO, dimethyl sulfoxide), 50 μM Z-VAD-fmk (zVAD), or 50 μM Z-LEHD-fmk (zLEHD) and assayed for apoptosis by Cell Death ELISA. Results represent the average of two replicates plus the S.D. This experiment was repeated twice with similar results.
phosphate-buffered saline, re-suspended in phosphate-buffered saline, and morphology of GFP-positive cells was assessed by fluorescent microscopy. Condensed cells were scored as apoptotic.

Drug-induced Apoptosis Assay—10^4 cells were plated in 60-mm dishes and treated with various concentrations of drugs for 24 h. Culture medium was then changed to fresh medium. Cells were allowed to form colonies for 7 days, colonies were visualized by Crystal Violet staining and counted.

Cell Death ELISA—Cells growing in monolayer or in suspension culture were 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.

Soft Agar Colony Formation Assay—This assay was described elsewhere (51).

RESULTS

Caspase-9 Antagonists Do Not Block Anoikis of Intestinal Epithelial Cells—Recent findings indicate that embryonic fibroblasts derived from caspase-9 and Apaf-1 knock-out mice are as prone to die by anoikis as their wild type counterparts (45). Likewise, it has been shown that Z-LEHD-fmk, an established inhibitor of caspase-9 (46), does not block anoikis of human keratinocytes (44). Taken collectively, these data indicate that caspase-9, a target of cytochrome c, is not involved in anoikis of fibroblasts and skin epithelial cells. In search for mitochondrial factor(s) responsible for anoikis of intestinal epithelial cells we decided to test whether caspase-9 plays a causal role in detachment-induced apoptosis of these cells. To this end, we first measured the presence of cytochrome c in the cytosolic extracts of non-malignant anoikis-susceptible rat intestinal epithelial cells, IEC-18 (1, 10), that were cultured in monolayer (attached to the ECM) or suspension (detached from the ECM). We found that detachment of these cells results in the release of cytochrome c into the cytoplasm (Fig. 1A). We further assayed IEC-18 cells for caspase-9 activity by measuring the ability of lysates derived from these cells to cleave LEHD-pNA tetrapeptidyl, a known substrate of caspase-9 (52). As shown in Fig. 1B, detachment triggered transient activation of caspase-9 around 14 h after loss of adhesion to the ECM. It has to be noted here that previously we observed a significant degree of anoikis of these cells as early as 4 h upon detachment (~10 h prior to caspase-9 induction) (13), which indicates that caspase-9 might not play a causal role in this type of apoptosis. To address the role of caspase-9 in anoikis of intestinal epithelial cells in a more definitive way we transfected IEC-18 cells with 2 different caspase-9-specific siRNAs, caspase-9 siRNA1 and caspase-9 siRNA2. We used a published nonspecific RNA (50) as a negative control. As shown in Fig. 1C and D, transfection with each caspase-9-specific siRNA resulted in a significant reduction of caspase-9 levels in IEC-18 cells. Enforced down-regulation of caspase-9, however, did not lead to a significant increase in the viability of these cells after they were cultured in the absence of adhesion to the ECM for 24 h (Fig. 1, C and D) or 48 h (not shown). Conversely, in agreement with what we found before, we observed that IEC-BclX3, a representative clone of several

FIGURE 2. Detachment of intestinal epithelial cells does not result in the release of Smac into the cytoplasm. A, IEC-18 cells were cultured being attached (att) to or detached (det) from the ECM for the indicated times, cytosolic fractions were obtained from these cells and analyzed for the presence of Smac by Western blot. B, Jurkat cells were treated for 7 h with 250 nM staurosporine and assayed for the presence of Smac in the cytoplasm as in A. β-Actin was used as a loading control in A and B.

