Down-regulation of Death-associated Protein Kinase-2 Is Required for β-Catenin-induced Anoikis Resistance of Malignant Epithelial Cells*

Hongbing Li1, Gibanananda Ray1, Byong Hoon Yoo, Mete Erdogan, and Kirill V. Rosen2

From the Departments of Pediatrics & Biochemistry and Molecular Biology, Atlantic Research Centre, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

Resistance of solid tumor cells to anoikis, apoptosis induced by cell detachment from the extracellular matrix, is thought to be critical for the ability of these cells to grow anchorage-independently within thee-dimensional tumor masses and from metastases. β-Catenin, a major oncoprotein, can inhibit anoikis of cancer cells via unknown mechanisms. In an effort to identify these mechanisms we found that β-catenin blocks anoikis of malignant kidney and intestinal epithelial cells and promotes their anchorage-independent growth by down-regulating death-associated protein kinase-2 (DAPk-2), a pro-apoptotic protein whose cellular functions have so far remained unexplored. We found that β-catenin-induced down-regulation of DAPk-2 requires the presence of the transcription factor Tcf-4, a known mediator of β-catenin signaling. We also observed that DAPk-2 contributes to the execution of anoikis of the non-malignant epithelial cells. Thus, β-catenin-induced down-regulation of DAPk-2 represents a novel signaling mechanism by which β-catenin promotes the survival of malignant epithelial cells following their detachment from the ECM and enables these cells to grow in an anchorage-independent manner.

Epithelial cells of many organs grow in vivo as monolayers that are attached to a form of the extracellular matrix (ECM) called the basement membrane (BM). Detachment from the ECM triggers apoptosis of these cells (1, 2), a phenomenon known as anoikis (3). By contrast, carcinomas, cancers of epithelial origin, represent three-dimensional disorganized multicellular masses in which cell-BM contacts are significantly altered. It is known in this regard that during tumor progression cancer cells often secrete BM-degrading enzymes, and this allows tumors to invade adjacent tissues (4). Furthermore, at advanced stages of the disease clumps of cancer cells detach from the tumor and migrate to other organs where they give rise to metastases (5, 6). Despite the fact that carcinoma cells tend to be deprived of normal contacts with the BM during tumor progression, a significant fraction of these cells remains viable (5, 6). Numerous studies indicate that this viability is a critical prerequisite for carcinoma progression. First, cancer cells can typically survive and grow without adhesion to the ECM as colonies in soft agar, and this ability represents one of the most stringent criteria for malignant transformation that are presently used (7, 8). Second, we and others found that activation of major oncoproteins, such as Ras (9, 10) or β-catenin (11), can block anoikis of various types of cancer cells. Third, several studies, including ours, indicate that treatments reversing anoikis resistance of cancer cells suppress their ability to form primary tumors (12–14) and metastases (6, 14). Finally, we found that spontaneous acquisition of anoikis resistance by the non-malignant epithelial cells is sufficient for their in vivo tumorigenicity (15). Thus, anoikis resistance of tumor cells represents a potential therapeutic target. However, molecular mechanisms that are responsible for anoikis resistance of these cells are understood poorly.

Signaling regulator β-catenin is a major oncoprotein that is thought to contribute to the progression of colorectal, ovarian, kidney, and other cancers (16). It is now known that β-catenin plays a significant role in tumor initiation (16). Furthermore, activation of β-catenin in cancer cells was demonstrated to promote metastasis (17, 18). β-Catenin was found to be able to inhibit anoikis and trigger anchorage-independent growth of cancer cells (11, 19), but the mechanisms by which β-catenin exerts these effects are presently not known.

In normal cells β-catenin typically interacts with proteins involved in cell-cell contact (16). The excess of β-catenin is removed from such cells by a complex composed of a tumor suppressor protein APC and other proteins. After binding to this complex β-catenin is targeted for proteasomal degradation due to its phosphorylation by kinases casein kinase-1α and glycogen synthase kinase 3β (16). APC is thought to ensure proper presentation of β-catenin to glycogen synthase kinase 3β (16). In carcinomas, β-catenin degradation is often blocked by either loss-of-function mutations of APC or by β-catenin mutations that render β-catenin stable (16). Consequently, excessive β-catenin moves to the nucleus of cancer cells where it is thought to contribute to tumor initiation (16) and metastasis (17, 18) by binding and activating transcription factors of the Lef/Tcf family (16). The indicated factors are known to exert
their effects on cells by altering the expression of genes that control diverse cellular properties. Some of these changes, such as the elevation of the cellular levels of the cell cycle regulator cyclin D1 (20), or a transcription factor c-Myc (21), are associated with the ability of the Lef/Tcf proteins to promote gene expression. Other changes, such as the down-regulation of the cell-to-cell adhesion mediator E-cadherin (22) or that of the cell cycle inhibitor p16INK4A (23) are attributed to the ability the Lef/Tcf transcription factor to block gene expression.

The mechanisms by which β-catenin inhibits anoikis of cancer cells are presently not known. This is in contrast to other major oncogenes, such as for example Ras, that similar to β-catenin, is thought to play an important role in the progression of colorectal cancer (24) and whose anti-anoikis affects were studied by us in some detail (9, 10, 12, 13, 15). We found that ras oncogene blocks anoikis of colon cancer cells by altering the expression of well studied components of the cell death machinery. The ras-dependent anti-anoikis mechanisms that we have identified include ras-induced activation of phosphoinositide 3-OH kinase, an event that results in the down-regulation of the pro-apoptotic member of the Bcl-2 family of proteins Bak (13). This, in turn, leads to the inhibition of a well established pro-apoptotic phenomenon that is normally triggered by detachment of the non-malignant cells from the ECM, such as the release of a death-promoting protein Omi/HtrA2 from the mitochondria into the cytoplasm of detached colon cancer cells (10). Other anti-anoikis mechanisms induced by ras identified by us include the up-regulation of the anti-apoptotic member of the Bcl-2 family Bcl-XL (12) as well as that of ras oncogene inhibitors cIAP2 and XIAP (9). According to our studies, the effect of ras on cIAP2 requires ras-dependent autocrine production of transforming growth factor-α by the malignant cells (9).

