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Oncogenic *ras*-induced Down-regulation of Pro-apoptotic Protease Caspase-2 Is Required for Malignant Transformation of Intestinal Epithelial Cells*

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Byong Hoon Yoo[‡], Yanfei Wang[‡], Mete Erdogan[‡], Takehiko Sasazuki[§], Senji Shirasawa[¶], Laurent Corcos[‖], Kanaga Sabapathy[**], and Kirill V. Rosen[‡1]

*From the [‡]Departments of Pediatrics and Biochemistry and Molecular Biology, Atlantic Research Centre, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada, the [§]Department of Pathology, Research Institute, International Medical Center of Japan, Tokyo 163-8655, Japan, the [¶]Department of Cell Biology, School of Medicine, Fukuoka University, Fukuoka 814-0180, Japan, [‖]INSERM, Faculté de Médecine, Brest 29200, France, and the **Laboratory of Molecular Carcinogenesis, National Cancer Centre, 169610 Singapore*

Background: Many epithelial tumors consist of cells that, unlike normal epithelial cells, survive outside of their original location. This viability is required for tumor growth.
Results: *ras* oncogene promotes survival of cancer cells outside of their original location by down-regulating a cell death-promoting protein caspase-2.
Conclusion: *ras*-induced caspase-2 down-regulation is required for *ras*-driven tumor growth.
Significance: This is a novel mechanism of *ras*-dependent tumor progression.

Resistance of carcinoma cells to anoikis, apoptosis that is normally induced by loss of cell-to-extracellular matrix adhesion, is thought to be essential for the ability of these cells to form primary tumors, invade adjacent tissues, and metastasize to distant organs. Current knowledge about the mechanisms by which cancer cells evade anoikis is far from complete. In an effort to understand these mechanisms, we found that *ras*, a major oncogene, down-regulates protease caspase-2 (which initiates certain steps of the cellular apoptotic program) in malignant human and rat intestinal epithelial cells. This down-regulation could be reversed by inhibition of a protein kinase Mek, a mediator of Ras signaling. We also found that enforced down-regulation of caspase-2 in nonmalignant intestinal epithelial cells by RNA interference protected them from anoikis. Furthermore, the reversal of the effect of Ras on caspase-2 achieved by the expression of exogenous caspase-2 in detached *ras*-transformed intestinal epithelial cells promoted well established apoptotic events, such as the release of the pro-apoptotic mitochondrial factors cytochrome *c* and HtrA2/Omi into the cytoplasm of these cells, significantly enhanced their anoikis susceptibility, and blocked their long term growth in the absence of adhesion to the extracellular matrix. Finally, the blockade of the effect of Ras on caspase-2 substantially suppressed growth of tumors formed by the *ras*-transformed cells in mice. We conclude that *ras*-induced down-regulation of caspase-2 represents a novel mechanism by which oncogenic Ras protects malignant intestinal epithelial cells from anoikis, promotes their anchorage-independent growth, and allows them to form tumors *in vivo*.

Many normal epithelia are organized *in vivo* into cellular monolayers, which are attached to the form of the extracellular matrix (ECM)[2] referred to as basement membrane (BM). Detachment of epithelial cells from the ECM causes their apoptotic death (1, 2), a phenomenon termed anoikis (2). Unlike normal epithelia, carcinomas (cancers derived from epithelial cells) typically represent three-dimensional disorganized multicellular masses in which cell-ECM contacts are significantly changed. It is known in this regard that carcinoma cells typically grow as multilayers and at least some of these cells are detached from the BM. It is also well established that cancer cells often produce BM-degrading enzymes, and this allows tumors to invade adjacent tissues (3). Furthermore, at advanced stages of cancer, cellular aggregates detach from the primary tumor and seed in other organs where they give rise to metastases (4, 5). However, even though carcinoma cells are deprived of normal contacts with the BM during tumor progression, many of these cells do not undergo anoikis (4, 5).

Several lines of evidence support the notion that anoikis resistance represents a critical prerequisite for carcinoma progression. First, cancer cells can typically survive and grow being detached from the ECM as colonies in soft agar. This property represents one of the most stringent criteria for malignant transformations that are presently being used (6, 7). Second, we and others established that activation of oncoproteins, such as Ras (1), EGF receptor (8), and β-catenin (9) or loss of tumor suppressor genes, such as PTEN (10), can block anoikis of cancer cells. Furthermore, we and others found that treatments that reverse anoikis resistance of tumor cells also suppress their ability to form primary tumors (11–15) and metastases (5, 11, 14, 16, 17). In addition, we observed (18) that acquisition of anoikis resistance by carcinoma cells is sufficient for their abil-

* This work was supported by a grant from Canadian Institutes of Health Research.
[1] To whom correspondence should be addressed: Atlantic Research Centre, Room C-302, CRC, Dalhousie University, 5849 University Ave., Halifax, Nova Scotia B3H 4H7, Canada. Tel.: 902-494-7088; Fax: 902-494-1394; E-mail: kirill.rosen@dal.ca.

[2] The abbreviations used are: ECM, extracellular matrix; BM, basement membrane; MEF, mouse embryonic fibroblast; qPCR, quantitative PCR.

ity to grow as primary tumors. Thus, resistance of malignant cells to anoikis represents a major prerequisite for tumor progression (4, 19, 20). Hence, anoikis resistance of cancer cells may serve as a novel therapeutic target. However, molecular mechanisms that control anoikis in normal and cancer cells are only partly understood.

Adherent cells are attached to the ECM via integrin receptors (21). Detachment-induced disengagement of integrins causes changes in the activity of various protein kinases, such as inhibition of c-Src (8) or activation of p38 MAPK (22). These changes alter levels and/or activity of proteins that control cell survival, including proteins composing cellular apoptotic machinery.

One known apoptotic pathway involves the release of mitochondrial molecules such as cytochrome *c*, Smac, and Omi/HtrA2 into the cytoplasm, which results in the activation of cysteine proteases of the caspase family, such as the initiator caspase-9. Once activated, caspase-9 in turn triggers executioner caspases (23–28), which then cleave vital cellular targets and cause apoptosis (13, 29). Caspases can be inhibited by the IAP family members, such as cIAP1, -2, and XIAP (30–32). Upon release from the mitochondria, Smac and Omi inactivate IAPs and trigger caspases (23–27, 33, 34). The release of the mitochondrial factors can be stimulated or blocked by pro- (Bak, Bax, etc.) and anti-apoptotic (Bcl-2, Bcl-X_L, etc.) proteins of the Bcl-2 family, respectively (35). Caspases can also be activated by another pathway that is induced by the activation of death receptors, such as Fas, by their ligands (Fas ligand) (36–39). Death receptors in turn activate initiator caspase-8 and -10, which then trigger the effector caspases (depending on the circumstances, either directly or by promoting the release of mitochondrial factors into the cytoplasm) and thus induce apoptosis.

We found so far that anoikis of intestinal epithelial cells is driven by detachment-induced down-regulation of Bcl-X_L and subsequent release of Omi into the cytoplasm (28). We observed that, in addition, anoikis of these cells is mediated by detachment-dependent p38 MAPK-driven up-regulation of the Fas ligand (22).