FIGURE 3. Omi is required for anoikis of intestinal epithelial cells. IEC-18 cells (A) or mouse colonocytes (B) were cultured being attached (att) to or detached (det) from the ECM for the indicated times, cytosolic fractions were obtained from these cells and analyzed for the presence of Omi by Western blot. C, IEC-18 cells were transfected with a control RNA (cont RNA) or Omi-specific siRNA1 (Omi siRNA1) and assayed for Omi expression by Western blot. β-Actin was used as a loading control. Cells were subsequently placed in monolayer either immediately or after 24 h of suspension culture. Colonies formed by the cells that survived after being cultured in suspension were counted 7 days later. D, IEC-18 cells were transfected with a control RNA (cont RNA) or Omi-specific siRNA2 (Omi siRNA2) and Omi siRNA3 (Omi siRNA3) and processed as in C. E, IEC-18 cells were transfected with a control RNA (cont RNA) or Omi-specific siRNA3 (Omi siRNA3) and caspase-9 knock-out mouse (44). Taken collectively, these data indicate that caspase-9, a target of cytochrome c, is not involved in anoikis of fibroblasts and skin epithelial cells. In search for mitochondrial factor(s) responsible for anoikis of intestinal epithelial cells we decided to test whether caspase-9 plays a causal role in detachment-induced apoptosis of these cells. To this end, we first measured the presence of cytochrome c in the cytosolic extracts of non-malignant anoikis-susceptible rat intestinal epithelial cells, IEC-18 (1, 10), that were cultured in monolayer (attached to the ECM) or suspension (detached from the ECM). We found that detachment of these cells results in the release of cytochrome c into the cytoplasm (Fig. 1A). We further assayed IEC-18 cells for caspase-9 activity by measuring the ability of lysates derived from these cells to cleave LEHD-pNA tetrapeptidyl, a known substrate of caspase-9 (52). As shown in Fig. 1B, detachment triggered transient activation of caspase-9 around 14 h after loss of adhesion to the ECM. It has to be noted here that previously we observed a significant degree of anoikis of these cells as early as 4 h upon detachment (~10 h prior to caspase-9 induction) (13), which indicates that caspase-9 might not play a causal role in this type of apoptosis. To address the role of caspase-9 in anoikis of intestinal epithelial cells in a more definitive way we transfected IEC-18 cells with 2 different caspase-9-specific siRNAs, caspase-9 siRNA1 and caspase-9 siRNA2. We used a published nonspecific RNA (50) as a negative control. As shown in Fig. 1, C and D, transfection with each caspase-9-specific siRNA resulted in a significant reduction of caspase-9 levels in IEC-18 cells. Enforced down-regulation of caspase-9, however, did not lead to a significant increase in the viability of these cells after they were cultured in the absence of adhesion to the ECM for 24 h (Fig. 1, C and D) or 48 h (not shown). Conversely, in agreement with what we found before, we observed that IEC-BclX3, a representative clone of several
The Activity of Omi/HtrA2 Is Required for Anoikis of Intestinal Epithelial Cells—We further examined whether Omi/HtrA2 is released into the cytoplasm of intestinal epithelial cells upon loss of adhesion to the ECM. We found that detachment of both IEC-18 cells and mouse colonocytes resulted in a significant release of Omi into the cytoplasm of these cells (Fig. 3, A and B). We then tested whether Omi is required for anoikis. To this end, we transfected IEC-18 cells with 3 different Omi-specific siRNAs, Omi siRNA1, -2, and -3, one of which (Omi siRNA1) previously published clones of IEC-18 cells constitutively expressing exogenous Bcl-XL (13), displayed a significantly higher viability than IEC-18 cells when deprived of adhesion to the ECM for 24 h (Fig. 1E). Thus, our results indicate that detachment-induced apoptosis of IEC-18 cells can be blocked by suppressors of the release of mitochondrial death-inducing factors into the cytoplasm, such as Bcl-XL, but that this inhibition of anoikis is driven by caspase-9-independent mechanisms. In agreement with what was observed for the caspase-9–specific siRNAs, the caspase-9 inhibitor Z-LEHD-fmk did not prevent detachment-induced apoptosis of IEC-18 cells (Fig. 1F). By contrast, Z-VAD-fmk, a broad-spectrum caspase inhibitor, blocked anoikis significantly (Fig. 1F), suggesting that the activity of some caspase(s) that is (are) distinct from caspase-9 is, at least in part, involved in this process. Both Z-LEHD-fmk and Z-VAD-fmk were active in our experimental conditions as these inhibitors efficiently suppressed caspase-9–like activity in the positive control experiments in which IEC-18 cells were treated with adriamycin, an anti-cancer drug that is known to activate caspase-9 in a variety of cell lines (Fig. 1G) (53, 54). Lack of sensitivity to the caspase-9 inhibitor was not a unique property of IEC-18 cells as anoikis of non-malignant mouse colonocytes, another cell line highly susceptible to this type of death (36), could not be suppressed by Z-LEHD-fmk either (Fig. 1H). Based on these data, we concluded that similar to what was observed in fibroblasts and keratinocytes (44, 45), anoikis of intestinal epithelial cells does not require the activity of caspase-9, a target of cytochrome c.