We found in this study that β-catenin suppresses anoikis of colon cancer cells as well as that of malignant kidney epithelial cells via a novel mechanism that involves β-catenin-dependent down-regulation of Death-Associated Protein Kinase-2 (DAPk-2), a poorly studied pro-apoptotic protein that has never been implicated as a mediator of anoikis or a target of β-catenin and whose cellular functions have so far remained unexplored.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—RK3E cells and their β-catenin-transformed variants were provided by Dr. E. Fearon. RK3E and DLD-1 cells were cultured as described elsewhere (12, 25). For suspension cultures cells were plated above a layer of 1% sea plaque agarose and were cultured as described elsewhere (12, 25). For suspension cultures cells were plated above a layer of 1% sea plaque agarose and were cultured as described elsewhere (12, 25). For suspension cultures cells were plated above a layer of 1% sea plaque agarose and were cultured as described elsewhere (12, 25). For suspension cultures cells were plated above a layer of 1% sea plaque agarose and were cultured as described elsewhere (12, 25).

**Western Blot**—This assay was performed as previously described (1). The following antibodies were used: anti-DAPk-2 (Chemicon), anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-TCF-1 (Cell Signaling), anti-TCF-4 (Cell Signaling), anti-HA (Upstate), anti-CDK-4 (Santa Cruz Biotechnology), anti-caspase-3 (Cell Signaling), anti-cyclin D1 (Santa Cruz Biotechnology), anti-β-actin (Sigma), and anti-α-tubulin (Upstate).

**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.

Vectors—Expression vector pcDNA-3 carrying human HA-tagged DAPk-2 was provided by Dr. A. Kimchi (26). pcDNA-3 served as a control vector in the transient transfection assays. The T-REX system (Invitrogen) was used to generate cells expressing tetracycline-inducible DAPk-2. DAPk-2 cDNA was placed into XbaI and BamHI sites of the pcDNA4-T vector (a component of the T-REX system).

RNA Interference—RNA interference (RNAi) was performed as we described (10). In the case of β-catenin-directed RNAi experiments (Fig. 3), 5 × 10⁵ DLD-1 cells were transfected with 100 nM of each RNA. In the case of the RNAi experiments aimed at simultaneous ablation of β-catenin and DAPk-2 (Fig. 6), 5 × 10⁵ DLD-1 cells were transfected. In this case total RNA concentration in each transfection reaction was 50 nM. In the case of DAPk-2-directed RNAi experiments (Fig. 7), 10⁵ RK3E cells were transfected with 100 nM of each RNA. In the case of TCF-4-directed RNAi experiments (Fig. 8), 3.5 × 10⁵ DLD-1 cells were transfected with 100 nM of each RNA, siCONTROL non-targeting siRNA #1 (Dharmacon) was used as a control RNA. The sequences of the sense strands of the RNAs used in this study were as follows: control RNA (siCONTROL non-targeting siRNA #1 (Dharmacon)), UGUUGUUGGAGGGAGACGGTT; human-specific β-catenin siRNA-1, GAUAAAGGAUCUGGUUGGAUU; human-specific β-catenin siRNA-2, CCACUAAUGUCCACGCGUUUU; human-specific β-catenin siRNA-3, GCUAGAAACGUGCAUGUUAAU; human-specific DAPk-2 siRNA-1, GGAAUUUGUGGUCCAGAGUU; human-specific DAPk-2 siRNA-2, GAGAUGGGCCAGAGAAUUUU; rat-specific DAPk-2 siRNA-1, UGGAGAAGUAAAGAGCAUU; rat-specific DAPk-2 siRNA-2, UGCGUUGGCUCAGGAAUAUU; human-specific TCF4 siRNA-1, AAAGUGCGUUGCCAGCAUAUU; human-specific TCF1 siRNA-1, GAUGCUAGGUUCCAGUGAAUU; and human-specific TCF1 siRNA-2, CCAAGAAGCAGCCAGACUAUU.

**Terminal Transferase dUTP Nick End Labeling (TUNEL) Assay**—We performed this assay by using the TUNEL Apoptosis Detection kit from Millipore. Cells were processed and assayed for apoptosis by fluorescence microscopy following the manufacturer’s instructions.

The following assays were performed as previously described: Northern blot analysis, detection of apoptosis by monitoring changes in nuclear morphology, soft agar, the Cell Death enzyme-linked immunosorbent assay (1), membrane blebbing (27), and clonogenicity (10) assays.

**Chromatin Immunoprecipitation**—Cells were exposed to formaldehyde for cross-linking of proteins to chromosomal DNA as described by others (28) and submitted to Genpathway, Inc. (San Diego, CA) for further analysis, which was performed as per standard Genpathway protocol. In brief, cells were sonicated to fragment the DNA into 300- to 500-bp pieces.
and DNA-protein complexes were immunoprecipitated as described (28). Enrichment of specific genomic regions in immunoprecipitated DNA was determined by quantitative PCR (QPCR). Along with each experimental QPCR reaction (carried out in triplicates), a dilution series of three standards of known amounts of sonicated DNA was amplified with a “standard” set of primers (primers that are routinely used by Genpathway for QPCR calibration). Assuming that the mass of a haploid genome is 3.3 pg, the starting quantities of the experimental pair of primers to that of the QPCR signal observed for the “standard” set of primers. Antibodies used for the immunoprecipitation were rabbit IgG (Sigma), anti-β-catenin (Upstate), and anti-TCF-4 (Santa Cruz Biotechnology). The following primers were used for the QPCR reactions: for standards standard-a, CTGTACCTGGGTTCTCATTT; standard-b, CAGTAAGGGGTTTCTCCTACA; for negative controls negative control-a, TGCAGTTTTGGCTTTGTCTC; negative control-b, GATGCGGAGAGGTCAGAG; for the analysis of chromosomal DNA adjacent to the DAPk-2 transcription start site DAPK1-a, GGGGAGCAAGAGGTGAGAGAG; DAPK1-b, AGCGAGGGAGGACAG; DAPK2-a, TGACATGTGTGCACAACTC; DAPK2-b, AGACGGGCTTCTCGAGTGT; DAPK3-a, TGACAGCCACACTCCTC; DAPK3-b, TCACACGCTCATCTCCT; DAPK4-a, GGCATGCTCTTCTCTC; DAPK4-b, TGACATGCTTCTC; DAPK5-a, AATGCCTGGGAGAGAGTCT; DAPK5-b, TGCATGCAAGAACCCCTCCTAAG; DAPK6-a, AGGTTGTGCTGTTACAG; DAPK6-b, GACTGCTGCTGCTGCTGCTGCT; DAPK7-a, CACCACCTTCGGTGTTGATC; DAPK7-b, TGGCCTGAGAAAAGGAGAG; DAPK8-a, GAGTGGCTGAGAGAGAGAG.

