Detachment of normal epithelial cells from the extracellular matrix (ECM) triggers apoptosis, a phenomenon called anoikis. Conversely, carcinomas (cancers of epithelial origin) represent three-dimensional disorganized multicellular masses in which cells are deprived of adhesion to the ECM but remain viable. Resistance of cancer cells to anoikis is thought to be critical for tumor progression. However, the knowledge about molecular mechanisms of this type of resistance remains limited. Herein we report that ras oncogene, an established inhibitor of anoikis, triggers a significant up-regulation of anti-apoptotic proteins cIAP2 and XIAP in intestinal epithelial cells. We also observed that the effect of ras on cIAP2 requires ras-induced autocrine production of transforming growth factor α (TGF-α), a ligand for epidermal growth factor receptor, whereas ras-triggered up-regulation of XIAP is TGF-α-independent. Moreover, overexpression of either cIAP2 or XIAP in non-malignant intestinal epithelial cell was found to block anoikis. In addition, an established IAP antagonist Smac or Smac-derived cell-permeable peptide suppressed ras-induced anoikis resistance and subsequent anchorage-independent growth of ras-transformed cells. We conclude that ras-induced overexpression of cIAP2 and XIAP significantly contributes to the ability of ras-transformed intestinal epithelial cells to survive in the absence of adhesion to the ECM and grow in a three-dimensional manner.
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**FIGURE 1.** Activated H-ras triggers overexpression of cIAP2 in attached and detached intestinal epithelial cells. Attached (att) (A) and detached (det) (B) IEC-18 cells and an H-ras-transformed clone of these cells ras-3 were assayed for the expression of cIAP2 by Northern blot. 18 S and 28 S ribosomal RNAs were used as loading controls. Changes in the cIAP2 mRNA expression were quantified by densitometric analysis of the blots. Values were normalized for loading by the respective values observed for 18 S rRNA. Levels of cIAP2 mRNA expression in IEC-18 cells were arbitrarily defined as 1.0. The results of quantification represent the average of two independent experiments plus the S.D. C, detached IEC-18 cells and two independently derived H-ras-transformed clones of these cells ras-3 and ras-4 were assayed for the expression of cIAP2 by Western blot. D, detached MT-ras cells were assayed for the expression of cIAP2 by Western blot in the absence (−) and in the presence (+) of Cd^{2+}. E, detached IEC-18 cells were assayed for the expression of cIAP2 by Western blot in the absence (−) and in the presence (+) of Zn^{2+} and Cd^{2+}. Detached cells were cultured in suspension for 3 h. The membranes in C, D, and E were re-probed with an anti-β-actin antibody as a loading control. Changes in cIAP2 expression observed in C and D were quantified as in A and B, and the values were normalized by levels of β-actin for loading. The results of quantification in C and D represent the average of two independent experiments plus the S.D.

...FADD. This, in turn, results in the induction of effector caspases (26–29).

We and others found that anoikis of non-malignant cells is controlled by multiple mechanisms (30–39). For example, we established that anoikis of non-malignant intestinal epithelial cells occurs due to the simultaneous induction of two pro-apoptotic signaling pathways. The first pathway involves detachment-induced down-regulation of the anti-apoptotic protein Bcl-X\textsubscript{L} (6, 9). The second pathway is triggered by detachment-induced activation of p38 mitogen-activated protein kinase and subsequent overexpression of Fas ligand (34). Identification of some of the key pro-anoikis mechanisms allowed us to investigate which of these mechanisms may be altered in tumor cells by oncogenes, such as ras.

Ras is a small GTPase activated by receptor-tyrosine kinases in response to various mitogenic signals (40). Activated Ras, in turn, triggers multiple downstream pathways mediated by signaling molecules such as Raf, phosphatidylinositol 3-kinase, and Ral guanine nucleotide dissociation stimulator among others. Some of these events frequently result in activation of transcription factors, such as NF-κB (40) and subsequent changes in the expression of their target genes. Ultimately, Ras-induced signaling mechanisms control proliferation, survival, motility, and other critical cellular functions (41). Activating mutations of ras are among the most frequently occurring oncogenic events in human cancer (42, 43), including colorectal carcinoma. Thus far we found that oncogenic Ras blocks anoikis of intestinal epithelial cells by two mechanisms. One mechanism involves reversal of detachment-induced inhibition of Bcl-X\textsubscript{L} expression (33). The second one is driven by Ras-induced down-regulation of Bak (44).