Smac/Diablo Is Not Released into the Cytoplasm of Intestinal Epithelial Cells upon Detachment—We next tested whether Smac/Diablo, another mitochondrial pro-apoptotic factor, is released into the cytoplasm of IEC-18 cells upon detachment from the ECM and did not observe any significant release (Fig. 2A). This was in sharp contrast with what was found for the positive control, human B cell lymphoma Jurkat cells treated with a pro-apoptotic drug staurosporine (Fig. 2B). In the latter case a significant release of Smac into the cytoplasm was observed, indicating that we used appropriate conditions for detecting Smac in the cytoplasm. We thus concluded that Smac is unlikely involved in the induction of anoikis of intestinal epithelial cells.

The Activity of Omi/HtrA2 Is Required for Anoikis of Intestinal Epithelial Cells—We further examined whether Omi/HtrA2 is released into the cytoplasm of intestinal epithelial cells upon loss of adhesion to the ECM. We found that detachment of both IEC-18 cells and mouse colonocytes resulted in a significant release of Omi into the cytoplasm of these cells (Fig. 3, A and B). We then tested whether Omi is required for anoikis. To this end, we transfected IEC-18 cells with 3 different Omi-specific siRNAs, Omi siRNA1, -2, and -3, one of which (Omi siRNA1)
was previously published by others (26, 55). As shown in Fig. 3, C−E, transfection with each Omi-specific siRNA resulted in a noticeable reduction of Omi expression in IEC-18 cells and a significant protection of these cells from anoikis compared with the cells transfected with a control nonspecific RNA. Because transfection with caspase-9-specific siRNAs did not rescue cells from anoikis (Fig. 1, C and D) we used one of these RNAs (caspase-9 siRNA2) as an additional negative control for the Omi siRNA-based experiments. As might have been expected, transfection with a representative Omi-specific siRNA (Omi siRNA3) resulted in a substantial inhibition of Omi expression in IEC-18 cells and a noticeable protection of these cells from anoikis compared with the cells transfected with a caspase-9-specific siRNA (Fig. 3F). Collectively, our data indicate that the activity of Omi is required for anoikis of intestinal epithelial cells.

The effect of loss of Omi expression on the survival of detached cells could not be enhanced by enforced down-regulation of caspase-9 (Fig. 4, A−C), indicating that Omi- and caspase-9-dependent pro-apoptotic mechanisms do not cooperate during the execution of anoikis. We further observed that, unlike the case with anoikis, inhibition of Omi expression in IEC-18 cells resulted in a relatively weak protection of these cells from apoptosis induced by the antimitabolite drug 5-fluorouracil (56) (Fig. 5A). Furthermore, enforced down-regulation of Omi did not lead to a significant protection of these cells from apoptosis triggered by a topoisomerase I inhibitor irinotecan or a non-steroidal anti-inflammatory drug sulindac, two other compounds well known to induce programmed cell death (57, 58) (Fig. 5, B and C). Thus, the ability of Omi to contribute to the execution of apoptosis of intestinal epithelial cells depends on the nature of a death-inducing signal. This ability is clearly pronounced when apoptosis is triggered by loss of cell-ECM adhesion (Fig. 3, C−F).