Ras is a GTPase that is activated by receptor tyrosine kinases in response to diverse mitogenic signals (40). Activated Ras triggers multiple downstream pathways mediated by signaling molecules, such as Raf, Ral guanine nucleotide exchange factors (RalGEFs), and phosphoinositide 3-OH kinase (40). Some of these events promote changes in the expression of various genes. Ultimately, Ras-induced signaling mechanisms control proliferation, survival, and other critical cellular functions (41). Oncogenic mutations of *ras* often occur in numerous human cancers, including colorectal carcinoma (42, 43).

Oncogenic *ras* is an efficient inhibitor of anoikis (28, 44). According to our studies, Ras blocks anoikis of intestinal epithelial cells by triggering a network of anti-apoptotic signals, rather than by one mechanism. So far, we have been able to identify some of the elements of this network. We have found that Ras blocks anoikis of intestinal epithelial cells by preventing detachment-induced down-regulation of Bcl-X_L (12), by down-regulating Bak (13), and by up-regulating cIAP2 and XIAP (44). Importantly, we established that disruption of the effects of Ras on Bak and Bcl-X_L partially blocked anoikis resistance of *ras*-transformed cells *in vitro* and partly suppressed their tumorigenicity *in vivo* (12, 13).

Whether or not all critical elements of the *ras*-activated network of anti-anoikis signals have been identified is not known. Furthermore, mechanisms linking Ras with those components of this network that have already been identified are understood poorly. Thus, which mediators (or their combination) of the anti-anoikis effect of Ras represent optimal targets for treatment aimed at the suppression of *ras*-driven anoikis resistance of cancer cells remains to be established.

In an effort to further understand the mechanisms allowing Ras to block anoikis, we found that oncogenic Ras down-regulates the initiator caspase-2 in intestinal epithelial cells. Caspase-2 is known to mediate apoptosis triggered by diverse stimuli (45). The mechanisms by which this protease contributes to the execution of cellular apoptotic program are not well understood. It was proposed in this regard that caspase-2 can mediate apoptosis either downstream of the death receptors in complex with an adapter molecule RAIDD or as a part of a complex containing the protein PIDD (45). However, according to the studies based on cells derived from PIDD and RAIDD knock-out mice, activation of this caspase by various stimuli is not affected by complete loss of PIDD and RAIDD (46). Thus, the ability to form complexes with the indicated molecules does not seem to be essential for caspase-2-dependent apoptosis. One property of caspase-2 that many authors agree on is the ability of this caspase to induce cell death by facilitating (via poorly understood mechanisms) mitochondrial outer membrane permeabilization and thus stimulating the release of various mitochondrial factors into the cytoplasm (47–50).

Given that, to our knowledge, the effect of *ras*-induced down-regulation of caspase-2 on anoikis resistance of malignant intestinal epithelial cells has never been studied, we explored the role of this down-regulation in the ability of the *ras* oncogene-carrying cells to resist anoikis. We found that *ras*-dependent reduction of caspase-2 expression in these cells is required for their anoikis resistance and their ability to form tumors *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture—The generation of the IEC clones expressing activated H-*ras* has been described previously (1). Expression of H-*ras* in MT-*ras* cells was induced by adding 100 μM $ZnCl_2$ and 2 μM $CdCl_2$ to cells. Clones of ras-3 cells expressing exogenous caspase-2 were generated using methods that we described previously (51). All IEC clones were cultured in α-minimum essential medium containing 5% fetal bovine serum, 10 μg/ml insulin, and 0.5% glucose. The DLD-1, DKS-8, and DKO-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. For suspension, cultures cells were plated above a layer of 1% sea plaque-agarose polymerized in α-minimum essential medium or Dulbecco's modified Eagle's medium.

Expression Vectors—The expression vector pEGFP-N1 carrying green fluorescent protein (GFP) fused to the C terminus of caspase-2 was used for the transient transfection experiments (52). This vector was kindly provided by Dr. S. Kumar,

Centre for Cancer Biology, Adelaide, Australia. pGL3b expression vector carrying firefly luciferase gene under the control of the fragment of the caspase-2 gene containing the caspase-2 promoter (spanning the DNA fragment located between positions −3970 and −2595 of the caspase-2 gene) was described previously (53). pRL expression vector carrying the *Renilla* luciferase was kindly provided by Dr. P. Lee, Dalhousie University, Halifax, Nova Scotia, Canada. For the generation of clones of ras-3 cells constitutively expressing caspase-2, GFP-tagged caspase-2 cDNA was placed into BamHI/NotI sites of the pcDNA4-TO vector (Invitrogen).

qPCR—Total RNA was isolated by use of the RNeasy Plus mini kit (Qiagen). RNA (3 μg) was subsequently reverse-transcribed by using RNA to cDNA EcoDry kit (Clontech). The resulting cDNA was mixed with Brilliant SYBR Green qPCR master mix (Stratagene) and respective primers. qPCR and respective data analysis were performed as described previously (54) by use of MX3000P instrument under the following conditions. Samples were subjected to a 10-min pre-denaturation at 95 °C and then 40 cycles as follows: 30 s at 95 °C, 1 min at 55 °C, and 30 s at 72 °C, each cycle. Samples were further incubated for 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C for the dissociation curve generation. The data were analyzed by MxPro qPCR software (Stratagene). Primers used to amplify respective rat cDNA were as follows: caspase-2 forward primer, TACTGCTCACAACCCTCTCT, and reverse primer, TATAGGCCACGTAGTGT; 18 S rRNA forward primer, AGTTCGAGTTAAAAAGC, and reverse primer, ACTCAGCAGAGCATCGAG.

Caspase-2 Promoter Activity Assay—Iec-18 and ras-3 cells were co-transfected with an expression vector coding for firefly luciferase under the control of the caspase-2 gene fragment containing the caspase-2 promoter (1 μg) and an expression vector coding for *Renilla* luciferase (0.25 μg) for 24 h as described previously (28). Cells were then lysed, and respective lysates were assayed for luciferase activity by use of Dual-Luciferase report assay kit (Promega). In the experiments where only ras-3 cells were transfected (in case of treatment with Mek1 inhibitor PD98059), the cells were transfected with 1 μg of caspase-2 promoter-containing expression vector as indicated above for 24 h, after which cells were further cultured for an additional 24 h in the presence of 25 μM PD98059 and assayed for luciferase activity as described above.

Transient Transfection of ras-2 Cells with Caspase-2 Expression Vector—Transient transfection of ras-3 cells with a GFP-caspase-2 expression vector was performed as described previously (28).

Western Blot Analysis—Western blot analysis was performed as described elsewhere (44). The following antibodies were used in this study: anti-caspase-2 (Santa Cruz Biotechnology in Figs. 1, 2, and 6 and Alexis in Figs. 3 and 4); anti-α-tubulin (Upstate); anti-CDK4 (Santa Cruz Biotechnology); anti-β-actin (Sigma); anti-p38 MAPK (Santa Cruz Biotechnology); anti-GAPDH (Sigma), anti-cytochrome *c* (Cell Signaling), and anti-HtrA2/Omi (R & D Systems). When lanes were removed from Western blot images and separate parts of an image were joined together, a short vertical black line was used to indicate where the image was cut.

