PRAS40 Deregulates Apoptosis in Malignant Melanoma

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Abstract

Malignant melanoma is the most invasive and deadly form of skin cancer with no effective therapy to treat advanced disease, leading to poor survival rates. Akt3 signaling plays an important role in deregulating apoptosis in ~70% of melanomas. Thus, targeting Akt3 signaling in melanoma patients has significant therapeutic potential for inhibiting melanomas, but no Akt3-specific chemotherapeutic agent exists. Unfortunately, nonspecific Akt inhibitors can cause systemic toxicity or increase metastasis. Identifying and targeting the Akt3 substrate that deregulates apoptosis might circumvent these complications but would require demonstration of its functional importance in disrupting normal apoptosis. In this study, PRAS40 was identified as an Akt3 substrate that deregulated apoptosis to promote melanoma tumorigenesis. Levels of phosphorylated PRAS40 (pPRAS40) increased during melanoma tumor progression paralleling increasing Akt3 activity. Majority of melanomas from patients with elevated Akt activity also had correspondingly higher levels of pPRAS40. Targeting PRAS40 or upstream Akt3 similarly reduced anchorage-independent growth in culture and inhibited tumor development in mice. Mechanistically, decreased pPRAS40 increased tumor cell apoptosis as well as sensitivity of melanoma cells to apoptosis-inducing agents, thereby decreasing chemoresistance. Collectively, these studies provide a solid mechanistic basis for targeting PRAS40 to inhibit the Akt3 signaling cascade and thereby retard melanoma development. [Cancer Res 2007;67(8):3626–36]

Introduction

Despite many clinical trials in melanoma testing a wide variety of nontargeted therapeutic approaches involving immunotherapy, radiotherapy, and chemotherapy, no effective long-term treatment has been identified for advanced-stage patients (1–3). This has led to the realization that targeted therapeutics are needed to inhibit the activities of specific genes or signaling pathways involved in the development of this disease. The Akt3 signaling pathway is one important therapeutic target deregulated in ~70% of advanced-stage tumors (4, 5). The generic Akt pathway initiates at the cell surface after growth factors or other extracellular stimuli activate phosphatidylinositol 3-kinase (PI3K). Activated PI3K generates a lipid second messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP3), causing translocation of Akt to the plasma membrane where it is activated through phosphorylation (6, 7). Cellular levels of PIP3 that regulate Akt function are in turn regulated primarily by a phosphatase called PTEN, which serves to lower its levels, thereby decreasing Akt activity (8, 9). Akt in turn phosphorylates >20 different substrates, thereby regulating the activities of proteins and signaling cascades controlling diverse cellular processes, such as cell proliferation and survival (5, 10, 11). Therefore, validation of the functional role of any Akt substrate in a tumorigenic process requires genetic and pharmacologic inhibition and demonstration of its relevance to the particular cellular process (5, 12–14).

Three Akt kinase family members, Akt1/protein kinase B (PKB) α, Akt2/PKBβ, and Akt3/PKBγ, have been identified in human cells sharing extensive structural similarity (15, 16). However, Akt3 is the predominant isoform active in advanced-stage melanomas compared with melanocytes or early-stage lesions that have relatively low levels of activity (4). Although mechanisms of Akt3 activation in melanoma cells remain to be fully elucidated, overexpression of Akt3 and decreased PTEN activity have been identified as playing important roles in this process (4, 9). Reduction of Akt3 activity using small interfering RNA (siRNA) or through PTEN expression decreased the tumorigenic potential of melanoma cells by increasing cellular apoptotic sensitivity (4). Thus, therapeutically targeting Akt3 in melanoma patients has potential of shrinking tumors, but high degree of structural homology to Akt1 and Akt2 has hindered development of agents specifically inhibiting Akt3 rather than targeting all three isoforms (17, 18). Untargeted Akt inhibitors, such as API-2 (triciribine, 1,3-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphthylen-3-amine), would then have the drawback of causing systemic toxicity (5, 19, 20). Furthermore, Akt inhibition has been suggested to lead to increased metastasis due to inhibition of proteins, such as NFAT (21). To overcome these limitations, targeting a direct substrate of Akt3 in melanomas might circumvent this complication. An illustrative example is targeting the Akt substrate mammalian target of rapamycin (mTOR) with the drug rapamycin, which is a promising therapeutic for breast and prostate carcinomas (22–24). However, for this type of therapeutic strategy to be effective for melanomas, the Akt3 substrate regulating apoptosis would need to be identified, which until now remained to be undertaken.