The Release of Omi into the Cytoplasm of Intestinal Epithelial Cells Is Driven by Detachment-induced Down-regulation of Bcl-XL.—Previously we found that anoikis of intestinal epithelial cells occurs due to detachment-induced down-regulation of an anti-apoptotic protein Bcl-XL (13). This conclusion was based in part on the fact that three independently derived clones of IEC-18 cells, IEC-BclX-3, -11, and -27, in which this down-regulation was inhibited by the constitutive expression of exogenous Bcl-XL, were significantly more resistant to anoikis than the parental cells (13). Bcl-XL is known to be able to block the release of various mitochondrial pro-apoptotic factors into the cytoplasm (21). We, therefore, reasoned that if detachment-induced release of Omi is driven by detachment-dependent inhibition of Bcl-XL expression, then cells, in which this down-regulation was blocked by the expression of exogenous Bcl-XL, should not display the release of Omi into the cytoplasm upon loss of adhesion to the ECM. Indeed, we observed that all three Bcl-XL-overexpressing clones of IEC-18 cells contained significantly lower amounts of cytosolic Omi when cultured in the absence of attachment to the ECM than the parental IEC-18 cells (Fig. 6, A−C).

We further hypothesized that if exogenous Bcl-XL blocks anoikis by suppressing the pro-apoptotic activity of Omi, then the enforced increase in Omi activity should override the protective effect of Bcl-XL on detached cells. It has been shown that one way to achieve a high level of Omi-dependent apoptosis is to express this protein ectopically (17, 18, 26). We, therefore, transiently transfected IEC-BclX-3 cells, one of the clones carrying exogenous Bcl-XL, previously published by us (13), with an expression vector coding for Omi-GFP fusion protein whose various biological characteristics, including the ability to trigger apoptosis, were previously demonstrated to be similar to those of the wild type Omi (26) (Fig. 6D). We then measured the susceptibility of transfected cells to anoikis. As shown in Fig. 6E exogenous Omi triggered a signifi-
Omi Blocks ras-induced Transformation

cant increase of anoikis of IEC-BclX-3 cells compared with the cells transfected with a control vector. Thus, Omi suppressed the ability of Bcl-X<sub>L</sub> to protect cells from detachment-induced apoptosis. In conjunction with the Omi siRNA-based experiments (Fig. 3, C–F), our data indicate that anoikis of intestinal epithelial cells is triggered, at least in part, due to detachment-induced down-regulation of Bcl-X<sub>L</sub> and subsequent release of Omi into the cytoplasm of these cells.

ras Oncogene Blocks the Release of Omi into the Cytoplasm of Detached Intestinal Epithelial Cells—We demonstrated previously that oncogenic Ras strongly blocks anoikis of intestinal epithelial cells (10). As Omi appears to be an important mediator of anoikis we asked whether Ras is capable of blocking the release of this pro-apoptotic protein into the cytoplasm of intestinal epithelial cells upon detachment from the ECM. To this end, we compared the levels of Omi in the cytoplasm of detached IEC-18 cells to those of 3 previously published highly anoikis-resistant clones of these cells ras-3, ras-4, and ras-7 constitutively expressing the oncogenic form of H-ras (1, 13). As shown in Fig. 7, Ras strongly suppressed the release of Omi into the cytoplasm of detached cells.