RNA Interference—All transfections with siRNAs were performed by using Lipofectamine 2000 (Invitrogen) as described previously (44). The sequences of the sense strands of the RNAs used in this study were as follows: control RNA (siCONTROL nontargeting siRNA-1, Dharmacon, UAGCGACUAAACACAUCAAUU; caspase-2 siRNA-1, GCACUUCACUGGAGAGAAAUU; caspase-2 siRNA-2, UCACAACCCUCUCUGAUAUUU; FADD siRNA-1, GGAAAAGACUGGCCCGUGA; FADD siRNA-2, GGGAUUCAACUGUGUCUUU. All RNAs were from Dharmacon.

Gene Expression Array—The expression of mRNAs coding for regulators of apoptosis was assayed by the rat-specific array carrying respective cDNAs (SuperArray) according to manufacturer's instructions. Signals on the array were detected by ECL. The intensity of each signal was quantified by densitometry as described previously (44).

Isolation of GFP-positive Cells by Flow Cytometry—Cells were trypsinized and washed with and resuspended in a phosphate-buffered saline buffer containing 25 mM Hepes, 1% BSA, and 1 mM EDTA). FACSAria (BD Biosciences) instrument was used for the isolation of GFP-positive cells.

Analysis of Apoptosis by Flow Cytometry—Apoptosis detection kit from Chemicon was used in the assay. Cells were harvested, washed with PBS, and resuspended in binding buffer provided by the manufacturer at a concentration of 10^6 cells/ml. 200 μl of cell suspension was then mixed with 4 μl of annexin V conjugated to allophycocyanin and 2 μl of propidium iodide (20 μg/ml), and the resulting mixture was incubated for 15 min at room temperature. FACSCalibur system (BD Biosciences) was used for the analysis. AnnexinV-positive propidium iodide-negative cells were considered apoptotic.

The following assays were performed as we described previously: measurement of the ability of cells to form colonies in monolayer after being cultured in suspension (55), soft agar growth assay (13), and *in vivo* tumorigenicity assay (12).

Preparation of Cytosolic Fraction—Preparation of cytosolic fraction was performed as described by us and others (28, 56).

Statistical Analysis—Two-tailed Student's *t* test was used for assessing statistical significance of data.

RESULTS

Oncogenic Ras Blocks Caspase-2 Expression in Intestinal Epithelial Cells—In an effort to understand the mechanisms by which oncogenic Ras blocks anoikis, we compared the levels of mRNAs coding for 97 apoptosis regulators in the detached, spontaneously immortalized, nonmalignant, and highly anoikis-susceptible intestinal epithelial cells, IEC-18, and a previously published anoikis-resistant tumorigenic clone of these cells, ras-3 (1, 12), constitutively expressing oncogenic H-*ras* by using the array carrying respective cDNAs. One *ras*-induced change observed by us was the down-regulation of the mRNA coding for the pro-apoptotic protein caspase-2 (Fig. 1*A*).

Caspase-2 is an initiator caspase that mediates the execution of apoptosis through poorly understood mechanisms (45). This protease has been recently proposed to be able to act as a tumor suppressor in various contexts (45). Because the role of *ras*-dependent down-regulation of caspase-2 in the control of anoikis of intestinal epithelial cells by oncogenic Ras, to our knowledge,

