The Survival Kinase Mirk/Dyrk1B Is a Downstream Effector of Oncogenic K-ras in Pancreatic Cancer

Kideok Jin, Sunju Park, Daina Z. Ewton, and Eileen Friedman

Department of Pathology, Upstate Medical University, State University of New York, Syracuse, New York

Abstract

The kinase Mirk is overexpressed in many resected pancreatic adenocarcinomas and is amplified in a subset of pancreatic cancer cell lines. Depletion of Mirk has been shown to lead to apoptosis in pancreatic cancer cell lines, and thus to inhibit their clonogenic growth. Mirk is activated by signaling from activated Rac1 to MKK3 in MDCK cells, but the mechanism of activation of Mirk in pancreatic cancers is unknown. In this report, Mirk is shown to be a novel effector of K-ras, a gene mutated in ~90% of pancreatic cancers. Activation of Mirk signaling from oncogenic K-ras through Rac1 was shown in transient expression systems and reporter assays. Mirk activation in pancreatic cancer cells was blocked by RNA interference using three different synthetic duplex RNAs to K-ras, or two RNAs to Rac1, by pharmacologic inhibition of Rac1, or by expression of dominant negative K-rasS17N. Rac1 was activated in four out of five pancreatic cancer cell lines, and was activated by signaling from oncogenic K-ras. Mirk knockout does not induce embryonic lethality, and depletion of Mirk had no effect on the survival of normal diploid fibroblasts. In contrast, the clonogenic ability of Panc1 and AsPc1 pancreatic cancer cell lines was reduced 8- to 12-fold by the depletion of Mirk, with a greater reduction seen following the depletion of K-ras or both genes. Mirk is a novel downstream effector of oncogenic K-ras and mediates some of the survival signals activated by ras signaling. [Cancer Res 2007;67(15):7247–55]

Introduction

Inappropriate control of cell survival can lead to the development of cancer. Pancreatic ductal adenocarcinomas are so lethal because they are highly resistant to apoptosis induced by chemotherapeutic drugs or by radiation. Cancers often amplify genes which give them a survival advantage. Eight different studies have found an amplicon in pancreatic cancers at 19q13.1–13.2, in which the serine/threonine kinase Mirk/dyrk1B is localized (reviewed in ref. 1). This amplicon was found in 9% of primary tumors (n = 33) as well as in a subset of pancreatic cancer cell lines (5 of 31) including the Panc1, SU86.86, and HPAC cell lines (2). High level Mirk protein levels were seen in Panc1 and SU86.86 cells (ref. 3; HPAC not tested), which is consistent with the Mirk gene residing within this amplicon. In a recent report (3), Mirk was shown to have a major role in mediating the survival of clonogenic pancreatic cancers. Mirk was expressed in the majority (89%) of resected pancreatic cancers, with elevated expression in 39%, and unexpectedly, depletion of Mirk increased apoptosis in AsPc1 pancreatic cancer cells with low Mirk expression, as well as increasing apoptosis in Panc1 and SU86.86 pancreatic cancer cells with higher Mirk expression. A second conclusion from these studies was that depletion of Mirk by synthetic duplex RNAs sensitized each of three pancreatic cancer cell lines to apoptosis induced by gemcitabine. The increase was 2- to 3-fold higher compared with drug treatment alone in each of the three cell lines. Thus, depletion of Mirk rendered pancreatic cancer cells with high (Panc1 and SU86.86) or low (AsPc1) Mirk expression more sensitive to gemcitabine, a drug commonly used clinically to treat pancreatic cancer.

Mirk was activated in each pancreatic cancer in which it was detected, but the mechanism of action was unknown. In earlier studies, Mirk had been shown to be activated by overexpressed, constitutively active mutant Rac1QL in transient transfection experiments and by endogenous Rac1 activated by cadherin ligation in nontransformed Madin-Darby canine kidney (MDCK) cells (4). K-ras is mutated to an oncogenic, active GTPase form in the vast majority of pancreatic adenocarcinomas, and K-ras is known to signal through Rac1 in some cell types. In the current study, the activation of Mirk in pancreatic cancer cells was shown to be mediated by a signaling pathway from oncogenic K-ras to Rac1.