To demonstrate that the effect of Ras on Omi was not unique to IEC-18 cells we used highly tumorigenic human colorectal carcinoma-derived cells, HCT-116, that carry one copy of oncogenic K-ras, and their poorly tumorigenic derivative Hkh-2, in which the oncogenic K-ras allele was disrupted by homologous recombination (49). We first confirmed that similar to what was previously published, Hkh-2 cells possessed a noticeably lower ability to grow in the absence of adhesion to the ECM as colonies in soft agar (Fig. 8A). This inhibition of anchorage-independent growth caused by ablation of mutant ras was paralleled by an increased susceptibility of ras-deprived cells to apoptosis in the absence of adhesion to the ECM (Fig. 8B). Furthermore, detachment of HCT-116 cells did not result in a significant release of Omi into the cytoplasm, whereas mutant ras knock-out Hkh-2 cells displayed a significant increase in the levels of cytosolic Omi upon loss of adhesion to the ECM (Fig. 8C).

We further hypothesized that if Ras blocks anoikis by suppressing the pro-apoptotic activity of Omi, then the artificial increase in this activity achieved by ectopic expression of Omi should reverse the anti-anoikis effect of Ras. We thus transiently transfected ras-3 cells, one of the previously published clones of IEC-18 cells expressing the ras oncogene (1), with exogenous Omi (Fig. 9A) and observed a significant increase in the susceptibility of these cells to anoikis compared with the cells transfected with a vector control (Fig. 9B). Collectively, our data indicate that Ras blocks anoikis of intestinal epithelial cells, at least in part, by preventing detachment-triggered release of Omi into cellular cytoplasm.

ras-induced Inhibition of the Release of Omi into the Cytoplasm of Intestinal Epithelial Cells Requires ras-dependent Down-regulation of Bak—We established in the past that the anti-anoikis effect of oncogenic Ras is mediated, at least in part, by a mechanism involving ras-induced inhibition of the expression of a pro-apoptotic protein Bak (10). As Bak is known to promote the release of mitochondrial death-inducing factors into the cytoplasm (59), we asked whether the effect of Ras on Bak is required for the ability of Ras to prevent the release of Omi upon the loss of cell-ECM adhesion. To address this question we used two previously published clones of ras-3 cells, Bak-45 and Bak-55, constitutively expressing exogenous Bak (10). These clones express Bak at levels significantly higher then those in ras-3 cells and comparable with those in the parental IEC-18 cells. We first confirmed that in agreement with
what we published before (10), the Bak-overexpressing clones maintained higher susceptibility to death by anoikis than ras-3 cells (Fig. 10A). We then compared the levels of Omi in the cytoplasm of detached ras-3 cells to those of Bak-45 and -55 cells and found that Bak-overexpressing clones display a significant increase in the amount of cytosolic Omi compared with the ras-3 cells (Fig. 10B). Thus, we concluded that ras-dependent inhibition of Bak expression leads to the suppression of the release of Omi into the cytoplasm of intestinal epithelial cells, which in turn results in the blockade of anoikis.

Oncogenic Ras is known to be able to trigger multiple signaling pathways, including those mediated by PI 3-kinase/protein kinase B and Raf/MEK/ERK kinases (12). Previously we demonstrated that inhibition of PI 3-kinase signaling in ras-transformed IEC-18 cells strongly blocks ras-induced resistance to anoikis (10). Conversely, suppression of the activity of MEK had a much weaker impact on this effect of Ras (10). Similar observations were made on ras-transformed kidney and breast epithelial cells by others (60, 61). We found previously that inhibition of PI 3-kinase activity in IEC-18 cells transformed by ras is followed by a significant reversal of ras-induced down-regulation of Bak, whereas suppression of MEK activity did not disrupt the effect of Ras on Bak expression (10). Based on these data, we reasoned that if ras-dependent down-regulation of Bak requires activation of the PI 3-kinase by Ras and if inhibition of Bak expression in turn results in the suppression of the release of Omi upon cell detachment, then inhibition of PI 3-kinase activity should block the effect of Ras on Omi. Indeed, we found that treatment of detached ras-3 cells with the widely used pharmacological inhibitor of PI 3-kinase LY 294002 (10, 60) resulted in a significant increase in the release of Omi into the cytoplasm of these cells (Fig. 10C). Conversely, treatment with an inhibitor of MEK PD 98059 had no effect on the levels of Omi in the cytoplasm of these cells. We further observed that PD 98059 did not enhance LY 294002-induced release of Omi (Fig. 10C). PD 98059, however, was active in our experimental conditions. This conclusion is based on the fact that even though this inhibitor, when used alone, had a relatively weak impact on ras-induced resistance to anoikis, it noticeably enhanced LY 294002-triggered apoptosis of detached ras-3 cells (Fig. 10D). These data suggest that apoptotic events triggered by MEK that are distinct from those leading to the inhibition of Omi release cooperate with molecular changes initiated by PI 3-kinase in blocking anoikis downstream of Ras. Thus, our results are consistent with a scenario, according to which ras-dependent activation of PI 3-kinase leads to the inhibition of Bak expression, subsequent suppression of the release of Omi into the cytoplasm of detached cells and inhibition of anoikis.