FIGURE 1. Oncogenic β-catenin blocks anoikis of malignant kidney epithelial cells. A, indicated cells were cultured for 24 h attached to the ECM and assayed for cyclin D1 expression by Western blot. α-Tubulin served as a loading control. B, indicated cells were cultured for 24 h attached to (att) or detached (det) from the ECM and assayed for apoptosis by Hoechst 33258 nuclei staining. Percent TUNEL-positive cells was calculated as a percentage of TUNEL-positive cells in a total cell population. Results represent the average of two independent experiments.* p value that was calculated by both t test and chi-square test for goodness-of-fit was <0.05 regardless of the method of calculation. C, cells were cultured for 17 h attached to (att) or detached (det) from the ECM and assayed for chromosomal DNA fragmentation by the cell death enzyme-linked immunosorbent assay. Percent cells with apoptotic nuclei was calculated as a percentage of cells with condensed and fragmented nuclei in a total cell population. Results represent the average of two independent experiments.*** p value that was calculated by both t test and chi-square test for goodness-of-fit was <0.01 regardless of the method of calculation. D, indicated cells were cultured for 17 h attached to (att) or detached (det) from the ECM and assayed for cyclin D1 expression by Northern (A) or Western (B and C) blot. 18 S and 28 S ribosomal RNAs (A) and α-tubulin (B and C) served as loading controls.

FIGURE 2. Oncogenic β-catenin down-regulates DAPk-2 expression in malignant kidney epithelial cells. A–C, cells were cultured attached to (attached) (A and B) or detached from (detached) (C) the ECM for 24 h and assayed for DAPk-2 expression by Northern (A) or Western (B and C) blot. 18 S and 28 S ribosomal RNAs (A) and α-tubulin (B and C) served as loading controls.
β-Catenin Blocks Anoikis by Down-regulating DAPk-2

RESULTS

Mutant β-Catenin Down-regulates Death-associated Protein Kinase-2 in Malignant Kidney Epithelial Cells—To explore the mechanisms by which β-catenin blocks anoikis of cancer cells we used non-malignant, anoikis-susceptible rat kidney epithelial cells RK3E (19) and two published (25) independently derived clones of these cells S33Y-A and -D that constitutively express an S33Y mutant of β-catenin, which cannot be phosphorylated by glycogen synthase kinase 3β and is thus abnormally stable. Unlike the parental RK3E cells, these clones can form tumors in immunodeficient mice (25). The indicated clones are known to display a significantly increased transcriptional activity of the Lef/Tcf factors compared with the parental cells (25). Indeed, we confirmed that both clones showed substantially higher levels of cyclin D1 protein (transcription of the mRNA encoding cyclin D1 is well known to be stimulated by β-catenin and Lef/Tcf proteins due to direct binding of the indicated factors to the Tcf recognition elements within the cyclin D1 promoter (20)) than the parental RK3E cells (Fig. 1A). We further verified that β-catenin can suppress apoptotic events, such as condensation and fragmentation of the nuclei and fragmentation of the chromosomal DNA in RK3E cells following their detachment from the ECM (Fig. 1, B–D).

To identify the mechanisms by which β-catenin blocks anoikis, we compared levels of mRNAs coding for 97 apoptosis regulators in detached RK3E and S33Y-A cells by using the array carrying respective cDNAs. The most significant β-catenin-induced change observed by us was the down-regulation (~10-fold) of the mRNA coding for the pro-apoptotic protein DAPk-2/DRP-1 (not shown). DAPk-2/DRP-1 is a cytosolic Ca2+/calmodulin-regulated Ser/Thr kinase that belongs to the DAPk family (26, 29). DAPk-2/DRP-1 has a well established ability to cause apoptosis via unidentified mechanisms requiring its kinase activity (26, 27, 29). Substrates and physiological functions of DAPk-2 are not known. We confirmed that β-catenin down-regulates DAPk-2 both at the mRNA (Fig. 2A) and the protein (Fig. 2, B and C) levels in both...
**B-Catenin Blocks Anoikis by Down-regulating DAPk-2**