Given that Ras has the ability to trigger multiple signaling pathways and, thus, change the expression and activity of numerous cellular proteins (41), it is highly likely that ras-induced anti-anoikis mechanisms are not limited to those regulated by Bak and Bcl-X\textsubscript{L}. We reasoned that, in addition to the Bcl-2 family members, other regulators of apoptosis, e.g. caspases and/or their inhibitors, such as the IAPs, could mediate the anti-anoikis effect of ras. The possibility of a role for caspases and the IAPs in ras-dependent anoikis resistance is supported by several observations. Thus, we and others showed that detachment of various types of epithelial cells results in the activation of initiator caspases-9, (31) and -8 (34) as well as effector caspase-3 (6). In addition, several studies have demonstrated that blockade of caspase activity by small molecule inhibitors or dominant negative caspase mutants results in the suppression of anoikis (6, 31, 45). Moreover, various IAPs are often overexpressed in numerous types of human malignancies (46–50). This overexpression likely contributes to cancer progression as small molecule- or antisense oligonucleotide-based IAP antagonists as well as peptides derived from an IAP inhibitor Smac possess a significant anti-tumor activity in vivo...
(51–53). An examination of the potential involvement of the IAPs in ras-dependent resistance to anoikis represented the main subject of this study. We now show that ras oncogene triggers up-regulation of cIAP2 and XIAP, two members of the IAP family, in intestinal epithelial cells and that the activity of these IAPs significantly contributes to ras-induced anoikis resistance.

EXPERIMENTAL PROCEDURES

Cell Culture—The generation of the IEC clones expressing activated H-ras and antisense TGF-α has been previously described (1, 54). Expression of H-ras in MT-ras cells was induced by adding 100 μM ZnCl2 and 2 μM CdCl2 to cells 48 h before the experiments involving the detection of cIAP2 or 24 h before the experiments involving the detection XIAP. 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 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.

Vectors—The generation of expression vectors carrying rat IAP-1 (rat analog of cIAP2) and mouse IAP-3 (mouse analog of XIAP) has been described elsewhere (55, 56). Smac expression vector has been provided by Dr. X. Wang (Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas).

Reagents—EGF receptor inhibitor gefitinib (Iressa) was provided by AstraZeneca.

Western Blot Analysis—Cells were lysed for 30 min on ice in a buffer containing 50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 100 mM NaF, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 10 μg/ml leupeptin. After removing the insoluble material, aliquots of supernatant containing 20–30 μg of protein were run through a 10% polyacrylamide gel under reducing conditions. Proteins were transferred to a nylon membrane that was subsequently incubated for 1 h at room temperature in TBST buffer (125 mM Tris–HCl, pH 8.0, 625 mM NaCl, and 0.5% Tween 20) containing 4% skim milk. The membrane was incubated with one of the following antibodies: anti-RIAP1 (rat analog of cIAP2), anti-RIAP-3 (rat analog of XIAP) (the generation of these two antibodies has been described elsewhere (55)), anti-β-actin (Sigma), anti-Myc (Santa Cruz Biotechnology), and anti-FLAG (Santa Cruz Biotechnology). Incubation with antibodies was performed in a TBST buffer containing 4% skim milk overnight. Binding of the antibodies was quantified as in Fig. 1. The results of quantification represent the average of two independent experiments plus the S.D.

FIGURE 2. Activated H-ras triggers overexpression of XIAP in attached and detached intestinal epithelial cells. Attached (A) (att) and detached (B) (det) IEC-18 and ras-3 cells were assayed for the expression of XIAP by Northern blot. C, detached IEC-18, ras-3, and ras-4 cells were assayed for the expression of XIAP by Western blot. D, detached MT-ras cells were assayed for the expression of XIAP by Western blot in the absence (−) and in the presence (+) of Zn2+ and Cd2+. E, detached IEC-18 cells were assayed for the expression of XIAP by Western blot in the absence (−) and in the presence (+) of Zn2+ and Cd2+. Detached cells were cultured in suspension for 3 h. 18S and 28S ribosomal RNAs were used as loading controls in A and B. The membranes in C, D, and E were re-probed with an anti-β-actin antibody as a loading control. Changes in XIAP mRNA and protein expression were quantified as in Fig. 1. The results of quantification represent the average of two independent experiments plus the S.D.
detected with the enhanced chemiluminescence system (PerkinElmer Life Sciences).