Materials and Methods

Materials. Antibodies to Rac1 and the PAK-1 PBD (Pak-binding domain)-agarose for GST binding experiments were from Upstate Biotechnology. Rabbit polyclonal antibody to a unique sequence at the COOH terminus of Mirk was raised as described (5). The specific Rac1 inhibitor NSC23766 was purchased from Calbiochem. All other reagents were from Sigma.

Cell culture. All pancreatic ductal adenocarcinoma cell lines and the BJ normal diploid fibroblast cell strain were obtained from American Type Culture Collection and maintained in DMEM containing either 7% (tumor cells) or 10% (fibroblasts) fetal bovine serum and modified and supplemented as described (6).

Plasmids. The pcDNA-Myc-Tiam1 and pcDNA-Tiam1-RBD(KTHQ → ADEE) mutant were obtained from C. Der (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC). The K-ras2G12V, K-ras2S17N, and H-rasG12V expression plasmids were from the UMR CDNA Resource Center (Rolla, MO). All other plasmids have been described previously (3–6).

RNA interference. Synthetic duplex RNAs 25mers directed to the K-ras mRNA sequences starting at base 57, 387, and 454 (s11, s12, s13) were from Invitrogen, as were the RAC1 validated stealth RNAi DuoPak. A duplex corresponding to the Mirk mRNA sequence initiated at base 840 (Invitrogen) was directed against a sequence within exon 6 and unique to Mirk by Blast search. Controls were GC-matched scrambled sequences.

Transient transfections. Cells were transfected by incubating with a complex of PLUS reagent (3 μL/μg DNA) and LipofectAMINE (2 μL/μg DNA) in serum-free medium for 18 to 24 h in a CO2 incubator. The amount of total DNA used was kept constant by the addition of vector DNA, and luciferase activities were calibrated by cotransfected β-galactosidase activity.
activity to normalize the transfection efficiency. These assays were carried out in triplicate, and the data shown are representative of two independent experiments.

**Immunoprecipitations.** An aliquot of total cell lysate of 500 μg was immunoprecipitated with 5 μL of anti-Mirk rabbit polyclonal antibody overnight at 4°C; the complexes were then collected by the addition of 20 μL of protein A-agarose, incubated for 1 h at 4°C, washed thrice with lysis buffer, and separated by SDS-PAGE.

**In vitro kinase assay.** The kinase activity of Mirk was tested exactly as described (4) with the myelin basic protein from Upstate Biotechnology, or with a 283 amino acid fragment of recombinant histone deacetylase 5 (HDAC5) prepared as described (7). Immunodetection and affinity precipitation of GTP-bound Rac1 were done exactly as described (4).

**Results**

**Oncogenic K-ras proteins activate Mirk.** Mutation of a Ras protein at one of a set of specific amino acids, such as G12, makes that Ras protein a constitutively active GTPase. Such mutant Ras proteins are found in ~30% of all carcinomas, and in ~90% of pancreatic cancers (8, 9). The expression of such mutant Ras proteins has been shown to contribute to oncogenic transformation in many cell types. The ability of one oncogenic mutant K-ras (K-rasG12V) to activate coexpressed Mirk was assayed. Mirk kinase activity was measured in the presence of increasing concentrations of K-rasG12V (Fig. 1A). Mirk was coexpressed in 293T cells with increasing amounts of K-rasG12V expression plasmid. Mirk was then immunoprecipitated and its kinase activity measured in an *in vitro* kinase assay using a known *in vivo* substrate of Mirk, HDAC5. An NH$_2$-terminal fragment of HDAC5, which contains one Mirk phosphorylation site at Ser279, was used with the casein kinase II site of Ser$^\text{259}$ mutated to alanine (7). The expression of increasing amounts of K-rasG12V led to a dose-dependent increase in Mirk kinase activity (Fig. 1A). The relative abilities of mutant K-ras and wild-type K-ras to activate coexpressed Mirk were then compared. K-rasG12V increased Mirk activity a mean of 5-fold more than wild-type K-ras in transient transfection experiments in Cos1 cells and 293T cells (Fig. 1C).