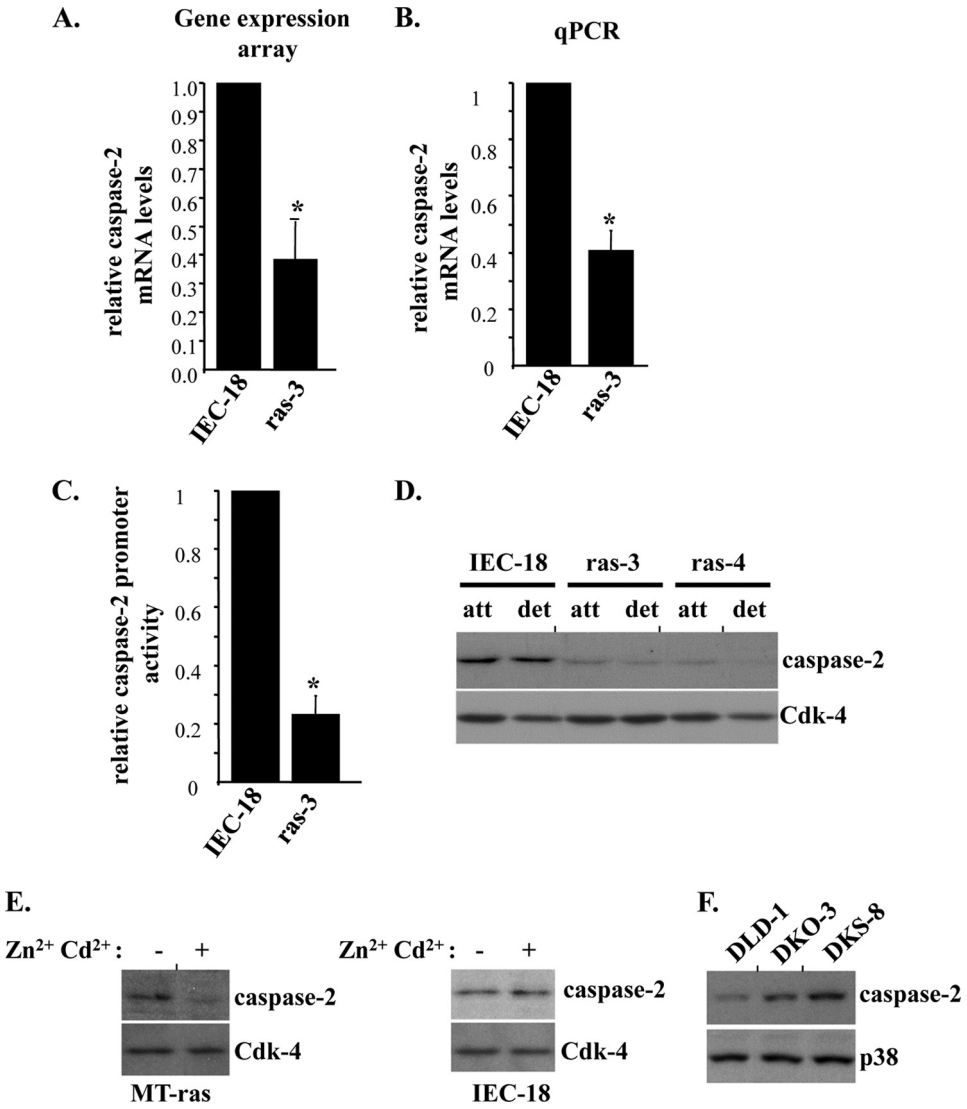

FIGURE 1. **Oncogenic *ras* down-regulates caspase-2 in intestinal epithelial cells.** *A*, IEC-18 and an H-*ras*-transformed clone of these ras-3 cells were cultured detached from the ECM for 24 h, and the expression of caspase-2 mRNA (along with other regulators of apoptosis) was analyzed in these cells by use of the rat-specific array carrying respective cDNAs (SuperArray). Signals on the array were detected by ECL. The intensity of each signal was quantified by densitometry and normalized by the levels of Ube2i mRNA that served as a loading control. The data represent the average of two independent experiments plus the S.D. *B*, indicated cell lines were analyzed for caspase-2 mRNA expression by qPCR. The observed caspase-2 mRNA levels were normalized by the levels of 18 S rRNA that were also determined by qPCR. The resulting levels of caspase-2 mRNA in IEC-18 cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *C*, indicated cell lines were transiently transfected with expression vector coding for firefly luciferase under the control of the caspase-2 gene fragment containing caspase-2 promoter and an expression vector coding for *Renilla* luciferase. The intensity of respective signals corresponding to firefly luciferase activity was normalized by those of *Renilla* luciferase. The resulting numbers obtained for IEC-18 cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. The values observed for IEC-18 cells and marked with an *asterisk* in *A–C* were significantly ($p < 0.05$) different from those observed for ras-3 cells. *D*, IEC-18 cells and two independently derived H-*ras*-transformed clones of these ras-3 and ras-4 cells were cultured attached (*att*) to and detached (*det*) from the ECM for 24 h and assayed for the expression of caspase-2 by Western blot. *E*, attached MT-*ras* cells (*left panel*) or IEC-18 cells (*right panel*) were cultured in the absence (−) and in the presence (+) of 100 μM Zn^{2+} and 2 μM Cd^{2+} for 24 h (*left panel*) and assayed for the expression of caspase-2 by Western blot. *F*, attached human colorectal carcinoma cells DLD-1 and their K-*ras* knock-out derivatives DKO-3 and DKS-8 were assayed for the expression of caspase-2 by Western blot. The membranes in *D* and *E* were re-probed with a CDK-4 antibody and the membranes in *F* with an anti-p38 MAPK as loading controls.

has never been investigated, we decided to explore this role in this study.

We confirmed by qPCR that caspase-2 mRNA levels are significantly lower in ras-3 cells than in IEC-18 cells (Fig. 1*B*). We further found that ras-3 cells transfected with an expression vector carrying a luciferase gene under the control of a previously characterized (53) fragment of a caspase-2 gene containing a caspase-2 promoter (spanning the DNA fragment located between positions −3970 and −2595 of the caspase-2 gene) (53) displayed a noticeably lower luciferase activity than the parental IEC-18 cells transfected with the same vector (Fig. 1*C*). Collectively, the data presented above (Fig. 1, *A–C*) indicate that Ras blocks transcription of the caspase-2 gene in the intestinal epithelial cells.

We further observed that caspase-2 expression is significantly lower at the protein level in ras-3 and ras-4 (another published anoikis-resistant tumorigenic clone of IEC-18 cells) cells (1, 12) compared with the parental IEC-18 cells regardless of whether these cells were attached to or detached from the ECM (Fig. 1*D*). To confirm that the down-regulation of

caspase-2 represents a direct consequence of the presence of oncogenic *ras* in IEC-18 cells, we utilized a published clone of IEC-18 cells MT-*ras* that harbors exogenous activated mutant of H-*ras* under the control of Zn^{2+}- and Cd^{2+}-inducible metallothionein promoter (57). We found that treatment of MT-*ras* cells with Zn^{2+} and Cd^{2+} results in a significant inhibition of caspase-2 expression (Fig. 1*E*, *left*). By contrast, treatment of the parental IEC-18 cells with the indicated metal ions did not cause any down-regulation of caspase-2 (Fig. 1*E*, *right panel*).

To establish whether Ras can promote caspase-2 down-regulation in human colon cancer cells, we utilized highly tumorigenic human colon carcinoma-derived cells DLD-1 carrying one allele of oncogenic K-*ras* and derivatives of these cells DKO-3 and DKS-8, in which the mutant K-*ras* allele had been disrupted by homologous recombination (58). We and others found previously that both oncogenic K-*ras*-deprived variants of DLD-1 cells are significantly more anoikis-susceptible (12) and much less tumorigenic (58) than oncogenic *ras*-harboring DLD-1 cells. As shown in Fig. 1*F*, we observed that DLD-1 cells carry much lower amounts of caspase-2 than the mutant K-*ras*-knock-out cells DKS-8 and DKO-3. Thus, *ras* oncogene down-regulates caspase-2 in malignant intestinal epithelial cells.

Caspase-2 Contributes to Execution of Anoikis of Intestinal Epithelial Cells—To test whether caspase-2 plays a role in the execution of anoikis of intestinal epithelial cells, we ablated this caspase in IEC-18 cells by using two separate small interfering RNAs (siRNAs) targeted to different regions of caspase-2 mRNA (Fig. 2, *A* and *D*). To assess the effect of enforced caspase-2 down-regulation on the viability of detached cells, we used a clonogenic cell survival assay that we often utilized in the past for measuring anoikis (28, 55). In the course of the assay cells transfected with control or caspase-2-specific siRNAs were cultured detached from the ECM, re-plated in a monolayer, cells that remained viable after detachment were allowed to form colonies, and the resulting colonies were counted. We found that loss of caspase-2 significantly increases the viability of IEC-18 cells following detachment from the ECM (Fig. 2, *B* and *E*). We further observed that enforced caspase-2 down-regulation noticeably reduces the ability of detached IEC-18 cells to bind annexin V (Fig. 2, *C* and *F*) (this ability is one of the well established hallmarks of apoptosis (59)). Thus, caspase-2 contributes to the execution of anoikis of nonmalignant intestinal epithelial cells.

Ras-induced Down-regulation of Caspase-2 Is Required for Anoikis Resistance of Ras-transformed Intestinal Epithelial Cells—To address the role of *ras*-induced down-regulation of caspase-2 in anoikis resistance of *ras*-transformed intestinal epithelial cells, we decided to reverse this down-regulation. To this end, we transiently transfected ras-3 (an oncogenic *ras*-expressing derivative of IEC-18 cells, see Fig. 1) with GFP-tagged caspase-2 (others established that caspase-2 remains active in the presence of the GFP tag (52)). We then isolated GFP-positive ras-3 cells by flow cytometry and confirmed that the total amount of caspase-2 in the resulting cells is significantly higher than that in the cells transfected with a control vector carrying GFP alone and does not exceed that in the parental IEC-18 cells (Fig. 3*A*). Thus, the subsequently observed effects of caspase-2 on apoptosis of ras-3 cells were