**FIGURE 4. B-Catenin-induced down-regulation of DAPk-2 is required for anoikis resistance of kidney epithelial cells.** A, S33Y-A cells were transiently transfected with a control expression vector (vector) or an expression vector carrying HA-tagged DAPk-2 (HA-DAPk-2) and assayed for DAPk-2 expression by Western blot as in Fig. 3A by using an HA-specific antibody. B, S33Y-A cells treated as in A were assayed for nuclear morphology as in Fig. 18. Results represent the average of three independent experiments ± S.D. C, tet-DAPk-2 cells were cultured in the absence (−) or in the presence (+) of 50 ng/ml tetracycline (tet) for 24 h and assayed for membrane blebbing by Western blot by using an HA-specific antibody as in Fig. 3A. D, tet-DAPk-2 cells were assayed for DAPk-2 expression as in C by using the DAPk-2-specific antibody. Positions of endogenous (end) DAPk-2 and exogenous (ex) HA-tagged DAPk-2 on the gel are indicated. E, vector control cells (vector) were treated as in D. F, cells treated as in D and E were assayed for nuclear morphology as in Fig. 18. Results represent the average of two independent experiments ± S.D. G, cells treated as in D and E were assayed for membrane blebbing. Percent cells displaying membrane blebbing was calculated as a percentage of cells displaying membrane blebbing in a total cell population. Results represent the average of three independent experiments ± S.D. H, cells treated as in D and E were assayed for the presence of fragmented chromosomal DNA by the TUNEL assay as in Fig. 1C. Results represent the average of two independent experiments ± S.D. I, to confirm these results by a complementary method we generated a variant of S33Y-A cells that we named tet-DAPk-2 in which DAPk-2 could be expressed in a tetracycline-inducible manner (Fig. 4C). We induced the expression of this kinase in tet-DAPk-2 cells by using a tetracycline concentration at which the resulting total DAPk-2 levels in these cells did not exceed those in the parental RK3E cells (Fig. 4D). We found that ectopic DAPk-2 caused at least three types of apoptotic events, such as nuclear condensa-
tion and fragmentation, membrane blebbing, as well as chromosomal DNA fragmentation (Fig. 4, F–H), at a substantially higher degree in detached than in the attached tet-DAPk-2 cells. This increase of apoptosis did not occur in response to tetracycline treatment of S33Y-A cells transfected with a control expression vector (Fig. 4, E–H). Given that activation of caspase-3, a protease that triggers a number of key apoptotic events (such as chromosomal DNA fragmentation (33)) in cells that have been stimulated to die, was previously found by us to occur during anoikis (34), we tested whether caspase-3 is activated by DAPk-2 in detached tet-DAPk-2 cells. In further support of the data indicating that β-catenin-induced down-regulation of DAPk-2 plays a causal role in anoikis resistance of cancer cells (see Fig. 4, E–H) we found that exogenous DAPk-2 does trigger the activation of caspase-3 (such activation is characterized by the emergence of cleaved fragments of caspase-3 that are derived from the enzymatically inactive pro-caspase-3) in the indicated cells following their detachment from the ECM (Fig. 4I). Of note, in all cases (see Fig. 4, F–I) the presence of apoptotic features was much more pronounced in the detached than that in the attached tetracycline-treated tet-DAPk-2 cells. Furthermore, we found (data not shown) that the ability of anoikis-susceptible RK3E cells (that display relatively high levels of DAPk-2, see Fig. 2) to form colonies in monolayer during a 7-day time period is not significantly different from that of oncogenic β-catenin-expressing S33Y-A and -D cells (that display low levels of DAPk-2, see Fig. 2). Thus, our data indicate that when DAPk-2 is expressed in cells at levels observed in the RK3E cells (or when cellular DAPk-2 levels are relatively close to but do not exceed those observed in RK3E cells as is the case with tetracycline-treated tet-DAPk-2 cells, see Fig. 4D), this kinase is not by itself toxic to cells if these cells are attached to the ECM but sensitizes them to apoptosis once they detach from the ECM.

Interestingly, the ability of DAPk-2 to promote apoptosis was relatively unique to anoikis, because DAPk-2 did not enhance death of tet-DAPk-2 cells in response to treatment with an apoptosis-promoting drug staurosporine (Fig. 5). These results suggest that apoptosis induced by loss of cell-ECM adhesion and that triggered by drugs, such as staurosporine, are controlled by different mechanisms.

We further investigated whether β-catenin-induced down-regulation of DAPk-2 in colon cancer cells DLD-1 (see Fig. 3) lacking functional APC and carrying abnormally active wild-type β-catenin (30) contributes to their anoikis resistance. In agreement with this possibility, we observed that transient expression of ectopic DAPk-2 triggers anoikis of these cells (Fig. 6, A and B). To confirm the causal role of β-catenin-induced down-regulation of DAPk-2 in anoikis resistance of colon carcinoma cells by a complementary approach we tested whether DAPk-2 up-regulation induced by loss of β-catenin in DLD-1 cells (see Fig. 3A) is the cause of their increased susceptibility to detachment-induced apoptosis and of their reduced ability to grow anchorage independently (see Fig. 3, B and C). We found the average of two independent experiments ± S.D. *, in A–C indicates that p value that was calculated by both t test and chi-square test for goodness-of-fit was <0.05 regardless of the method of calculation.
**β-Catenin Blocks Anoikis by Down-regulating DAPk-2**

**FIGURE 6.** β-Catenin-induced down-regulation of DAPk-2 is required for anoikis resistance of colon carcinoma cells. A and B, DLD-1 cells were treated as in Fig. 4 (A and B). C and D, DLD-1 were transfected (+) or not transfected (−) with a 50 nM control RNA (cont RNA) (C and D, left lane) or 25 nM control RNA (cont RNA) together with 25 nM β-catenin siRNA-2 (C and D, middle lane) or 25 nM β-catenin siRNA-2 together with 25 nM human DAPk-2-specific siRNA (DAPk-2 siRNA)-1 (C, right lane) or 25 nM β-catenin siRNA-2 together with 25 nM human DAPk-2-specific siRNA (DAPk-2 siRNA)-2 (D, right lane) and assayed for β-catenin and DAPk-2 expression as in Fig. 3A. E, DLD-1 cells processed as in C and D were cultured attached to or detached from the ECM for 48 h and assayed for apoptosis as in Fig. 18. F, DLD-1 cells processed as in C and D were assayed for soft agar growth as in Fig. 3C. Results represent the average of the triplicates (B and E) or the duplicates (F) ± S.D. Experiments in E and F were repeated twice with similar results. ** or * in B and E indicates that p value that was calculated by both t test and chi-square test for goodness-of-fit was <0.05 or 0.01, respectively, regardless of the method of calculation. ** or * in F indicates that the p value that was calculated by t test was <0.05 or 0.01, respectively.