The results of these direct kinase assays were confirmed by using an HNF1 reporter assay. Mirk has been shown to increase HNF1 transcriptional activity 2- to 3-fold (6). HNF1 and the HNF1 reporter were cotransfected into Cos1 cells with either the active K-rasG12V mutant or a dominant negative K-rasS17N mutant. Both of these K-ras mutants induced the same slight increase in reporter activity over background in the absence of exogenous Mirk (Fig. 1D), which showed that the modest increase in HNF1 activity was unrelated to the GTPase, oncogenic functions of K-rasG12V. Coexpressed wild-type Mirk introduced an additional 8-fold increase in reporter activity only in the presence of K-rasG12V. The 2- to 3-fold increase in HNF1 reporter activity observed in cells expressing both Mirk and dominant negative K-rasS17N was no more than that induced by Mirk alone in earlier studies (6). Thus, this reporter assay and direct kinase activity assays showed that oncogenic K-ras proteins lie upstream of Mirk in a signaling cascade.

**Depletion of K-ras or expression of a dominant negative K-ras inhibits Mirk activity in Panc1 and SU86.86 pancreatic carcinoma cells.** K-ras genes are mutated in ~90% of all pancreatic adenocarcinomas. Panc1 cells exhibit such an endogenous activating mutation in K-ras and also express activated Mirk (3). We questioned whether the depletion of oncogenic K-ras in these cells would inhibit the activity of endogenous Mirk. K-ras mRNA was depleted in Panc1 pancreatic cancer cells by transfection of three synthetic duplex RNAs to different regions of the K-ras mRNA. The extent of depletion of K-ras caused by Si1, Si2, and Si3 was 61%, 54%, and 70%, respectively (Fig. 1B, bottom). Analysis of the cell lysates also showed a dramatic decrease in activation of one effector of oncogenic K-ras, the survival kinase Akt, as determined by Western blotting for Akt phosphorylated at Ser$^\text{473}$. Equivalent amounts of endogenous Mirk were immunoprecipitated from each culture and Mirk kinase assay was determined in an *in vitro* kinase assay on a fragment of HDAC5. Depletion of K-ras by each RNAi was enough to decrease Mirk kinase activity to undetectable levels although there was no decrease in Mirk protein levels, as determined by Western blotting of total cell lysates (Fig. 1B). Therefore, even partial depletion of endogenous K-ras levels in a cell line expressing oncogenic K-ras was enough to dramatically decrease Mirk kinase activity.

Expression of a dominant negative K-ras gene, K-rasS17N, was done by transient transfections of two pancreatic cancer cell lines with endogenous K-ras mutations, Panc1 and SU86.86 (Fig. 2). Overexpression of total K-ras proteins in the cells because of the transfection was detected by Western blotting (Fig. 2B, lysate). Equivalent amounts of endogenous Mirk were immunoprecipitated from each culture and Mirk kinase assay was determined in an *in vitro* kinase assay on HDAC5. In both cell lines, increased expression of the dominant negative S17N mutant led to a dose-dependent decrease in Mirk activity of 10% to 20% of control levels in Panc1 cells and to 20% to 30% of control levels in SU86.86 cells (Fig. 2). Therefore, Mirk activity was strongly inhibited either by the depletion of endogenous oncogenic K-ras from each of three different synthetic RNAs or by inhibition of endogenous oncogenic mutant K-ras from transient overexpression of a dominant negative construct. These experiments provide evidence that Mirk is a novel downstream effector of endogenous oncogenic K-ras in two pancreatic cancer cell lines.