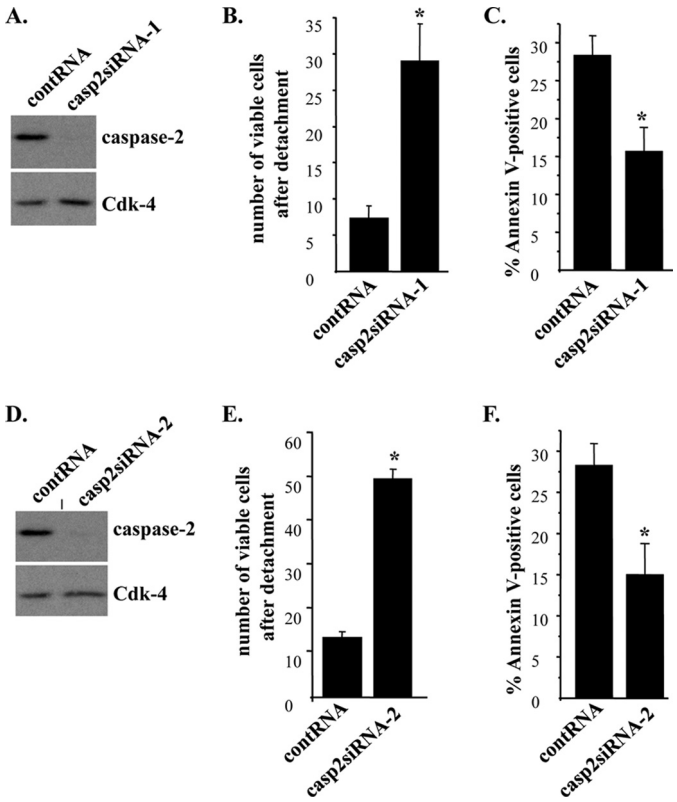

FIGURE 2. **Caspase-2 is required for anoikis of intestinal epithelial cells.** *A* and *D*, IEC-18 cells were transfected with a control RNA (*contRNA*) or caspase-2-specific siRNA1 (*casp2siRNA-1*, *A*) or caspase-2-specific siRNA2 (*casp2siRNA-2*, *D*) and assayed for caspase-2 expression by Western blot. CDK-4 was used as a loading control. *B* and *E*, cells were subsequently placed in suspension for 24 h and then re-plated in monolayer. The number of viable cells after detachment was calculated as a number of colonies formed 7 days later by the cells that survived after being cultured in suspension. The data represent the average of the triplicates plus the S.D. This experiment was repeated twice with similar results. *C* and *F*, cells transfected as in *A* and *D*, respectively, were placed in suspension for 24 h and assayed for annexin V binding by flow cytometry. The data represent the average of three experiments plus the S.E. The values marked with an *asterisk* were significantly ($p < 0.05$) different from those derived from the respective control experiments.

not due to the presence of abnormally high amounts of this caspase in the indicated cells. We then tested the ability of GFP- and caspase-2-GFP-transfected cells to bind annexin V and found that exogenous caspase-2 significantly increases the susceptibility of detached (Fig. 3*C*) but not that of the attached (Fig. 3*B*) ras-3 cells to apoptosis.

The ability of cells to grow in the absence of adhesion to the ECM as colonies in soft agar represents one of the most stringent criteria for malignant transformation that are presently being used (6, 7). We thus tested whether the reversal of the effect of oncogenic Ras on caspase-2 blocks the long term growth of the *ras* transformed in soft agar. Because transient transfection of ras-3 cells with a caspase-2 expression vector was not optimal for these type of studies, we generated three clones of ras-3 cells, ras-casp2-2, ras-casp2-3, and ras-casp2-4, expressing ectopic caspase-2 in a constitutive manner. Again, we found that the total amount of caspase-2 in each of these clones was significantly higher than that in a vector control clone (*ras*-control) but did not exceed that in the parental IEC-18 cells (Fig. 4*A*). We further observed that exogenous caspase-2 had a relatively small effect on the ability of attached

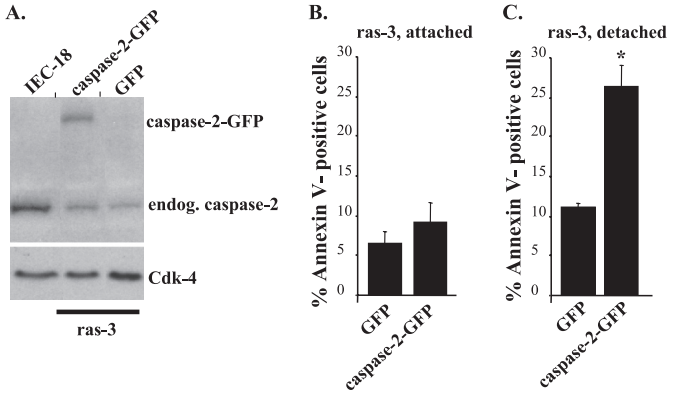

FIGURE 3. ***ras*-induced down-regulation of caspase-2 is required for anoikis resistance of malignant intestinal epithelial cells.** *A*, ras-3 cells were transfected either with a control vector carrying GFP alone or with an expression vector carrying GFP-tagged caspase-2 and assayed for caspase-2 expression along with the parental IEC-18 cells by Western blot. A caspase-2-specific antibody was used in the assay. The positions of endogenous (*endog.*) caspase-2 and exogenous GFP-tagged caspase-2 (*caspase-2-GFP*) on the gel are indicated. CDK-4 was used as a loading control. *B* and *C*, cells processed as in *A* were cultured attached to (*B*) detached from (*C*) the ECM for 48 h and analyzed for annexin V binding by flow cytometry. The data represent the average of three (*B*) and two (*C*) independent experiments plus the S.D. The value marked with an *asterisk* was significantly ($p < 0.05$) higher than that derived from the respective control experiments.