In this regard that anoikis of these cells triggered by RNAi-induced ablation of β-catenin was inhibited when the up-regulation of DAPk-2 (this up-regulation was promoted by loss of β-catenin) was blocked by RNAi (Fig. 6, C–E). Furthermore, suppression of anchorage-independent growth of these cells in soft agar induced by the ablation of β-catenin was reversed when the up-regulation of DAPk-2 in the indicated cells (triggered by loss of β-catenin) was prevented by RNAi (Fig. 6F).

Based on the data presented above, we concluded that the ability of DLD-1 cells to survive without being attached to the ECM and grow anchorage independently can, at least in part, be explained by the fact that DAPk-2 levels in these cells are constitutively low due to the presence of β-catenin. Collectively, our results (see Figs. 4 and 6) indicate that β-catenin-induced down-regulation of DAPk-2 significantly contributes to anoikis resistance of malignant kidney and intestinal epithelial cells.

**Endogenous DAPk-2 Contributes to Detachment-induced Death of Anoikis-susceptible Cells**—The fact that siRNA-induced ablation of the endogenous DAPk-2 blocked anoikis of β-catenin-deprived DLD-1 cells (Fig. 6, C–F) indicates that DAPk-2 became a component of anoikis-promoting machinery of these cells once they had lost β-catenin. In further support of the possibility that DAPk-2 can act as a component of such machinery in anoikis-susceptible cells, we found that RNAi-induced DAPk-2 down-regulation protected from anoikis non-malignant kidney epithelial cells RK3E (Fig. 7). Even though the rescue from anoikis of RK3E cells that was induced by rat DAPk-2-directed RNAi (Fig. 7B) was well noticeable, this rescue was not as pronounced as that observed by us in the case of human DAPk-2-specific RNAi in β-catenin-deprived DLD-1 cells (see Fig. 6, C–F), possibly due to the fact that the efficiency of RNAi directed toward DAPk-2 in DLD-1 cells was higher than that in RK3E cells (compare Figs. 6C, 6D, and 7A). Collectively, these data (Figs. 6 and 7) indicate that DAPk-2 represents a component of anoikis-promoting machinery in anoikis-susceptible cells, such as β-catenin-deprived DLD-1 cells or RK3E cells.

**β-Catenin-dependent Down-regulation of DAPk-2 Is Mediated by Transcription Factor Tcf-4**—β-Catenin is thought to promote cell transformation by activating transcription factors of the Lef/Tcf family (16). Increased activity of β-catenin and that of the indicated transcription factors can lead to the up-regulation of certain proteins, such as cyclin D1 (20) or c-Myc (21). In addition, β-catenin, and its partners Lef-1 and Tcf-4, can directly repress the expression of genes, such as those encoding proteins E-cadherin, p16INK4A, and Nkx2.2 (22, 23, 35), by binding to the Tcf recognition sites within promoters of these genes. We thus decided to test whether any of the Lef/Tcf proteins are involved in the effect of β-catenin on DAPk-2. We found in this
and Tcf-4 to the cyclin D1 promoter region that harbors four
other methods (31, 36)). As expected, the binding of
cells by the chromatin immunoprecipitation assay as well as by
accompanying binding of both
start site. To validate the assay in our model system (DLD-1
tion of binding of the indicated tran-
mediated by Tcf-4.
FIGURE 7. DAPk-2 contributes to anoikis of the non-malignant kidney
epithelial cells. A, RK5E cells were transfected with a 100 nm control RNA
(cont RNA) or rat DAPk-2-specific siRNA (Dapk2 siRNA) -1 or -2 and assayed for
DAPk-2 expression by Western blot as in Fig. 2A. B, cells treated as in A were
placed in monolayer immediately or after being detached for 96 h. Colonies
formed by the viable cells were counted 7 days later. An increase in the per-
centage of the number of colonies obtained after culturing cells in monolayer
was then calculated for each DAPk-2-specific siRNA relative to that observed
in case of transfection with the control RNA was arbitrarily designated
as 1.0). The results represent the average of two independent experiments ±
S.D. The average percentage of survival was 18% in the case of the control
RNA, 27% in case of Dapk2 siRNA-1 and 27% in case of Dapk2 siRNA-2. *** or *
indicates that p value that was calculated by t test was <0.05 or 0.01,
respectively.

FIGURE 8. Ablation of Tcf-4 in colon carcinoma cells results in the up-regu-
luation of DAPk-2. A and B, DLD-1 cells were transfected with a 100 nm
control RNA (cont RNA) or Tcf-4-specific siRNA (TCF4 siRNA)-1 (A) or -2 (B) and
assayed for Tcf-4 or DAPk-2 expression by Western blot. C, DLD-1 cells pro-
cessed as in A and B were cultured detached from the ECM for 48 h and
assayed for apoptosis as in Fig. 1A. The results represent the average of two
independent experiments the S.D. **) or *, p value that was calculated by both
t test and chi-square test for goodness-of-fit was <0.05 or 0.01, respectively,
regardless of the method of calculation. D and E, DLD-1 cells were transfected
with a 100 nm control RNA (cont RNA) or Tcf-1-specific siRNA (TCF1 siRNA)-1
(D) or -2 (E) and assayed for Tcf-1 or DAPk-2 expression by Western blot. CDK-4
served as a loading control in A, B, D, and E.

regard that RNAi-induced ablation of Tcf-4, a member of the LEF/
Tcf family (Fig. 8, A and B), but not that of Tcf-1, another member
of the family (Fig. 8, D and E), in DLD-1 cells triggered DAPk-2
up-regulation. As might have been expected, ablation of Tcf-4 triggered
apoptosis of DLD-1 cells in the absence of adhesion to the
ECM (Fig. 8C). Our observations, according to which transfection
with a control siRNA promotes lower background levels of apop-
tosis in case of the Tcf-4-directed RNAi experiments compared with
those observed during RNAi experiments aimed at β-catenin
(see Figs. 6 and 8), can likely be explained by the fact that cell
densities and siRNA concentrations were different in the two sets
of transfections (see “Experimental Procedures” and respective fig-
ure legends). Thus, β-catenin-dependent down-regulation of
DAPk-2 is mediated by Tcf-4.