Northern Blot Analysis—Northern blot analysis was performed on total RNA. cDNAs coding for rat IAP-1 (rat analog of cIAP2) (55) and mouse IAP-3 (mouse analog of XIAP) (56) labeled with [32P]dCTP by random priming were used as probes.

Densitometric Analysis of Western and Northern Blots—Respective films were scanned, and Quantity One software (Bio-Rad) was used for densitometric analysis of the resulting digital images.

IAP-induced Survival Assay—5 × 10^5 IEC-18 cells grown in 60-mm dishes were incubated for 4 h with either 2 μg of pcDNA3 or 2 μg of RIAP-1 (rat analog of cIAP2) or mIAP-3 (mouse analog of XIAP) expression vector in the presence of 2 μl of Lipofectamine 2000 (LF2000, Invitrogen) in Opti-MEM (Invitrogen) medium. The transfection mixture was subsequently replaced with regular IEC-18 medium, and the incubation was continued for another 24 h. Cells were trypsinized, washed with phosphate-buffered saline, re-suspended in phosphate-buffered saline, and morphology of GFP-positive cells was then assessed by fluorescent microscopy. Condensed cells were scored as apoptotic.

XIAP RNA Interference—5 × 10^5 ras-3 cells grown in 60-mm dishes were incubated for 17 h with either 100 nM control non-targeting siRNA (siCONTROL non-targeting siRNA 1, Dharmacon) or 100 nM XIAP-specific siRNA in the presence of 0.4 μg of pEGFP-C1 expression vector (Clontech) and 2 μl of Lipofectamine 2000 (LF2000, Invitrogen) in Opti-MEM (Invitrogen) medium. The transfection mixture was subsequently replaced with regular IEC-18 medium, and the incubation was continued for another 30 h. Cells were further plated in monolayer suspension culture for 24 h, trypsinized, washed with phosphate-buffered saline, re-suspended in phosphate-buffered saline, and the morphology of GFP-positive cells was then assessed by fluorescent microscopy.

FIGURE 3. Targeted disruption of the activated K-ras allele in cells derived from human colorectal carcinoma results in the down-regulation of cIAP2 and XIAP. A and B, human colorectal carcinoma cells DLD-1 and their K-ras knock-out derivatives DKS-8 (A) and DKO-3 (B) were cultured being attached to or detached from the ECM for 24 h and assayed for apoptosis by the cell death ELISA that detects oligonucleosomes in the cytoplasm of apoptotic cells. Levels of apoptosis observed in attached cells were similar for all cell lines and were subtracted from the respective numbers obtained in case of detached cells as background. Results represent the average of the triplicates plus the S.E. C–F, indicated cell lines were cultured being detached from the ECM for 3 h and assayed for the expression of cIAP2 (C and D) and XIAP (E and F) by Western blot. The membranes were re-probed with an anti-β-actin antibody as a loading control. Levels of cIAP2 and XIAP expression were quantified as in Fig. 1C. The results of quantification represent the average of two independent experiments plus the S.D.
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RESULTS