**Depletion of either Mirk or K-ras decreases anchorage-dependent colony formation in pancreatic cancer cells.** Colony formation assays test for the most aggressive cells within a tumor cell line. In earlier studies, our group had shown that depletion of Mirk from Panc1 cells by a plasmid-based RNAi vector decreased anchorage-dependent colony formation by 3- to 5-fold, whereas depletion of Mirk by two different RNAi duplex oligonucleotides induced apoptosis in Panc1 and two other pancreatic cancer cell lines (3). Apoptosis of the clonogenic cancer cells following loss of Mirk thus seemed to be responsible for the decrease in clonogenicity. However, in the prior study, the colonies which arose after drug selection for transfected cells expressed as much Mirk as the controls. These data suggested that the Panc1 tumor cells with Mirk depletion were not viable, and as a result, tumor cells with stable depletion of Mirk could not be isolated even though the selection pressure for drug resistance was present throughout the cloning procedure. This hypothesis was addressed in the current study by measuring the amount of Mirk protein which was depleted culturewide in Panc1 cells transfected with the pSilencer plasmid encoding RNAi to Mirk in a time course experiment during drug selection for the coexpressed G418 resistance marker (Fig. 3A). The ratio of pSilencer DNA to pcDNA3 DNA was 10:1, so it was very likely that the cells expressing the transfected drug resistance marker also had been transfected with the pSilencer plasmid. Levels of Mirk were compared with those expressed in cultures transfected with a mutant Mirk RNAi pSilencer construct or the vector alone. Both sets of measurements
gave similar results (Fig. 3A). Mirk levels in the cultures decreased about 60% after 4 days of selection and were maintained at this level for another 4 days. These measurements included the non-transfected cells which had not yet been killed by G418. Mirk levels were found to be depleted 60-fold when cells transfected with this pSilencer construct were selected by cell sorting in earlier studies (7), thus, the observed 60% decrease in Mirk levels was an average from both transfected and nontransfected cells. Cell lysis was apparent after ~7 to 10 days of drug selection, so transfected cells expressing the G418 resistance gene and remaining after drug selection would be expected to also express the pSilencer RNAi to Mirk and exhibit very low Mirk levels. This did not occur. After 10 days of selection, the amount of Mirk increased in the cultures, which previously had shown depletion of Mirk, and Mirk levels reached control levels during the final 12 to 18 days of selection for drug-resistant cells. It is possible that the pSilencer RNAi expression plasmid was lost or inactivated during drug selection, even though the pSilencer plasmid was transfected at 10-fold the concentration of the pcDNA encoding the drug resistance marker. Alternatively, the transfected clonogenic cells with low Mirk levels were not viable even when the cells were maintained in growth medium.

Next, another approach was tried to deplete Mirk in the majority of Panc1 cells, and then to test for effects on clonogenicity. Mirk was depleted by RNA interference using a synthetic duplex RNAi directed to another mRNA sequence than that used in the pSilencer studies. The control was a GC-matched RNA duplex. Cells were maintained in serum-free medium for 2 days to decrease the activity of serum factor-initiated survival pathways such as IGF-I/Akt or IGF-I/STAT3. Mirk levels and Mirk activity increased in serum-free cultures because of the up-regulation of Mirk mRNA (10, 11), hence this stress condition might be expected to increase reliance on Mirk. Cells were then switched to serum-containing growth medium containing G418 for 3 weeks to allow the formation of colonies from the surviving cells. The transfected clonogenic cells also expressed the neomycin resistance gene because the pcDNA3 vector was cotransfected with the synthetic duplex RNAis at the time of Mirk depletion. Tumor colonies which arose were counted by an automated colony counter. Depletion of Mirk in Panc1 cells which were also deprived of serum-mediated survival pathways at the time of plating led to a 10-fold decrease in the number of colonies 10 to 20 mm in diameter and a 14-fold decrease in the larger colonies (Fig. 3B; one of two similar experiments shown). Thus, >90% of the clonogenic Panc1 cells depleted of Mirk died during the 2-day serum-free stress period before serum was replaced. This result was consistent with earlier studies in which stable Mirk transfectants of colon carcinoma cells were more...
Depletion of Mirk or K-ras without selection for the transfected cells led to less of an effect, a 2- to 3-fold loss of colony formation, respectively, whereas depletion of both genes reduced colony formation 11-fold. Thus, even under conditions in which Mirk was depleted for a short time and nontransfected cells remained in the cultures, a reduction in clonogenicity was seen. It is possible that drug selection provided an additional stress for transfected Panc1 cancer cells following depletion of either Mirk or K-ras, which enhanced the reduction in colony formation caused by the depletion of either protein (Fig. 3D).

Mirk/dyrk1B knockout mice are viable, which suggests that Mirk is not essential for the survival of nontransformed cells (12). To test this hypothesis, Mirk was depleted from human normal diploid fibroblasts, and cell growth and survival was then assayed under anchorage-dependent conditions at low plating densities. Treatment of BJ human diploid fibroblasts with the synthetic duplex RNAi led to a 25-fold decrease in Mirk protein levels (data not shown). However, this large loss in Mirk levels caused no apparent decrease in the growth of normal diploid fibroblasts (Fig. 3D). The BJ fibroblasts do not form colonies like the tumor cells, but grow into small cell clusters. Thus, depletion of Mirk did not inhibit the survival of diploid fibroblasts plated at low cell densities, although it inhibited the clonal growth of pancreatic cancer cells. Possibly diploid fibroblasts depend for survival under the stress conditions of low plating density on many antiapoptotic signals, including those initiated by integrins which mediate cell attachment. In contrast, the survival of clonogenic pancreatic tumor cells was largely dependent on oncogenic K-ras and Mirk kinase.