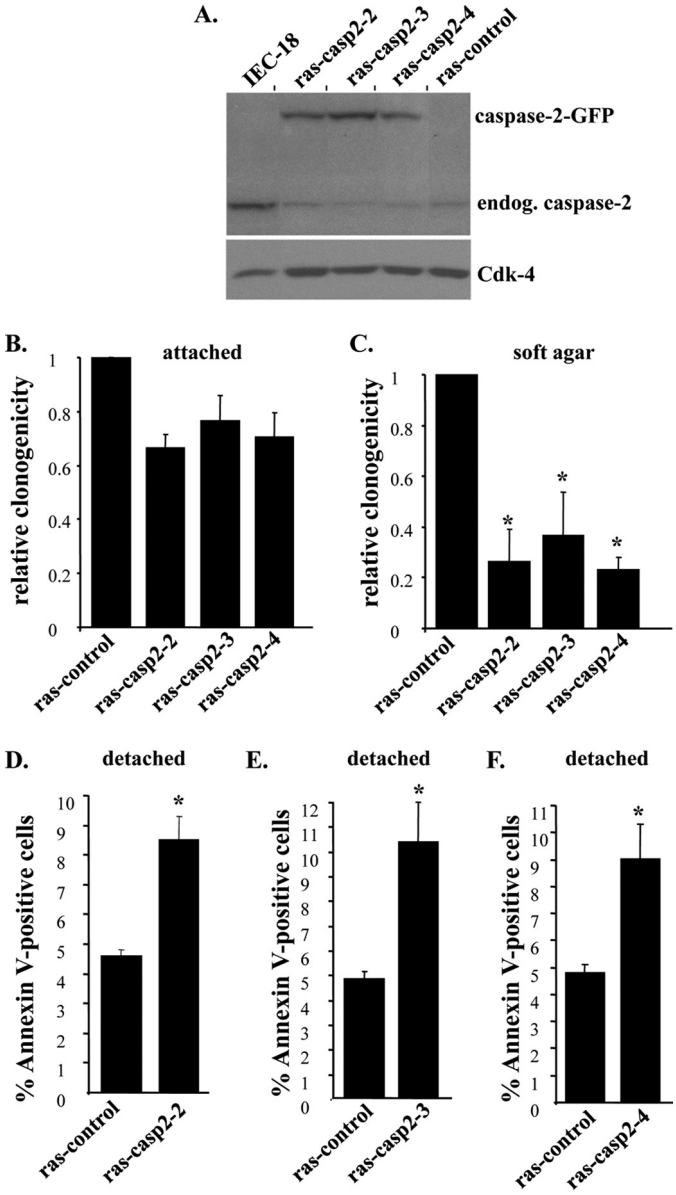

FIGURE 4. ***ras*-induced down-regulation of caspase-2 is required for the ability of malignant intestinal epithelial cells to grow in an anchorage-independent manner.** *A*, IEC-18 cells, clones of ras-3 cells ras-casp2-2, ras-casp2-3, and ras-casp2-4 (generated by transfection of ras-3 cells with a caspase-2-GFP expression vector), expressing ectopic caspase-2 in a constitutive manner and a control clone of ras-3 cells (generated by transfection of ras-3 cells with a control vector) were assayed for caspase-2 expression by Western blot. A caspase-2-specific antibody was used in the assay. The positions of endogenous (*endog.*) caspase-2 and exogenous GFP-tagged caspase-2 (*caspase-2-GFP*) on the gel are indicated. CDK-4 was used as a loading control. *B* and *C*, indicated cell lines were plated in monolayer (*B*) or in soft agar (*C*), and colonies formed by these cells were counted 7–10 days later. The number of colonies formed by the *ras*-control cells was arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *D--F*, indicated cell lines were assayed for annexin V binding by flow cytometry. The data in *D* represent the average of two independent experiments plus the S.D. The data in *E* and *F* represent the average of three independent experiments plus the S.E. Values marked with an *asterisk* were significantly ($p < 0.05$) different from those derived from the respective control experiments.

cells to form colonies (Fig. 4*B*) but noticeably blocked their clonogenicity in soft agar, when these cells were detached from the ECM (Fig. 4*C*). As expected, ectopic caspase-2, when expressed in ras-3 cells in a constitutive manner, also significantly increased their ability to bind annexin V following detachment (Fig. 4, *D–F*). Based on the data presented above, we concluded that *ras*-induced down-regulation of caspase-2 is required for the ability of oncogenic Ras to protect intestinal epithelial cells from anoikis.

We found previously that one of the mechanisms of anoikis of intestinal epithelial cells, including IEC-18 cells, is mediated by the Fas ligand, a pro-apoptotic protein that exerts its effect on cells via an adapter molecule FADD (22). We observed in this study that ablation of FADD (Fig. 5*A*) in ras-casp2-4 cells (a clone of ras-3 cells expressing ectopic caspase-2, see Fig. 4) did not block their apoptosis following detachment (Fig. 5, *B* and *C*). Thus, the pro-apoptotic signaling pathway driven by Fas ligand and FADD does not appear to be required for the ability of caspase-2 to induce anoikis of *ras*-transformed intestinal epithelial cells.

Ras-induced Down-regulation of Caspase-2 Prevents the Release of Mitochondrial Apoptosis-inducing Proteins into the Cytoplasm of Intestinal Epithelial Cells following Detachment—Molecular events involved in caspase-2-initiated apoptosis are not well understood (45). It is, however, thought that one way by which this initiator caspase promotes cell death is via facilitating (through yet unknown mechanisms) the release of the pro-apoptotic mitochondrial factors into the cytoplasm, where they activate various elements of the cellular pro-apoptotic program (47–50). We found previously in this regard that detachment of intestinal epithelial cells (including IEC-18 cells) promotes the release of the mitochondrial proteins, such as cytochrome *c* and Omi/HtrA2 in the cytoplasm and that oncogenic Ras inhibits these events (28). Furthermore, we demonstrated that Ras-induced inhibition of detachment-induced release of the mitochondrial proteins, such as Omi, is required for the ability of oncogenic Ras to suppress anoikis (28). We thus tested whether the reversal of *ras*-induced down-regula-

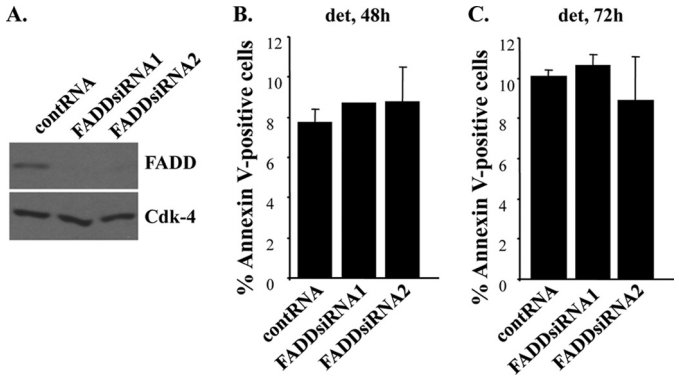

FIGURE 5. **Apoptosis of detached (*det*) *ras*-transformed intestinal epithelial cells expressing exogenous caspase-2 cannot be blocked by the ablation of FADD.** ras-casp2-4 cells (a clone of ras-3 cells expressing exogenous caspase-2) were transfected with a control RNA (*contRNA*) or FADD-specific siRNA1 (*FADDsiRNA-1*) or FADD-specific siRNA2 (*FADDsiRNA-2*) and assayed for FADD expression by Western blot. CDK-4 was used as a loading control. Cells transfected as in *A* were placed in suspension for 48 h (*B*) or 72 h (*C*) and assayed for annexin V binding by flow cytometry. The data represent the average of two independent experiments plus the S.D.

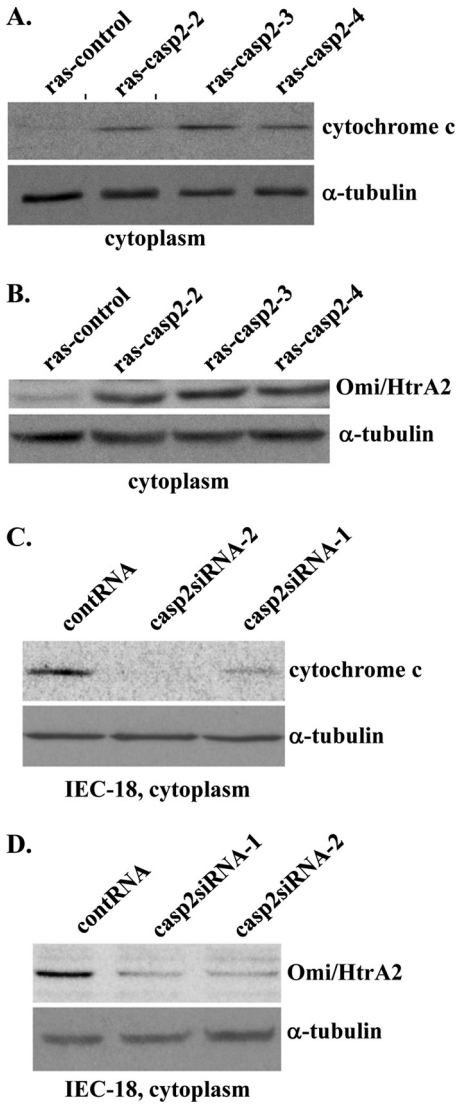

FIGURE 6. ***ras*-induced down-regulation of caspase-2 prevents the release of cytochrome *c* and Omi/HtrA2 into the cytoplasm following detachment of intestinal epithelia cells.** *A* and *B,* indicated cell lines were cultured in suspension for 24 h, and cytosolic material was isolated from these cells and assayed for the presence of cytochrome *c* (*A*) and Omi (*B*) by Western blot. *C* and *D,* IEC-18 cells were transfected as in Fig. 2 with a control RNA (*contRNA*) or caspase-2-specific siRNA1 (*casp2siRNA-1*) or caspase-2-specific siRNA2 (*casp2siRNA-2*) and cultured in suspension for 2 h; cytosolic material was isolated from these cells and assayed for the presence of cytochrome *c* (*C*) and Omi (*D*) by Western blot. α-Tubulin was used as a loading control.