 Oncogenic ras Triggers Up-regulation of cIAP2 and XIAP in Intestinal Epithelial Cells—In an effort to identify molecular mechanisms of ras-induced resistance to anoikis we compared the expression of anti-apoptotic protein cIAP2 in non-malignant highly anoikis-susceptible intestinal epithelial cells IEC-18 to that in ras-3, a previously published highly anoikis-resistant clone of IEC-18 cells stably expressing oncogenic H-ras (1). As shown in Fig. 1, oncogenic ras triggers a noticeable increase in expression of cIAP2 in IEC-18 cells at the RNA level regardless of whether or not these cells are attached to the ECM (Fig. 1, A and B). ras-induced overexpression of cIAP2 was also observed at the protein level (Fig. 1C). To ensure that the differences in the efficiency of cIAP2 expression detected in IEC-18 and ras-3 cells do not represent the result of clonal variation, we compared cIAP2 protein levels between IEC-18 cells and ras-4, another previously published H-ras expressing clone of IEC-18 cells derived independently of ras-3 (1). Similar to what was observed in the ras-3 clone, ras-4 cells displayed a significant increase in cIAP2 levels compared with the parental IEC-18 cells (Fig. 1C). To confirm that overexpression of cIAP2 is directly triggered by ras, we used a published clone of IEC-18 cells MT-ras carrying exogenous activated H-ras under the control of metallothionein promoter, which is inducible by Zn$^{2+}$ and Cd$^{2+}$ (33, 57). In agreement with what we observed in clones that expressed ras oncogene in a constitutive manner, we found that induction of ras in MT-ras cells leads to a noticeable up-regulation of cIAP2 (Fig. 1D). Conversely, control experiments demonstrated that treatment of the parental IEC-18 cells with Zn$^{2+}$ and Cd$^{2+}$ did not result in accumulation of this IAP (Fig. 1E). We further tested the effect of ras on XIAP, another established inhibitor of apoptosis, and found that ras triggers increased expression of this molecule at the RNA (Fig. 2, A and B) and protein (Fig. 2C) levels both in attached and detached cells (Fig. 2, A–C). We observed a similar effect of ras on XIAP in MT-ras cells (Fig. 2D) but not IEC-18 cells (Fig. 2E) in response to treatment with Zn$^{2+}$ and Cd$^{2+}$.

To test whether Ras can trigger overexpression of the IAPs in colorectal cancer cells, we used a highly tumorigenic anoikis-resistant human colorectal carcinoma-derived cells DLD-1 that carried one copy of oncogenic form of K-ras and two non-tumorigenic derivatives of these cells DKS-8 and DKO-3, in which K-ras allele has been disrupted by homologous recombination (58). In agreement with what we published before (33), both ras-knock-out cell lines were significantly more susceptible to anoikis than the parental DLD-1 cells (Fig. 3, A and B). Furthermore, ablation of oncogenic ras resulted in a noticeable down-regulation of both cIAP2 (Fig. 3, C and D) and XIAP (Fig. 3, E and F). Collectively, our data indicate that the expression of cIAP2 and XIAP can be enhanced by Ras in intestinal epithelial cells.

Increased Expression of cIAP2 and XIAP in Intestinal Epithelial Cells

Results in the Suppression of Anoikis—Because Ras is capable of both triggering the up-regulation of cIAP2 and XIAP and blocking anoikis, we asked whether increased expression of these IAPs can by itself lead to the suppression of detachment-induced apoptosis. To this end we measured the ability of exogenous cIAP2 and XIAP to prevent anoikis of IEC-18 cells upon transient transfection of these cells with the respective expression vectors. We found that increased expression of cIAP2 (Fig. 4, A and B) or XIAP (Fig. 4, C and D) noticeably increased survival of these cells upon detachment from the ECM. It has to be noted that the actual proportion of IEC-18 cells that acquired anoikis resistance in response to overexpression of the IAPs was likely significantly higher.
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FIGURE 5. IAP antagonists reverse anoikis resistance of IEC-ras cells. A, ras-3 cells were transiently transfected with a non-targeting control siRNA (control RNA) or XIAP-specific siRNA1 (XIAP siRNA1) in the presence of a GFP (pEGFP-C1) expression vector and assayed for XIAP expression by Western blot. β-Actin was used as a loading control. Levels of XIAP expression were quantified as in Fig. 1C. The results of quantification represent the average of two independent experiments plus the S.D. B, cells were transfected as in A and cultured being detached from or attached to the ECM for 24 h. GFP-positive cells were then assayed for apoptosis. Condensed cells were scored as apoptotic. Levels of apoptosis in monolayer culture were similar for both RNAs and were subtracted from the respective numbers obtained in case of detached cells as background. Results represent the average of two independent experiments plus the S.D. C, ras-3 cells were transiently transfected with a control RNA or XIAP-specific siRNA2 (XIAP siRNA2) and assayed for XIAP expression by Western blot as in A. Levels of XIAP expression were quantified as in Fig. 1C. The results of quantification represent the average of two independent experiments plus the S.D. D, ras-3 cells were transfected with a control RNA or XIAP siRNA2 as in A and assayed for apoptosis as in B. Results represent the average of two independent experiments plus the S.D. E, ras-3 cells were transiently transfected with pcDNA-3 (control vector) or pcDNA-3-encoded FLAG-tagged Smac (Smac vector) in the presence of a GFP expression vector, and the expression of Smac was assayed in these cells by Western blot. F, ras-3 cells were transfected with a control vector or Smac vector as in E and assayed for apoptosis as in B. Levels of apoptosis in monolayer culture were similar for both vectors and were subtracted from the respective numbers obtained in the case of detached cells as background. Results represent the average of two independent experiments plus the S.D. G, ras-3 and IEC-18 cells were transiently transfected with pcDNA-3 in the presence of a GFP expression vector as in E and assayed for apoptosis as in F. Levels of apoptosis detected in monolayer cultures were similar for both cell lines and were subtracted from the respective numbers obtained in case of detached cells as background. Results represent the average of two independent experiments plus the S.D.