Mirk can be activated in pancreatic adenocarcinoma cells through a K-ras to Rac1 signaling pathway. In earlier studies, Mirk was shown to be activated by overexpressed constitutively active mutant Rac1QL and by endogenous Rac1 activated by cadherin ligation (4). The hypothesis that oncogenic K-ras activated Mirk through Rac1 was tested by first measuring the activation state of Rac1 in pancreatic cell lines which were known to exhibit active Mirk (ref. 3; Fig. 4A). The GTP-Rac1 forms which bound to the Rac binding domain of Pak1 coupled to agarose beads were quantified and compared with total Rac1 in cell lysates by Western blot analysis. Rac1 was activated in the three pancreatic carcinoma cell lines with oncogenic K-ras genes and in BxPc3 cells with wild-type K-ras genes. Thus, activated GTP-Rac1 was found in Panc1, SU86.86, BxPc3, and AsPc1 cells which exhibit active Mirk, but not in MiaPaCa cells, which exhibited very low Mirk levels (Fig. 5A), and no detectable Mirk activation (3).

Rac1 itself can be activated by Ras proteins through the Rac-specific guanine nucleotide exchange factor Tiam1 (13). Tiam associates with activated, oncogenic Ras-GTP to stimulate Rac activity. Tiam1 is a Wnt-responsive gene up-regulated in human colon adenomas (14) and is believed to play a role in oncogenesis. Tiam1 is widely expressed in many tissues including the pancreas, and suppression of Tiam1 by a constitutive shRNA vector decreased the growth of Capan1 pancreatic cancer cells in soft agar (15). The capacity of exogenous Tiam1 to activate Mirk in HNF1 reporter assays in 293T transformed kidney epithelial cells was measured by an in vitro kinase reaction on HDAC5, as in Fig. 1A. The amount of Mirk in each immunoprecipitate was determined by Western blotting. B, Western blot of total cell lysate for K-ras.
possible that the high levels of exogenous Tiam1 introduced in this reporter assay activated endogenous Rac1 which in turn activates Mirk. Because Tiam1 (15) and Rac1 (Fig. 4A) are active in the majority of pancreatic cancer cells, Tiam1 might mediate the activation of Rac1.

Because Rac1 had been found to be activated in the majority of pancreatic cancer cell lines examined (Fig. 4A), the hypothesis was next tested that oncogenic K-ras found in Panc1 cells was responsible for the activation of Rac1 seen in these cells. Three synthetic duplex RNAs to different regions of the K-ras mRNA, si1, si2, and si3, which had been used in our prior experiments (Fig. 1B), were now used to determine whether depletion of K-ras would extinguish Rac1 activation. Each of the three RNAs depleted K-ras a mean of 14-fold in Panc1 cells (Fig. 4B). In each case, this substantial depletion of K-ras was followed by a mean 5-fold decrease in the activation of Rac1. Therefore, oncogenic K-ras activated Rac1 in Panc1 pancreatic cancer cells, and was probably responsible, at least in part, for the activation of Rac1 seen in the other pancreatic cancer cell lines with mutant K-ras genes.

Mirk activity in Panc1 pancreatic cancer cells is inhibited by either depletion of Rac1 by RNA interference or pharmacologic inhibition of Rac1. Rac1 mRNA was depleted in Panc1 pancreatic cancer cells and in SU86.86 pancreatic cancer cells by two synthetic duplex RNAs to different regions of the Rac1 mRNA (Fig. 6A). Analysis of total cell lysates by Western blotting showed that siA and siB depleted Rac1 protein levels. Depletion of Rac1 in Panc1 cells led to a marked decrease in activity of a downstream effector, Akt, as shown by a decrease in Akt phosphorylation. Mirk was then immunoprecipitated from the Rac1-depleted cells and its kinase activity measured by an in vitro kinase reaction on HDAC5. Depletion of Rac1 levels by siA and siB led to a strong decrease in