We further performed chromatin immunoprecipitation to
test whether β-catenin and Tcf-4 are capable of binding any of
the chromosomal regions located near the DAPk-2 transcrip-
tion start site. To validate the assay in our model system (DLD-1
cells) we first confirmed that this technique allows the detection
of binding of both β-catenin and Tcf-4 to the promoter
regions of established targets of these transcriptional regula-
tors, such as cyclin D1(20), uPA (31), and SGK1 (36) (Fig. 9,
A–E) (promoters of the latter two genes were previously dem-
onstrated to be the targets of β-catenin and Tcf-4 in DLD-1
cells by the chromatin immunoprecipitation assay as well as by
other methods (31, 36)). As expected, the binding of β-catenin
and Tcf-4 to the cyclin D1 promoter region that harbors four
Tcf-binding sites was noticeably stronger than that to the uPA
and SGK1 promoter regions that carry two and one Tcf-binding
sites, respectively (Fig. 9, A–E). However, in all cases the
binding of β-catenin and Tcf-4 to the respective fragments of
chromosomal DNA was clearly detectable. Therefore, the assay
does allow the detection of the binding of the indicated tran-
scription regulators to respective promoters in DLD-1 cells.
Thus, we used chromatin immunoprecipitation to screen the
chromosomal region adjacent to the DAPk-2 transcription
start site (by amplifying various DNA fragments within this
region with seven different pairs of primers) located approxi-
mately between positions ~3000 and +600 (where +1 corre-
sponds to the DAPk-2 transcription start site) for the ability to
bind β-catenin and Tcf-4. We found that a DNA fragment con-
taining the sequence GACAAAG between positions ~725 and
~718 that closely matches the consensus Tcf-binding site did
co-immunoprecipitate with both β-catenin (Fig. 9F) and Tcf-4
(Fig. 9G). Interestingly, this exact sequence was previously
found to be involved in the inhibition of expression of a protein
Nkx2.2 by Tcf-4 (35). Even though the “~3000 to +600” region
contains several other potential Tcf-binding sites, the binding
of any of which to β-catenin and Tcf-4 could in principle be
detected due to the choice of primers that we used to amplify
DNA segments within the indicated region of chromosomal
DNA, none of these other DNA fragments seemed to co-immu-
noprecipitate with β-catenin and Tcf-4 (not shown).


** DISCUSSION **

The ability of solid tumor cells to resist anoikis is thought to be critical for tumor growth and metastasis (5, 37), but the mechanisms of such resistance are not well understood. β-Catenin, a major oncoprotein, is known to be able to block anoikis of cancer cells (11, 19). However, molecular events that link β-catenin with anoikis-inducing cellular machinery have never been explored. We have demonstrated in this study that activation of β-catenin in cancer cells, either by an oncogenic mutation of β-catenin itself or by loss of APC, a negative regulator of β-catenin-dependent signaling, results in the inhibition of anoikis of these cells. We found that in both cases this inhibition of anoikis requires β-catenin-induced down-regulation of DAPk-2/DRP-1. Thus, our work for the first time provides a mechanistic link between β-catenin and the cellular machinery that is responsible for the execution of apoptosis that is induced by loss of cell-ECM adhesion.

Another important aspect of our findings is that they attribute a role to DAPk-2 (whose cellular functions have so far remained unknown) in a physiologically relevant process, such as anoikis. We have demonstrated in this regard that colon cancer cells that lack β-catenin are highly susceptible to this form of apoptosis (see Figs. 3 and 6) and that the execution of anoikis of such cells can to a significant degree be suppressed by DAPk-2-directed RNAi (see Fig. 6). Likewise, we have found that anoikis of the non-malignant

Thus, our results indicate that β-catenin and Tcf-4 can bind the chromosomal DNA region containing a TCF-binding element (see Fig. 9, F and G) and that both of these proteins contribute to the down-regulation of DAPk-2 in cancer cells (see Figs. 2, 3A, and 8A). Collectively, these data are consistent with the scenario, according to which the indicated transcriptional regulators act as direct repressors of DAPk-2 expression in the malignant cells.

In summary, we have shown here that β-catenin, a major human oncoprotein, blocks anoikis of malignant epithelial cells and promotes their growth in the absence of adhesion to the ECM by down-regulating DAPk-2.

** FIGURE 9.** β-Catenin and Tcf-4 bind to a chromosomal region located upstream of DAPk-2 transcription start site in colon carcinoma cells. DLD-1 cells were exposed to formaldehyde for cross-linking of proteins to chromosomal DNA, sonicated to fragment the DNA into 300- to 500-bp pieces, and DNA-protein complexes were immunoprecipitated (IP) with an anti-β-catenin (A, C, and F) (IP β-catenin) or anti-Tcf-4 (B, D, E, and G) (IP Tcf-4) antibody. Rabbit IgG was used as a negative control in all cases (IP IgG). DNA that was isolated from the immunoprecipitated complexes was then amplified by quantitative PCR using primer pairs specific for either a chromosomal DNA region devoid of genes and of known regulatory DNA elements (this reaction served as another negative control) (neg ctrl DNA), or promoter regions of cyclin D1 (D1 promoter) (A and B), uPA (uPA promoter) (C and D), and SGK1 (SGK1 promoter) (E) all of which contain Tcf-binding sites, as well as the region of chromosomal DNA located upstream of the DAPk-2 transcription start site and containing a Tcf-binding site between positions −725 and −718 (F and G) (DAPk−725-718). The results are expressed as detected number of copies of DNA bound to the respective protein per 1000 cells. The results represent the average of three independent experiments plus the S.E. ** or * p value that was calculated by t test was <0.05 or 0.01, respectively.

2020 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 284 • NUMBER 4 • JANUARY 23, 2009
kidney epithelial cells can be blocked by RNAi-mediated down-regulation of DAPK-2 (see Fig. 7). Given that physiological substrates of this kinase as well as the mechanisms by which it triggers cell death are presently not known, identification of the molecular events involved in DAPK-2-dependent anoikis represents an interesting direction of our ongoing research.