One recently identified Akt substrate is PRAS40 (proline-rich Akt substrate of 40 kDa; ref. 25). Although early studies are suggestive that it plays some role in cancer development, no study has shown its functional significance in tumorigenesis (26). Like the mTOR, PRAS40 is a cytosolic protein expressed by all tissues, with the highest levels occurring in the liver and heart (25). However, targeting mTOR with rapamycin has minimal manageable clinical side effects, raising the possibility that targeting PRAS40 might be equally effective (23, 27). PRAS40 contains ~15% proline residues in contrast to normal proteins having an average of 5% and has an Akt consensus phosphorylation site (RXRXXS/T) located at Thr246 (25). This site can be phosphorylated in vitro using purified Akt, undergoes enhanced phosphorylation when PRAS40 was transiently expressed with constitutively active Akt in HEK293 cells, and undergoes decreased phosphorylation in cells lacking Akt1 and Akt2 (25). Several stimuli enhance PRAS40 phosphorylation, including insulin,
nerve growth factor (NGF), and platelet-derived growth factor (28, 29). It also contains a consensus sequence for binding by 14-3-3 proteins (RXRPs/pT) within the Akt phosphorylation site, which may play a role in regulating its activity (25, 28). Inhibition of upstream PI3K using wortmannin or LY-294002 reduced PRAS40 phosphorylation, which could be reversed by expression of constitutively active Akt1 (25, 26, 29). Very little is currently known about the role of PRAS40 in cancer development, and it has no known role in melanoma.

In this study, we identified PRAS40 as an important substrate of the Akt3 kinase, which regulates the apoptotic sensitivity of melanoma cells, thereby promoting melanoma tumorigenesis. Levels of phosphorylated PRAS40 (pPRAS40) increased during melanoma tumor progression paralleling increasing Akt3 activity. The majority of melanomas from patients with high levels of phosphorylated (active) Akt also had correspondingly higher levels of pPRAS40. Targeting PRAS40 or inhibiting upstream Akt3 similarly increased melanoma cell apoptotic sensitivity, causing reduced anchorage-independent growth in culture and delayed tumor development in mice. Thus, PRAS40 is an important downstream target of Akt3 in melanomas, whose phosphorylation leads to pathway signaling that deregulates apoptosis.

Materials and Methods

Cell lines and culture conditions. The human melanoma cell line UACC 903, variants of this cell line expressing a chromosomal copy of PTEN (36A, 29A, and 37A), and revertant cell lines that had lost the introduced chromosomal PTEN (36A-R1 and 36A-R2) were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT; ref. 30). Melanocytes and melanoma cell lines WM35, WM321, WM981, WM115, and WM278 were maintained as described previously (4).