Figure 3. Depletion of either Mirk or K-ras inhibits anchorage-dependent colony formation of pancreatic cancer cell lines. A, depletion of Mirk in Panc1 pancreatic cancer cells by RNAi encoded by the pSilencer plasmid was not maintained. Cells were cotransfected with pcDNA3 encoding the G418 resistance gene to serve as the selection marker and pSilencer encoding either Mirk RNAi, a mutant Mirk RNAi, or vector alone. After 48 h of expression, cells were switched to medium containing 400 μg/mL of G418 and selection continued for 18 d. Parallel wells were lysed at the times indicated and the amount of Mirk protein normalized to actin was determined. The percentage of Mirk remaining after RNAi treatment was calculated compared with Mirk levels in cells transfected with either the mutant control or the vector control and plotted versus the number of days in the selection medium. B, Panc1 pancreatic cancer cells, which express mutant K-ras, were depleted of either Mirk, K-ras, or both Mirk and K-ras by RNA interference, and pcDNA3 was cotransfected with each synthetic duplex RNAi to enable selection of the transfected cells by expression of its neomycin resistance gene. The transfected cells were maintained in serum-free medium for 2 d as a stress condition, then allowed to proliferate in growth medium plus G418 for 3 wks for colony growth. Columns, mean; bars, SD (n = 3). C, AsPC1 pancreatic cancer cells, which express mutant K-ras, were depleted of either Mirk, K-ras, or both Mirk and K-ras by RNA interference, and pcDNA3 was cotransfected with each synthetic duplex RNAi to enable selection of the transfected cells by expression of its neomycin resistance gene. The transfected cells were maintained in serum-free medium for 2 d as a stress condition, then allowed to proliferate in growth medium plus G418 for 3 wks for colony growth. Columns, mean; bars, SD (n = 3). D, Panc1 pancreatic cancer cells were depleted of either Mirk, K-ras, or both Mirk and K-ras by RNA interference, maintained in serum-free medium for 2 d as a stress condition, then allowed to proliferate in growth medium for 3 wks for colony growth. Normal diploid BJ fibroblasts were depleted of Mirk, plated at 2,000 cells per 100 mm dish, then allowed to grow into cell clusters for 3 wks. Approximately 1,500 cell clusters were seen in the control. G418 selection was not employed for either cell type. Data were normalized to values obtained after treatment with the control, GC-matched RNAi.
Mirk activity in both cell lines (Fig. 6A, top lanes). Linear regression analysis was used to show the relationship between the depletion of Rac1 and the inhibition of Mirk activity (Fig. 6C) by pooling the data from depletion of Rac1 in both Panc1 and SU86.86 cells. Depletion of Rac1 was linearly related to a decrease in Mirk activity. Therefore, Rac1 is an upstream activator of Mirk in pancreatic cancer cells, and can be activated by signaling initiated by oncogenic K-ras (Fig. 5).

The cell-permeable Rac1 inhibitor NSC23766 was shown in earlier studies to partially block Mirk activity in confluent MDCK cells (4). A dose-response curve determined that 200 to 1,000 μmol/L of NSC23766 inhibited the activation of Mirk by K-rasG12V in the HNF1 reporter assay (data not shown). A time course study was then done to compare the inhibition of Rac1 with the inhibition of Mirk kinase activity (Fig. 6D) using the 200 μmol/L concentration, which had been shown to be specific for Rac1 (16). In a 16-h time course study, the amount of activated GTP-Rac1 (top) was determined by GST-binding assays and Mirk kinase activity was measured at each time point (bottom). With increasing time of exposure to the inhibitor, Rac1 activity was progressively inhibited to 30% of control levels, and Mirk kinase activity declined to 3%, with the data showing a sigmoidal dose-response relationship by nonlinear regression analysis (Fig. 6D). Therefore, both depletion of Rac1 by RNA interference and pharmacologic inhibition of Rac1 inhibited Mirk kinase activity in pancreatic cancer cells.