Of note, we have observed here that treatments resulting in the increased expression of DAPK-2 in cancer cells induced a much more pronounced degree of apoptosis of these cells when they were detached from the ECM compared with that observed in the attached cells (see Figs. 4 and 6). Thus, it seems likely that a certain threshold level of DAPK-2 expression is required for the execution of apoptosis triggered by detachment-induced inhibition of survival signals that are normally generated by cell-ECM adhesion. In this case, when levels of DAPK-2 in cells are reduced, for example as a result of the activation of β-catenin, signals that are triggered by detachment of these cells from the ECM could by themselves be expected to be incapable of causing apoptosis.

Also, interestingly, the involvement of DAPK-2 in the anti-anoikis effect of β-catenin could not be predicted based on what is presently known about the mechanisms by which other oncoproteins, such as, for example Ras, block anoikis of tumor cells. We found in this regard that Ras, which similar to β-catenin, is frequently activated in colorectal cancer, blocks anoikis of tumor cells by down-regulating the pro-apoptotic protein Bak (13), by up-regulating the anti-apoptotic protein Bcl-XL (12) as well as by increasing cellular levels of two other apoptosis inhibitors, such as cIAP2 and XIAP (9). Also importantly, none of the signaling events that are presently known to control the changes in the expression of the indicated regulators of anoikis in ras oncogene-carrying cancer cells have so far been shown to involve DAPK-2. For example, we found that Ras-induced down-regulation of Bak in the malignant intestinal epithelial cell is mediated by phosphoinositide 3-OH kinase (13). We also observed that Ras-induced up-regulation of cIAP2 requires Ras-dependent autocrine production of transforming growth factor-α by the malignant intestinal epithelial cells (9). All of the proteins mentioned above represent well characterized elements of the cellular apparatus that controls apoptosis and are the conceivable candidates for the mediators of anoikis resistance of cancer cells triggered by oncoproteins, such as β-catenin. Even though the involvement of these proteins in β-catenin-induced inhibition of anoikis of malignant cells cannot be excluded, we have found so far that it is DAPK-2 that plays a significant role in the anti-anoikis effect of β-catenin. Thus, the present study introduces DAPK-2 as a novel component of the cellular machinery that is responsible for the regulation of anoikis of normal and cancer cells.

It is noteworthy that oncogenic mutations resulting in the activation of β-catenin and Ras often co-exist in colorectal cancer cells (38), as is the case with colon carcinoma cells DLD-1 that were used in this study (30, 39). We previously demonstrated that ras oncogene contributes to anoikis resistance of these cells (12). According to the results of the present study, in addition to Ras, β-catenin (that in case of DLD-1 cells is activated by a loss-of-function mutation of APC tumor suppressor gene (30)) also contributes to the ability of the indicated cells to resist anoikis by down-regulating DAPK-2. What advantage (with regard to their ability to resist anoikis) could colon cancer cells possibly gain from acquiring both types of oncogenic mutations? It is possible that anti-apoptotic signals generated by Ras and β-catenin cooperate in protecting colon cancer cells from anoikis, so that pro-survival events triggered by each oncoprotein alone are necessary but not by themselves sufficient for rescuing such cells from detachment-induced death. We found in this regard oncogenic ras blocks anoikis of DLD-1 cells by up-regulating the anti-apoptotic protein Bcl-XL (12) and that Bcl-XL, when its expression is increased in intestinal epithelial cells, prevents detachment-dependent activation of caspase-3, one of the key mediators of apoptosis, in these cells (34). Likewise, according to the data presented in this study (see Fig. 6), β-catenin-dependent down-regulation of DAPK-2 and subsequent inhibition of anoikis is associated with the suppression of detachment-induced caspase-3 activation. Thus, it is possible that signaling events triggered by both oncogenic Ras and β-catenin are necessary for preventing detachment-induced activation of caspases, such as caspase-3, beyond the level that is required for the induction of anoikis. In this case, inhibition of either Ras- or β-catenin-dependent anti-anoikis signals could be expected to result in the activation of caspases, such as caspase-3, to a degree sufficient for the execution of the pro-apoptotic program in detached cells.

We have shown here that β-catenin-induced down-regulation of DAPK-2 requires the presence of transcription factor Tcf-4. Others observed in this regard that increased binding of β-catenin and that of its partners transcription factors Lef-1 and Tcf-4 to respective promoter regions can result in the direct inhibition of expression of genes, such as those encoding proteins E-cadherin, p16INK4A, and Nkx2.2 (22, 23, 35). Furthermore, we have shown here that both β-catenin and Tcf-4 can bind the chromosomal DNA fragment located upstream of DAPK-2 transcription start site and carrying a Tcf-binding site. Taken together, these data are consistent with a scenario, in which β-catenin and Tcf-4 act as direct repressors of Dapk2 expression.

In summary, we have identified a novel mechanism by which a major oncoprotein, such as β-catenin, allows malignant epithelial cells to survive and grow without being attached to the ECM. This mechanism is driven by β-catenin-induced Tcf-4-dependent down-regulation of DAPK-2. Our work for the first time implicates DAPK-2/DRP-1, whose cellular functions have so far remained unknown, as a mediator of anoikis and a suppressor of β-catenin-dependent anchorage-independent growth of malignant epithelial cells.

Acknowledgments—We are grateful to Drs. E. Fearon and A. Kimchi for the materials provided to us. We are also grateful to Dr. Holly Etchegary, a statistician at the Isaac Walton Killam Health Centre (Halifax, Nova Scotia), for assistance with statistical analysis of our results.