Western blot analysis. For Western blot analysis, cell lysates were harvested by addition of lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 0.1 mmol/L sodium molybdate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, and 5 µg/ml leupeptin. Whole-cell lysates were centrifuged (≥10,000 x g) for 10 min at 4°C to remove cell debris. Protein concentrations were quantitated using the bicinchoninic acid assay from Pierce (Rockford, IL), and 30 µg of lysate were loaded per lane onto NuPage gels from Life Technologies, Inc. (Carlsbad, CA). Following electrophoresis, samples were transferred to polyvinylidene difluoride membrane (Pall Corp., Pensacola, FL). The blots were probed with antibodies according to each supplier’s recommendations: antibodies to PRAS40 and pPRAS40 from Biosource International (Camarillo, CA); antibodies to hemagglutinin (HA), cyclin D1, p27, C-Raf, extracellular signal-regulated kinase (ERK) 2, and α-enolase from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies to Akt3, phosphorylated Akt (p-Akt; Ser473), phosphorylated ERK1/2, and cleaved and uncleaved caspase-3 from Cell Signaling Technology (Danvers, MA). Secondary antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology. Immunoblots were developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Ectopic expression studies using Akt3 constructs. HA-tagged wild-type (WT-Akt3), E40K (E40K-Akt3), myristoylated (myr-Akt3), and dead (Dead-Akt3) Akt3 constructs were generated as detailed previously and directionally subcloned into the pcDNA 3.1(+) puro vector between EcoRI (5’) and XhoI (5’) sites (4, 31). Constructs were introduced into 1 x 10⁶ WM35 cells via nucleofection using an Amaza Nucleofector (Amaza, Koeln, Germany) with the NHM-Neo selection and electroporation program U-20. For Western blot analysis of nucleofected WM35 cells, 1 x 10⁶ cells were seeded into 60-mm Petri dishes precoated with polyHEMA (Sigma Chemical Co., St. Louis, MO) and whole-cell lysates were collected in lysis solution 3 days later as described above.

siRNA protein knockdown studies. siRNA (50–300 pmol) was introduced into 1 x 10⁶ UACC 903 or WM115 cells via nucleofection with an Amaza Nucleofector using Solution R/program K-17 or NHM-Neo Solution/U-20 program, respectively. Transfection efficiency was >90%. Following nucleofection, cells were replated in culture dishes for 48 h after which protein lysates were harvested for Western blot analysis. Duplexed Stealth siRNA (Invitrogen) was used for these studies. The siRNA sequences used were as follows: PRAS40 #1, CCCUACCCACACGACGACCCCAA; PRAS40 #2, CCAGAAGCUGGAGGCGAUAUGA; C-RAF, GGCUAAUGGGAAUUGAGC; AKT3, GCCACGUCAAAUUCGGCGAGAG; AKT2, GGCCGGCGCAUGUGACGUAUUACGUAU; and SCRAMBLED, AAUCGACAGGUGUCAGUGAGA.

To show down pAkt-mediated protein knockdown in cultured cells, 1 x 10⁶ UACC 903 cells were nucleofected with 100 pmol of siPRAS40 #1 or siPRAS40 #2 and replated in culture dishes and protein lysates were harvested 2, 4, 6, 8, and 10 days later for Western blot analysis to determine PRAS40 protein levels in cells. ERK2 served as a control for protein loading on Western blots. Cells nucleofected with a scrambled siRNA served as control.

Anchorage-independent growth and apoptosis studies. For anchorage-independent growth and apoptosis studies, 200 pmol of siRNA against PRAS40 or Akt3 were nucleofected into UACC 903 cells and replated in DMEM supplemented with 10% FBS. Forty-eight hours later, cells were trypsinized and plated into polyHEMA-coated 96-well plates at a density of 2-3 x 10⁴ per well in 100 µL serum-free medium. Number of viable cells after 3 days of growth or serum-free medium was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenoxyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI). For Western blot analysis, 1 x 10⁶ cells were harvested in serum-free medium for 3 days after nucleofection and protein lysates were collected as described above. Protein lysates obtained from buffer and scrambled siRNA-nucleofected cells served as controls. To measure apoptosis rates of cells growing anchorage independently, a caspase-3/7 assay was carried out using the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega). Three days after plating 2 x 10⁴ cells per well in a polyHEMA-coated 96-well plate, 100 µL of Apo-ONE Caspase-3/7 reagent substrate (Z-DEVD-Rhodamine 110, diluted 100 times in Apo-ONE Caspase-3/7 buffer) were added. After a light-protected 4-h room temperature incubation, liberated Rhodamine 110 product was measured using the SpectraMax M2 plate reader operating with SoftMax Pro version 5 software (Molecular Devices Corp., Sunnyvale, CA) at an excitation wavelength of 485 nm and emission wavelength of 520 nm. Results were expressed as relative fluorescent units. Cells transfected with buffer only or scrambled siRNA served as controls.