than that shown in Fig. 4. This is due to the fact that the efficiency of transient transfection of these cells, as determined by assessing the fraction of fluorescent cells in response to transfection with a green fluorescent protein expression vector, typically constitutes 20–30% (data not shown). Thus, we conclude that cIAP2 and XIAP, each, are capable of blocking anoikis once their expression is increased in detached intestinal epithelial cells.

IAP Activity Contributes to the Resistance of ras-transformed Cells to Anoikis—Because both cIAP2 and XIAP are overexpressed in response to the activation of ras (Fig. 1–3) and both are capable of blocking anoikis of intestinal epithelial cells (Fig. 4), we reasoned that these proteins could act as mediators of ras-induced anoikis resistance. To test this possibility we initially transfected ras-3 cells with two different XIAP-specific siRNAs (XIAP siRNA1 and XIAP siRNA2). As shown in Fig. 5A, XIAP siRNA1 triggered a 2–3-fold down-regulation of XIAP in these cells, whereas the effect of XIAP siRNA2 was less pronounced in that this RNA caused ~1.5-fold down-regulation of XIAP (Fig. 5C). Both RNAs triggered noticeable levels of anoikis of ras-3 cells (Fig. 5, B and D), and as might have been expected, levels of anoikis triggered by XIAP siRNA1 were approximately twice higher then those induced by XIAP siRNA2.

Down-regulation of XIAP alone (even by XIAP siRNA1) resulted in a relatively low level of anoikis (approximately <12% of cells). We, therefore, asked whether simultaneous inhibition of the activity of both XIAP and cIAP2 can result in enhanced susceptibility of ras-transformed cells to anoikis. To this end, we decided to take advantage of the fact that a direct IAP inhibitor Smac has a well established ability to block the activity of both XIAP and cIAP2 (12). Thus, we transiently transfected ras-3, a representative clone of ras-transformed IEC-18 cells, with a Smac expression vector (Fig. 5E) and assessed the susceptibility of transfected cells to anoikis. As shown in Fig. 5F, expression of exogenous Smac significantly (~4-fold) increased apoptosis of IEC-ras cells upon detachment from the ECM. Understandably, Smac, which is capable of neutralizing both XIAP and cIAP2, triggered a noticeably higher level of anoikis than down-regulation of XIAP alone (28 versus 12%; compare Fig. 5, B and F).

Smac-dependent reversal of the anti-anoikis effect of ras was significant but partial in that the degree of Smac-triggered anoikis of IEC-ras...
cells was approximately twice lower than that of the parental IEC-18 cells (28 versus 56%, respectively; compare Fig. 5, F and G). This was expected as we have already demonstrated that, in addition to inducing overexpression of the IAPs, ras triggers resistance to anoikis by other mechanisms such as down-regulation of Bak and up-regulation of Bcl-X, (33, 44). Similar to what has been observed for the IAPs, inhibition of each of these two mechanisms resulted in a partial suppression of the survival of detached ras-transformed cells (33, 44). Establishing whether or not simultaneous inhibition of IAP-, Bak-, and Bcl-X,-dependent anti-apoptotic mechanisms in ras-transformed cells has a synergistic effect on ras-induced anoikis resistance represents the subject of our ongoing research.