REFERENCES
1. Rosen, K., Shi, W., Calabretta, B., and Filmus, J. (2002) J. Biol. Chem. 277, 46123–46130
2. Liu, Z., Li, H., Wu, X., Yoo, B. H., Yan, S. R., Stadnyk, A. W., Sasazuki, T.,
β-Catenin Blocks Anoikis by Down-regulating DAPk-2

Shirasawa, S., LaCasse, E. C., Korneluk, R. G., and Rosen, K. V. (2006) Oncogene 25, 7680–7690
3. Frisch, S. M., and Sprent, R. A. (2001) Curr. Opin. Cell Biol. 13, 555–562
4. Ljubimov, A. V., Bartek, J., Couchman, J. R., Kappler, L. L., Veselov, V. V., Kovarik, I., Perevozchikov, A. G., and Krutovskikh, V. A. (1992) Int. J. Cancer 50, 562–566
5. Douma, S., Van Laar, T., Zevenhoven, J., Meuwissen, R., Van Garderen, E., and Peepere, D. S. (2004) Nature 430, 1034–1039
6. Berezovskaya, O., Schimmer, A. D., Glinskii, A. B., Pinilla, C., Hoffman, R. M., Reed, J. C., and Glinsky, G. V. (2005) Cancer Res. 65, 2378–2386
7. Freedman, V. H., and Shin, S. I. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1033–1037
8. Freedman, V. H., and Shin, S. I. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1033–1037
9. Freedman, V. H., and Shin, S. I. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1033–1037
10. Liu, Z., Li, H., Derouet, M., Berezkin, A., Sasazuki, T., Shirasawa, S., and Rosen, K. V. (2005) J. Biol. Chem. 280, 37383–37392
11. Orford, K., Orford, C. C., and Byers, S. W. (1999) Cell 100, 447–456
12. Rosen, K., Rak, J., Leung, T., Dean, N. M., Kerbel, R. S., and Filmus, J. (1998) J. Cell Biol. 140, 855–868
13. Rosen, K., Rak, J., Leung, T., Dean, N. M., Kerbel, R. S., and Filmus, J. (2000) J. Cell Biol. 149, 447–456
14. Duxbury, M. S., Itou, H., Zinner, M. J., Ashley, S. W., and Whang, E. E. (2004) Oncogene 23, 1448–1456
15. Derouet, M., Wu, X., May, L., Hoon, Y. B., Sasazuki, T., Shirasawa, S., Rak, J., and Rosen, K. V. (2007) Neoplasia 9, 536–545
16. Giles, R. H., van Es, J. H., and Clevers, H. (2003) Biochim. Biophys. Acta 1653, 1–24
17. Stein, U., Arlt, F., Walther, W., Smith, J., Waldman, T., Harris, E. D., Mertins, S. D., Heizmann, C. W., Allard, D., Birchmeier, W., Schlag, P. M., and Shoemaker, R. H. (2006) Gastroenterology 131, 1486–1500
18. Baltint, K., Xiao, M., Pinnix, C. C., Soma, A., Veres, I., Juhasz, I., Brown, E. J., Capobianco, A. J., Herlyn, M., and Liu, Z. J. (2005) J. Clin. Invest. 115, 3166–3176
19. Weng, Z., Xin, M., Pablo, L., Grueneberg, D., Hagel, M., Bain, G., Muller, T., and Papkoff, J. (2002) J. Biol. Chem. 277, 18677–18686
20. Tetsu, O., and McCormick, F. (1999) Nature 398, 422–426
21. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Science 281, 1509–1512
22. Jamora, C., DasGupta, R., Kocieniewski, P., and Fuchs, E. (2003) Nature 422, 317–322
23. Delmas, V., Beermann, F.,Martinozzi, S., Carreira, S., Ackermann, J., Kumasaka, M., Denat, L., Goodall, J., Luciani, F., Viro, S., Remini, N., Bastian, B., Goding, C. R., and Larue, L. (2007) Genes Dev. 21, 2923–2935
24. Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J., and Vogelstein, B. (1987) Nature 327, 293–297
25. Kolligs, F. T., Hu, G., Dang, C. V., and Fearon, E. R. (1999) Mol. Cell. Biol. 19, 5696–5706
26. Inbal, B., Shani, G., Cohen, O., Kissil, J. L., and Kimchi, A. (2000) Mol. Cell. Biol. 20, 1044–1054
27. Inbal, B., Blalik, S., Sabanay, I., Shani, G., and Kimchi, A. (2002) J. Biol. Cell. Biol. 157, 455–468
28. Labhart, P., Karmakar, S., Salicrue, E. M., Egan, B. S., Alexiadis, V., O’Malley, B. W., and Smith, C. L. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1339–1344
29. Kawai, T., Nomura, F., Hoshino, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Akira, S. (1999) Oncogene 18, 3471–3480
30. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) Science 275, 1787–1790
31. Haendeler, E., Regus, S., Wassermann, S., Hubak, F., Hayn, A., Dimmler, A., Koch, C., Knoll, C., van Beest, M., Reuning, U., Brablett, T., Kirchner, T., and Jung, A. (2004) Cancer Res. 64, 1209–1214
32. Chen, S., Guttridge, D. C., You, Z., Zhang, Z., Friebly, A., Mayo, M. W., Kitajevski, J., and Wang, C. Y. (2001) J. Cell Biol. 152, 87–96
33. Wolf, B. B., Schuler, M., Echeverri, F., and Green, D. R. (1999) J. Biol. Chem. 274, 30561–30566
34. Rosen, K., Coll, M. L., Li, A., and Filmus, J. (2001) J. Biol. Chem. 276, 37273–37279
35. Lei, Q., Jeong, Y., Misra, K., Li, S., Zelman, A. K., Epstein, D. J., and Matise, M. P. (2006) Dev. Cell 11, 325–337
36. Dehner, M., Hadjihannas, M., Weiske, J., Huber, O., and Behrens, I. (2008) J. Biol. Chem. 283, 14201–14210
37. Jacks, T., and Weinberg, R. A. (2002) Cell 111, 923–925
38. Fearon, E. R., and Vogelstein, B. (1990) Cell 61, 759–767
39. Shirasawa, S., Furuse, M., Yokoyama, N., and Sasazuki, T. (1993) Science 260, 85–88