We conclude that detachment of non-malignant intestinal epithelial cells results in the down-regulation of Bcl-XL expression, release of Omi into the cytoplasm, and apoptosis. Conversely, in the case of ras-transformed cells the release of Omi and subsequent anoikis are suppressed, at least in part, because of ras-induced down-regulation of Bak.

**DISCUSSION**

We have demonstrated in this study that anoikis of intestinal epithelial cells is mediated by the release of the mitochondrial pro-apoptotic protein Omi/HtrA2 into the cytoplasm, which is caused by detachment-induced inhibition of Bcl-XL expression. We further demonstrated that oncogenic Ras prevents the release of Omi and apoptosis of detached cells by blocking the expression of Bak. These data, to our knowledge for the first time implicate Omi as an important regulator of anoikis in normal and cancer cells.

Our results are consistent with observations made by others, according to which anoikis is caspase-9-independent but can be blocked by exogenous Bcl-XL or Bcl-2. In this regard, the present as well as other studies
demonstrated that even though activation of caspase-9 does occur upon detachment of intestinal epithelial cells (Fig. 1B) and keratinocytes (44), anoikis of these cells cannot be suppressed by caspase-9-specific siRNAs or a caspase-9 inhibitor Z-LEHD-fmk. Likewise, embryonic fibroblasts derived from caspase-9-deficient mice were reported to be as susceptible to anoikis as their wild type counterparts (45). Thus, activation of this caspase appears to be one of several consequences of detachment-induced activation of the pro-apoptotic program rather than a cause of anoikis. On the other hand, anoikis of various types of non-malignant adherent cells, including intestinal epithelial cells, keratinocytes, and fibroblasts, can be inhibited by Bcl-X	extsubscript{l} or Bcl-2 (13, 62, 63). The ability of Bcl-2 to prevent the release of Omi from the mitochondria has been demonstrated in the past (18, 64). As, according to our data, Omi plays a causal role in the induction of anoikis, Bcl-X	extsubscript{l} and Bcl-2 understandably act as efficient inhibitors of this form of apoptosis.

It has to be noted here that independence on caspase-9 activity paralleled by a significant dependence on signaling mediated by various Bcl-2 family members is not a unique feature of anoikis. It has been demonstrated that mature T cells, pro-B cells, or promyeloid cells derived from caspase-9 or Apaf-1 knock-out mice are as sensitive to diverse pro-apoptotic stimuli as their wild type counterparts. Conversely, Bcl-2 was shown to significantly protect these cells against the same apoptotic stimuli (45, 65). Likewise, death of mice injected with an activating anti-Fas antibody was demonstrated to be dependent on the presence of pro-apoptotic Bcl-2 family member Bid, an antagonist of Bcl-X	extsubscript{l} and Bcl-2, but did not require caspase-9 activity (66). Collectively, these data suggest that, at least in certain circumstances, apoptosis can be driven by mitochondria-dependent events, such as translocation of Omi, that are distinct from cytochrome c-induced activation of caspase-9.