tion of caspase-2 contributes to the ability of Ras to block the release of cytochrome *c* and Omi into the cytoplasm of detached cells. As shown in Fig. 6, *A* and *B*, detached cells ras-casp2-2, ras-casp2-3, and ras-casp2-4 expressing ectopic caspase-2 displayed significantly higher amounts of both cytochrome *c* and Omi in the cytoplasm than the respective control clone *ras*-control. In agreement with a notion that caspase-2 can mediate the release of the indicated pro-apoptotic factors in detached cells, we found that ablation of caspase-2 in the parental IEC-18 cells by RNAi (see Fig. 2) blocks such release (Fig. 6, *C* and *D*). In summary, our data indicate that *ras*-induced down-regulation of caspase-2 prevents the release of cytochrome *c* and Omi into the cytoplasm of intestinal epithelial cells following their detachment.

Activity of Protein Kinase Mek Is Required for Ras-induced Down-regulation of Caspase-2 in Intestinal Epithelial Cells—Ras is known to be able to activate numerous signaling pathways, including those mediated by the sequential induction of protein kinases Raf, Mek, and Erk (40). In an effort to identify the signaling mechanism by which Ras down-regulates caspase-2 in intestinal epithelial cells, we found that treatment of ras-3 cells with PD98059, a specific and widely used small molecule inhibitor of Mek (60), resulted in a significant increase of the caspase-2 promoter activity (Fig. 7*A*), a noticeable up-regulation of caspase-2 mRNA (Fig. 7*B*), and that of caspase-2 protein (Fig. 7*C*). By contrast, treatment with LY294002, an inhibitor of phosphoinositide 3-OH kinase (61), another major mediator of Ras signaling (40), did not trigger caspase-2 protein up-regulation in these cells (data not shown). We reasoned that if the effect of Ras on caspase-2 is mediated by Mek and if this effect contributes to *ras*-induced anoikis resistance of intestinal epithelial cells, then inhibitors of Mek, such as PD98059, should promote anoikis of *ras*-transformed cells. Indeed, we found that treatment with PD98059 did not result in significant apoptosis of attached ras-3 cells but caused a noticeable increase of death of these cells when they were detached from the ECM (Fig. 7*D*). Collectively, these data are consistent with a scenario, according to which *ras*-induced down-regulation of caspase-2 is mediated by Mek.

Ras-induced Down-regulation of Caspase-2 Is Required for the Ability of Ras-transformed Intestinal Epithelial Cells to Form Tumors in Vivo—Normal intestinal epithelium exists *in vivo* as a single layer, whereas primary tumors as well as tumors formed by cancer cells subcutaneously injected in mice, a model that is often used for studying tumorigenesis, tend to grow as three-dimensional masses. The results of several studies, including ours, indicate that anoikis resistance of cancer cells is required for the ability of these cells to form tumors following subcutaneous injection in mice (11–15). Given that *ras*-induced down-regulation of caspase-2 is required for the ability *ras*-transformed cells to resist anoikis and grow without

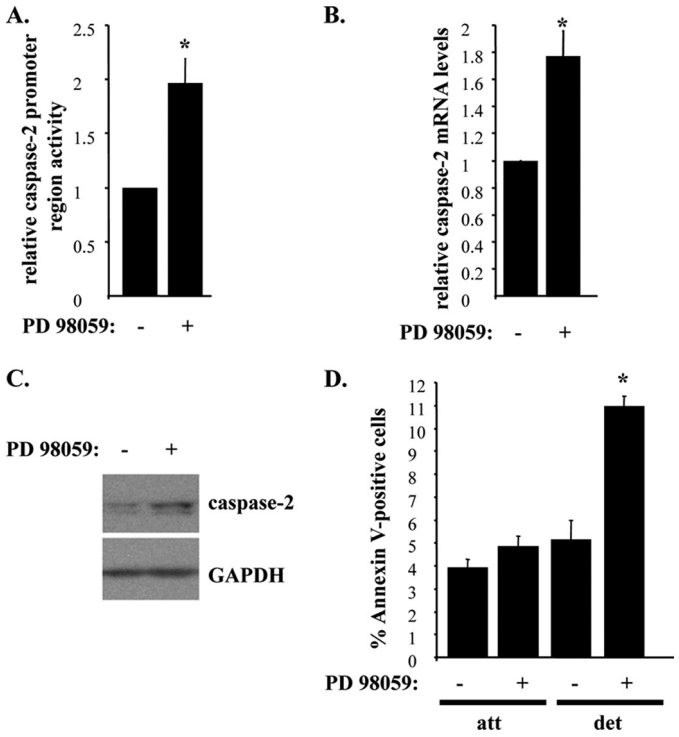

FIGURE 7. **Activity of protein kinase Mek is required for *ras*-induced down-regulation of caspase-2 in intestinal epithelial cells.** *A,* cells were transiently transfected with expression vector coding for firefly luciferase under the control of the caspase-2 gene fragment containing caspase-2 promoter, treated with either DMSO (−) or 25 μM PD98059 (+) for 24 h, and assayed for luciferase activity. The resulting numbers obtained for the untreated cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *B,* ras-3 cells were treated with PD98059 as in *A* and caspase-2 mRNA levels were measured in these cells by qPCR. The observed caspase-2 mRNA levels were normalized by the levels of 18 S rRNA, which were also determined by qPCR. The resulting levels of caspase-2 mRNA in DMSO-treated cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *C,* cells treated with PD98059 as in *A* were assayed for caspase-2 expression by Western blot. GAPDH was used as a loading control. *D,* cells were cultured in monolayer or suspension for 24 in the presence of either DMSO (−) or 25 μM PD98059 (+) and analyzed for annexin V binding by flow cytometry. The data represent the average of two independent experiments plus the S.D. The values marked with an *asterisk* were significantly ($p < 0.05$) different from those derived from respective control experiments.