Because Smac-derived cell-permeable peptides have recently been shown to act as potent inhibitors of tumor growth in vivo (51), we decided to test whether these peptides can block growth of ras-transformed intestinal epithelial cells in the absence of adhesion to the ECM. To this end, we used a recently published cell-permeable Smac-derived peptide that consisted of six N-terminal amino acids of Smac fused to the Drosophila Antennapedia penetratin sequence (cell permeability signal) (59). This N-terminal fragment of Smac/Diablo is capable of binding IAPs in vivo, blocking their activity, and causing apoptosis in a manner similar to that of a full-length Smac (59). We used a mutant Smac peptide in which the first alanine was replaced with methionine as a control. This mutation completely abrogates binding of both full-length Smac and cell-permeable Smac peptides to the IAPs (59). In agreement with what was observed for the full-length Smac, the Smac peptide noticeably suppressed growth of detached IEC-ras and DLD-1 cells (Fig. 6, A and C) but had no impact on growth of these cells in monolayer culture (Fig. 6, B and D). Collectively, our data indicate that ras-dependent overexpression of cIAP2 and XIAP is required for ras-induced anoikis resistance of intestinal epithelial cells. These findings are consistent with observations, according to which various IAP antagonists significantly suppress in vivo growth of numerous types of human solid tumor cells and, thus, represent a novel type of prototypic anticancer drugs (51–53).

ras-induced Overexpression of cIAP2 Requires ras-dependent Autocrine Production of Transforming Growth Factor-α (TGF-α)—One mechanism that was previously shown to contribute to the ability of ras-transformed intestinal epithelial cells to grow in the absence of adhesion to the ECM involves ras-induced overproduction of TGF-α, a ligand for EGFR (54). An important role for TGF-α in ras-driven transformation of intestinal epithelial cells has also been proposed by others (60). Furthermore, we found recently that TGF-α can strongly suppress anoikis of IEC-18 cells (6). This ability of activated EGFR to block anoikis was demonstrated in other types of cells (61). We, therefore, asked whether the effect of ras on the expression of the IAPs involves ras-triggered secretion of TGF-α. To this end, we studied the expression of cIAP2 in ras-3 cells and two previously published independently derived clones of these cells, asTGF-α-10 and asTGF-α-12, in which the expression of TGF-α was suppressed by transfection with an expression vector coding for a full-length antisense TGF-α RNA (54). These clones express substantially lower amounts of TGF-α and possess a significantly lower ability to grow anchorage-independently as colonies in soft agar than the parental ras-3 cells (54). We found that both clones express noticeably lower levels of cIAP2 than the parental cells (Fig. 7A). Conversely, the levels of XIAP in these clones were not significantly
different from those in ras-3 cells (Fig. 7B). We, thus, concluded that ras-induced overexpression of cIAP2 requires overproduction of TGF-α by the ras-transformed cells, whereas the effect of ras on XIAP is TGF-α-independent. To confirm our data by an independent method, we treated ras-3 cells with gefitinib (Iressa), a specific small molecule inhibitor of EGFR (62). Similar to what was observed with the antisense TGF-α clones, this inhibitor noticeably suppressed cIAP2 expression in ras-3 cells (Fig. 7C). We further asked whether TGF-α by itself is capable of up-regulating cIAP2 in intestinal epithelial cells. To address this question, we treated detached parental non-malignant IEC-18 cells with TGF-α and assayed these cells for cIAP2 expression. We found that exogenous TGF-α does up-regulate cIAP2 in IEC-18 cells (Fig. 7D).

We further reasoned that if the effect of ras on cIAP2 occurs in a TGF-α-dependent manner and cIAP2 in turn blocks anoikis of IEC-ras cells, then antisense TGF-α RNA and gefitinib should both trigger anoikis of these cells. Indeed, we found that detached antisense TGF-α clones were significantly more susceptible to anoikis than the parental ras-3 cells (Fig. 8A). Likewise, we observed that gefitinib-induced apoptosis of detached ras-3 cells was noticeably more pronounced than that of attached cells (Fig. 8B). These data place cIAP2 downstream of EGFR signaling and indicate that autocrine production of TGF-α by ras-transformed cells is required for their anoikis resistance.

In summary, we identified a novel mechanism of ras-induced anoikis resistance of intestinal epithelial cells in the present study. This mechanism involves ras-dependent up-regulation of anti-apoptotic proteins cIAP2 and XIAP. We demonstrated here that the effect of ras on these IAPs significantly contributes to the ability of ras-transformed cells to survive in the absence of adhesion to the ECM and grow in a three-dimensional manner.