We have shown here that detachment of intestinal epithelial cells leads to the release of Omi but not Smac/Diablo into the cytoplasm. These data suggest that detachment-induced translocation of Omi is regulated by mechanisms distinct from those that control the localization of Smac. In this regard, it was previously shown that the release of Smac induced by various stimuli is highly caspase-dependent (67). Based on this, it was proposed that Smac amplifies apoptotic signals downstream of caspase activation, rather than initiates apoptosis. Conversely, the release of Omi was reported to be caspase-independent (26). Therefore, it is conceivable that detachment-induced apoptosis that is mediated by the release of Omi occurs before Smac-dependent amplification of death signals becomes possible.

What pro-apoptotic events could be triggered by Omi after cell detachment? One mechanism, by which this protein can induce cell death, involves neutralization of caspase inhibitors of the IAP family. Previously we found that in intestinal epithelial cells one of the anoikis-inducing pathways is driven by detachment-induced overexpression of Fas ligand and subsequent activation of caspases 8 and 10 (36, 42). Even though these caspases are not the targets of the IAPs (68), they are capable of activating caspases 3 and 7 (69, 70), whose activity can be blocked by the IAPs (68). It is possible that detached intestinal epithelial cells contain levels of IAPs that preclude efficient activation of caspases 3 and 7 by Fas ligand and subsequent anoikis in the absence of Omi release. However, once Omi is released, IAPs could be neutralized. This could in turn cause Fas ligand-dependent caspase activation and apoptosis. Furthermore, in addition to being able to inactivate IAPs, Omi can cause apoptosis via its protease activity in a caspase-independent manner (17, 18), a mechanism that could also contribute to anoikis. Identification of the pro-apoptotic events triggered by Omi after cell detachment represents the subject of our ongoing research.

We showed here that oncogenic Ras blocks anoikis of intestinal epithelial cells by preventing the release of Omi into the cytoplasm and that this effect of Ras requires Ras-induced down-regulation of Bak. These results are in agreement with our previous findings, indicating that inhibition of Bak expression significantly contributes to Ras-dependent suppression of anoikis and induction of in vivo tumorigenicity of ras-transformed intestinal epithelial cells (10). These results also agree with observations, according to which Bak is down-regulated in a high proportion of colorectal cancers (71).

We found previously that, in addition to down-regulating Bak, Ras blocks anoikis of intestinal epithelial cells by preventing detachment-induced down-regulation of Bcl-X	extsubscript{l} (13). As, according to our data, this down-regulation strongly contributes to translocation of Omi into the cytoplasm, it is likely that in addition to the effect on Bak, the effect of Ras on Bcl-X	extsubscript{l} expression also contributes to Ras-triggered suppression of Omi release. We are currently exploring this possibility.

We and others found that Ras blocks anoikis by several mechanisms that involve changes in the expression of Bcl-2 family members, such as Bak, Bcl-X	extsubscript{l}, and possibly Bim (10, 13, 39). Therapeutic targeting of ras-induced anoikis resistance of colorectal cancer cells based on simultaneous reversal of these changes might represent a practically difficult task. However, the known activities of these Bcl-2 family members converge on the regulation of the release of mitochondrial factors into the cytoplasm. We demonstrated here that in the case of anoikis resistance of ras-transformed intestinal epithelial cells Omi is one of these factors. Therefore, delivery of Omi into the cytoplasm of ras-transformed cells or mimicking the consequences of Omi activation by other means, such as neutralization of the IAPs, may represent an efficient strategy for bypassing the effect of Ras on multiple Bcl-2 family members. Perhaps not by coincidence, small molecule and antisense oligonucleotide-based IAP antagonists have recently been demonstrated to block the tumorigenicity and metastatic potential of numerous types of solid tumor cells, including those carrying oncogenic ras (9, 72, 73). These agents are presently being used as lead compounds in pre-clinical and clinical cancer-directed studies. Importantly, some of them have already been shown to trigger anoikis of carcinoma cells (9). Thus, simulation of the effects of Omi on the anoikis machinery of tumor cells may represent a novel strategy for blocking three-dimensional tumor growth.

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