Analysis of the K-ras to Mirk signaling pathway. Oncogenic K-rasG12V initiates pathways which activate PI-3K and the mitogen-activated protein (MAP) kinase kinase MEK. The contribution of such pathways to the activation of Mirk by coexpressed K-rasG12V was tested in HNF1 reporter assays by the use of pharmacologic inhibitors (Fig. 5C). Both the PI-3K inhibitor LY294002 and the MEK inhibitor PD98059 blocked reporter activation in a dose-dependent manner. PI-3K transmits signals to Akt, and depletion of Rac1 by RNA interference was shown to block the activation of Akt, as well as to inhibit Mirk activation in pancreatic cancer cells (Fig. 6A). It is possible that PI-3K is also an upstream activator of Mirk through its association with Ras proteins, or its activation of Rac1. Microinjection of physiologic levels of oncogenic K-rasG12V into quiescent pancreatic ductal epithelial cells in primary culture led to an increase in entry into S phase and an increase in cell size dependent on the PI-3K/mTOR signaling pathway (17). The PI-3K inhibitor LY294002 prevented the increase in cell size induced by K-rasG12V/PI-3K/mTOR signaling (17), as well as inhibiting the activation of Mirk by K-rasG12V in this reporter assay (Fig. 5C).

The inhibition of reporter activity by PD98059 suggested that Mirk might be activated through a Ras to MEK pathway. We next tested whether cotransfection of the MAP kinase kinases MEK2, MKK6, or MKK7 would enhance Mirk transcriptional activation of HNF1 in transient transfection experiments. Mirk increased the activity of HNF1 - 3-fold (Fig. 5D, compare lanes 1 and 2), whereas cotransfection of the constitutively active form of the jnk kinase MKK7 (18) had no stimulatory effect. In contrast, the constitutively active form of the p38 MAP kinase kinases MKK6, MKK6E (19), synergized with Mirk to increase the activation of HNF1 - 35-fold, more than Mirk alone or MKK6E alone (Fig. 5D). The constitutively active form of the Erk kinase MEK2, MEK2E (20, 21), also enhanced Mirk activation of HNF1 - 12-fold. This lower stimulatory effect was consistent with a small increase of Mirk's transcriptional coactivator activity induced by the MEK2 upstream activators 12-O-tetradecanoylphorbol-13-acetate and platelet-derived growth factor (data not shown). However, these data show that the MAP kinase kinases which activate p38, MKK6 (Fig. 5D) and MKK3 (6), are major activators of Mirk, whereas the MAP kinase kinases which activates the Erks, MEK2, can also activate Mirk.

Discussion

Several genes have been associated with the development of pancreatic cancer including mutations in K-ras2, deletion in the INK4A locus encoding the p16 cyclin-dependent kinase inhibitor, mutations in p53 and BRAC2, deletions or truncating mutations in smad4/dpc, up-regulation of members of the sonic hedgehog signaling system, up-regulation of Her2/neu, and mutations in mismatch repair genes and in STK11/LKB1 (summarized in refs. 22–24). The most prevalent mutations in pancreatic cancer, 70% to 90% of cases, occur in K-ras (8, 9). K-ras mutations have been found in neoplastic precursor lesions called pancreatic intraepithelial neoplasms (PanINs; ref. 25). Studies employing mice engineered to exhibit pancreas-specific Cre-mediated activation of a mutant K-ras allele (K-rasG12D) have clearly shown that physiologic levels of mutant K-ras induce the full spectrum of PanINs found in humans (26, 27). At low frequencies, these lesions progress spontaneously to invasive and metastatic adenocarcinomas, and so establish PanINs as the definite precursors to pancreatic cancer (26). These important studies clearly show that K-ras mutations are the initiating lesions in pancreatic cancer, therefore, pathways activated by mutant K-ras, such as Rac1 → Mirk, may offer good targets for intervention in this disease.

Another effector of Ras-mediated tumorigenesis is RalA, which is activated in the majority of pancreatic cancer cell lines (28). RalA was not investigated in the current study for its capacity to activate Mirk. Some investigators have identified a Ral effector with
Rac-activating activity (29), so Mirk could possibly be a downstream effector of RalA. In the current study, the Ras effector Rac1 was shown to mediate signals initiated by K-ras to Mirk. Rac1 was activated in four of five pancreatic cancer cell lines, and in each of these four lines, Mirk had been found to be activated in earlier studies (3). Rac1 had been shown to activate Mirk in nontransformed MDCK cells through MKK3 (4), therefore, Rac1 is a likely upstream activator of Mirk in pancreatic cancer cells. The observation that Rac1 mRNA levels were elevated at least 2-fold in 70% of pancreatic cancers compared with samples of normal pancreas (30), also suggested that Rac effectors might play a role in pancreatic cancers.