**DISCUSSION**

We have demonstrated here that oncogenic ras blocks anoikis of intestinal epithelial cells by triggering TGF-α-dependent up-regulation of the anti-apoptotic protein cIAP2 and TGF-α-independent up-regulation of apoptosis inhibitor XIAP. To our knowledge this is the first study that links Ras with these IAP family members and places cIAP2 downstream of TGF-α and EGFR. Our data are consistent with observations, according to which cIAP2 and XIAP are often overexpressed in human tumors that frequently display activation of Ras- or EGFR-dependent signaling pathways. For example, both IAPs are often up-regulated in lung cancer (46, 47), a human malignancy associated with frequent oncogenic mutations of ras (63) and overexpression of EGFR (64) as well as activating EGFR mutations (65). Furthermore, the expression of cIAP2 and XIAP is typically elevated in prostate cancer (48), a
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disease characterized by overexpression of TGF-α (66) and increased levels of ErbB2 (67, 68), a common signaling partner of EGFR. In addition, variants of human prostate carcinoma cells derived on the basis of an increased ability to form metastasis have recently been shown to possess elevated levels of XIAP and display increased XIAP-dependent anoikis resistance compared with their parental poorly metastatic counterparts (69). Human carcinomas are known to grow, invade surrounding tissues, and metastasize to distant organs as three-dimensional disorganized multicellular masses in which cells are deprived of contacts with a properly organized basement membrane. This ability of solid tumor cells to survive in the absence of adhesion to the ECM represents a critical step in cancer progression. Therefore, it is conceivable that Ras-induced up-regulation of the IAPs prevents anoikis of those carcinoma cells that carry activating mutations of ras and/or display aberrant EGFR activity. This up-regulation would be expected to contribute to the ability of tumor cells to grow in a three-dimensional manner.

We found that the effect of Ras on cIAP2 is driven at least in part by ras-autodestruct production of TGF-α and subsequent activation of EGFR in intestinal epithelial cells. Our data agree with observations according to which EGFR antagonists prevent anchorage-independent growth of ras-transformed intestinal (54) and breast (62) epithelial cells as well as various cancer-derived cell lines (62). What molecular mechanisms could be responsible for the effect of EGFR on cIAP2 expression? Recently it has been shown that the inhibition of protein kinase C activity results in a reduced cIAP2 expression (70). Furthermore, EGFR and TGF-α are known to activate protein kinase C in various types of cells (71, 72). Thus, it is tempting to speculate that Ras-induced overproduction of TGF-α in intestinal epithelial cells results in an EGF receptor-triggered activation of one or several protein kinase C isoforms and subsequent induction of cIAP2 expression. We are presently exploring this possibility.

Two EGFR antagonists, gefitinib (Iressa) and erlotinib (Tarceva), are known to provide some benefit to patients in clinic and have recently been approved for use as drugs for treatment of lung cancer (64), a malignancy frequently associated with oncogenic mutations of ras (63). We found here that ras oncogene is capable of triggering both EGFR-dependent up-regulation of cIAP2 as well as EGFR-independent up-regulation of XIAP. Thus, our data suggest that EGFR antagonists might not be able to completely reverse all anti-anoikis mechanisms in those cancer cells that carry oncogenic forms of ras. Therefore, generation of compounds that block EGFR-independent mechanisms of ras-induced anoikis resistance and utilization of these agents in combination with the EGFR antagonists could increase the efficacy of the existing EGFR-directed therapies.

Several small molecule-, antisense oligonucleotide-, and Smac peptide-based IAP antagonists are presently being developed as lead compounds for cancer therapy (51–53, 73, 74). Some of these drugs alone or in combination with chemotherapeutic agents have already been shown to significantly suppress in vivo growth of numerous types of human tumor cells, including those that carry oncogenic mutations of ras (51–53). Our data agree well with these findings and suggest that the IAP antagonists exert their anti-tumor effect at least in part through triggering anoikis of cancer cells. We conclude that cIAP2 and XIAP are important novel mediators of Ras-induced transformation of intestinal epithelial cells that along with other effectors of the anti-anoikis effect of ras could serve as potential targets for treatment aimed at the suppression of three-dimensional tumor growth.

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