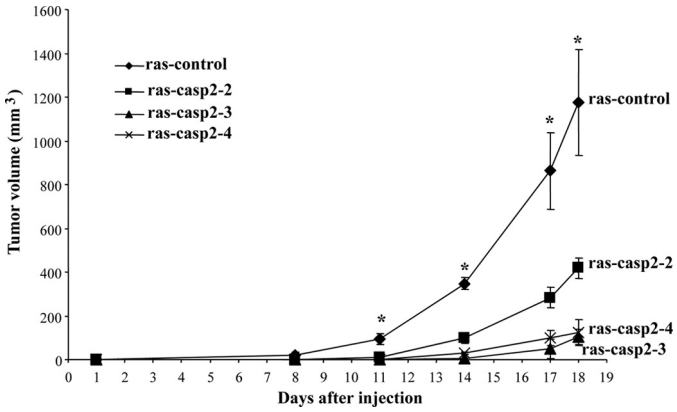

FIGURE 8. ***ras*-induced down-regulation of caspase-2 is required for the ability of malignant intestinal epithelial cells to form tumors in mice.** The indicated cell lines were injected subcutaneously into nude mice, and tumor volumes were measured at the indicated time points. Four mice were injected with each cell line. *Error bars* represent the S.E. This experiment was repeated twice with similar results. *Asterisks* indicate that values derived from the control experiments with *ras*-control cells were significantly ($p < 0.05$) higher than those observed for respective experiments with ras-casp2-2, ras-casp2-3, and ras-casp2-4 cells.

being attached to the ECM, we decided to test whether this down-regulation contributes to the ability of the indicated cells to form tumors in mice. As shown in Fig. 8, the sizes of tumors formed by cells ras-casp2-2, ras-casp2-3, and ras-casp2-4 expressing ectopic caspase-2 were significantly smaller at all times of the assay than those of tumors formed by respective control cells. Thus, *ras*-induced down-regulation of caspase-2 does contribute to the *in vivo* tumorigenicity of these cells.

In summary, we have identified a novel mechanism by which oncogenic Ras blocks anoikis of intestinal epithelial cells, allows them to grow in an anchorage-independent manner within three-dimensional multicellular masses, and enables them to form tumors. This mechanism is driven by *ras*-induced down-regulation of caspase-2.

DISCUSSION

We have identified in this study a novel mechanism by which oncogenic Ras promotes anoikis resistance of intestinal epithelial cells. This mechanism involves *ras*-dependent down-regulation of caspase-2. We found previously that anoikis of non-malignant intestinal epithelial cells is driven by detachment-induced down-regulation of Bcl-X_L (12) and subsequent release of the mitochondrial factors, such as HtrA2/Omi, into the cytoplasm (28). We have demonstrated in this study that, in addition, anoikis of these cells is mediated (via mechanisms that remain to be established) by caspase-2, a protease that according to this study (Fig. 6) and previous studies (47–50) does have the ability to increase the permeability of the mitochondria to the pro-apoptotic factors. This study as well as our previous studies indicate that oncogenic Ras has the ability to block this network of pro-anoikis signals in detached cells by activating a network of the anti-anoikis signals. We found in the past that two important elements of this network are the mechanisms involving *ras*-induced down-regulation of Bak (13) and inhibition of detachment-induced down-regulation of Bcl-X_L (12). We show here that one additional mechanism by which Ras prevents the release of cytotoxic factors, such as cytochrome *c* and Omi from the mitochondria, is driven by *ras*-dependent down-regulation of caspase-2.

Our data suggest that Ras down-regulates caspase-2 in intestinal epithelial cells by triggering a protein kinase Mek, an inducer of the MAPKs and a mediator of Ras signaling whose activity is known to be stimulated by a Ras binding partner protein kinase Raf (40). To our knowledge, this study for the first time demonstrates that the indicated signaling pathway can block anoikis downstream of Ras in intestinal epithelial cells. Of note, we observed in the past that *ras*-induced down-regulation of Bak, the second mechanism by which oncogenic Ras blocks the release of the pro-apoptotic mitochondrial factors in these cells and their subsequent anoikis, is driven by phosphoinositide 3-OH kinase (13), which represents another important mediator of Ras signaling (40). Therefore, it is the activation of both major Ras-induced signaling pathways, one mediated by Mek and another one controlled by phosphoinositide 3-OH kinase, that contributes to the indicated effects of Ras in intestinal epithelial cells.

Interestingly, we have found that exogenous caspase-2 promoted a much more noticeable apoptosis of the *ras*-transformed cells when they were detached from the ECM than in the attached cells (see Figs. 3 and 4). These data suggest that a threshold caspase-2 concentration is required for the induction of apoptosis by signals that are induced by loss of adhesion of intestinal epithelial cells to the ECM. However, it seems likely that when caspase-2 is down-regulated in these cells (*e.g.* in response to the expression of oncogenic *ras*) signals that are induced by detachment of the indicated cells become by themselves insufficient for stimulating apoptosis.

We found in this study that *ras*-induced down-regulation of caspase-2 is required for the ability of *ras*-transformed intestinal epithelial cells to form tumors *in vivo*. These findings agree well with what is known about the role of anoikis resistance of cancer in growth of tumors formed by malignant cells. It has been well established in this regard that normal intestinal epithelium exists *in vivo* as a single layer, whereas primary human tumors as well as tumors formed by cancer cells that have been subcutaneously injected into mice, a model that we have used in this study, typically form three-dimensional masses. Perhaps not by coincidence, the ability of cancer cells to grow in a three-dimensional anchorage-independent manner in soft agar has served as a "gold standard" for malignant transformation for several decades (6), and cells that are capable of this growth can usually form subcutaneous tumors (7, 12). Moreover, treatment that blocks resistance of cancer cells to detachment-induced death is known to inhibit their ability to form such tumors (11–14). We found in the past, for example, that the reversal of *ras*-induced down-regulation of Bak (13) or the ablation of Bcl-X_L (12) in the *ras*-transformed intestinal epithelial cells enhances their susceptibility to detachment-induced death and blocks their ability to form subcutaneous tumors in mice. In addition, we found that variants of the poorly tumorigenic intestinal epithelial cells selected for increased anoikis resistance acquire the capacity for forming such tumors (18). The results of this study indicate that the reversal of *ras*-induced down-regulation of caspase-2 in malignant intestinal epithelial cells represents a relatively efficient approach for blocking growth of tumors formed by these cells.

The fact that caspase-2 can suppress anoikis and three-dimensional tumor growth is consistent with several other studies pointing at the tumor suppression function for this caspase. It was shown in this regard that mouse embryonic fibroblasts (MEFs) derived from caspase-2 knock-out mice, when transformed with oncogenes, grow faster in monolayer culture as well as in soft agar and are more tumorigenic in mice than similarly transformed wild type MEFs (62). In this case, however, loss of caspase-2 seemed to accelerate proliferation of transformed MEFs, rather than block their anoikis (caspase-2 is known to have the ability to block the cell cycle progression under certain circumstances (45)). The fact that caspase-2 mediates proliferation, rather than anoikis, of MEFs is not surprising, in view of the fact that MEFs tend not to be prone to anoikis unless they are deprived of growth factors (63, 64). It is also known that caspase-2 expression is frequently reduced in human gastric tumors when compared with normal gastric mucosa (65). Furthermore, caspase-2 was found to be significantly underexpressed in metastatic brain tumors (66). Finally, caspase-2-deficient mice were demonstrated to be noticeably more susceptible to Myc-induced lymphoma than the respective wild type mice (62).

In summary, our data indicate that the anti-apoptotic mechanism triggered by *ras*-induced down-regulation of caspase-2 represents an important novel element of the signaling network by which oncogenic Ras blocks anoikis and promotes three-dimensional growth of tumors formed by malignant intestinal epithelial cells.

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