Other investigators examined the effects of oncogenic K-rasG12V in normal pancreatic ductal epithelial cells after microinjection of physiologic levels of the mutant ras proteins. The primary cultured cells were stimulated from a quiescent state to enter into S phase, their cell size increased, and they replaced their major intracellular adhesion protein E-cadherin with N-cadherin typically found in mesenchymal cells (17). Thus, mutant K-ras induced changes in cell morphology, and cadherin switching is thought to be responsible for the enhanced cell motility and invasive capacities of malignant cells. Mirk/dyrk1B was found to be one of the four most promigratory genes in highly motile SKOV3 tumor cells by an RNAi screen of >5,200 genes (31). It is possible that some of the motility induced by oncogenic K-ras was due to Mirk activity.

Tumor cell survival is mediated by several signaling pathways, such as those initiated by oncogenic ras, including the PI-3K/Akt pathway and the MEK/Erk pathway (32–35). Pharmacologic inhibition of either pathway inhibited Mirk activity in this study. Tumor cell survival can be measured by clonogenic assays which identify the most aggressive cells within a population, not the

---

**Figure 5.** Analysis of the K-ras → Rac1 → Mirk signaling pathway. A, the HNF1-responsive β-fibrinogen reporter construct (β-28)3-Luc was cotransfected overnight in transient transfection assays in 293T cells with expression plasmids for HNF1 (H), Mirk and either the guanine nucleotide exchange factor Tiam1 or Tiam1 mutant at its ras-binding domain (TiamRBD). Columns, mean of triplicate measurements, normalized for cotransfected β-galactosidase, and representative of duplicate experiments; bars, SE. B, reporter assay in (A) repeated in Panc1 cells. C, the HNF1-responsive β-fibrinogen reporter construct (β-28)3-Luc was cotransfected overnight in transient transfection assays in Cos1 cells with expression plasmids for HNF1, Mirk, and oncogenic K-rasG12V, then treated for 12 h with increasing concentrations of LY294002 or PD98059. Columns, mean of triplicate measurements, normalized for cotransfected β-galactosidase, and representative of duplicate experiments; bars, SD. D, the HNF1-responsive β-fibrinogen reporter construct (β-28)3-Luc was cotransfected overnight in transient transfection assays in 293T cells with expression plasmids for HNF1 (H), Mirk (M), the constitutively active form of the jnk kinase MKK7 (MKK7D), the constitutively active form of the p38 MAP kinase kinase MKK6 (MKK6E), the constitutively active form of erk kinase MEK2 (MEK2E). Columns, mean of triplicate measurements, normalized for cotransfected β-galactosidase, and representative of duplicate experiments; bars, SD.
differentiated tumor cells with limited proliferative capacities. Clonogenic tumor cells can be serially passaged, whereas the more differentiated tumor cells cannot (36). The current studies showed that depletion of Mirk by RNA interference, followed by culture in the absence of serum mitogens to eliminate IGF-initiated survival pathways, decreased the clonogenic ability of Panc1 and AsPc1 pancreatic cancer cells by 8- to 12-fold. Depletion of K-ras under the same conditions led to a somewhat larger decrease in clonogenic capacity, and depletion of both Mirk and K-ras dramatically reduced colony formation. Tumor cells in vivo also display a hierarchical organization, with a small subpopulation of renewing cells and a larger population of tumor cells with limited proliferative capacity. Mirk could maintain the survival of the clonogenic cells within pancreatic ductal adenocarcinomas.

Acknowledgments

Received 11/9/2006; revised 4/24/2007; accepted 5/16/2007.

Grant support: NIH RO1 CA67405 (E. Friedman), the Jones-Rohner Endowment, and a grant from Amgen, Thousand Oaks, California. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


The Survival Kinase Mirk/Dyrk1B Is a Downstream Effector of Oncogenic K-ras in Pancreatic Cancer


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/15/7247

Cited articles  This article cites 35 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/15/7247.full#ref-list-1

Citing articles  This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/15/7247.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.