Studies involving human melanoma tumors. Melanomas from human patients were collected according to protocols approved by the Penn State Human Subjects Protection Office and Cooperative Human Tissue Network. Protein lysates were extracted from tumors containing >60% tumor material as described previously (4) and analyzed by Western blotting to measure levels of pAkt and pPRAS40. Protein levels were normalized to α-enolase expression, and three independent blots were quantified using FujiFilm Science Lab 2001 Image Gauge (version 4.0; Fuji Photo Film Co. Ltd., Tokyo, Japan) to establish the average levels of phosphorylation, which are listed. Correlation between pAkt and pPRAS40 levels was assessed by Pearson's correlation analysis.

Pharmacologic inhibition of PI3K or Akt. PI3K was inhibited in UACC 903 cells using 50 µmol/L LY-294002 from Alexis Biochemicals (San Diego, CA). Cells (2.5 x 10⁵) grown in DMEM supplemented with 10% FBS were plated in 60-mm culture dishes, and after 48 h of growth, cells were serum starved for 24 h. Cells were then treated with either 50 µmol/L LY-294002 or DMVO vehicle in medium lacking serum for 1 h followed by serum stimulation for 30 and 60 min (9, 32). Akt was inhibited in UACC 903 and WM115 cells using API-2 from Tocris Bioscience (Ellisville, MO). For this study, UACC 903 cells (2.5 x 10⁵) were plated in 60-mm dishes in DMEM containing 10% FBS for 48 h and then serum starved for 24 h. Cells were then treated with 100 µmol/L API-2 or DMVO vehicle in medium lacking serum for 48 h followed by stimulation with serum-containing medium for 15 or 30 min (33). Similarly for studies involving the WM115 cell line,
2.5 \times 10^6 cells were grown in high-glucose DMEM supplemented with 10% FBS and treated with AP2 (4, 9). Protein lysates were analyzed by Western blotting. Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) was inhibited by treating UACC 903 with U0126 from Cell Signaling Technology. UACC 903 cells (2.5 \times 10^6) were plated in 60-mm dishes in DMEM containing 5% FBS and treated with 1, 5, 10, and 25 \mu M concentrations of U0126 for 48 h. Protein lysates were harvested and analyzed by Western blotting (34, 35).

**Apoptotic sensitivity assessments using staurosporine.** UACC 903 cells containing an introduced chromosomal copy of PTEN (29A, 36A, and 37A) and revertant 36A-R1 and 36A-R2 cells that had lost the introduced PTEN were treated with 1 \mu M staurosporine (BIOMOL International, Plymouth Meeting, PA) or with DMSO vehicle for 4 h and proteins were harvested for Western blotting (9). After 2 days of recovery in DMEM supplemented with 10% FBS, UACC 903 and WM115 cells nucleofected with siRNA against PRAS40 or Akt3 were treated with 0.4 \mu M/\text{L} staurosporine for 4 h. Protein lysates were collected for Western blotting to measure levels of cleaved caspase-3, a marker of apoptosis.

**Tumorigenicity assessments, knockdown of protein expression, and measurement of proliferation/apoptosis rates in tumors.** Animal experimentation was done according to protocols approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University College of Medicine. Tumor kinetics were measured by s.c. injection of 1 \times 10^6 UACC 903 cells nucleofected with siRNA (siScrambled, siAkt3, siC-Raf, siPRAS40 #1, and siPRAS40 #2) in 0.2 ml of DMEM supplemented with 10% FBS above both left and right cage rib caps of 4- to 6-week-old female nude mice (Harlan Sprague Dawley, Indianapolis, IN). Dimensions of developing tumors were measured using calipers on alternate days up to day 17.5. To measure siRNA-mediated knockdown of protein expression in tumors developing in animals, 5 \times 10^6 UACC 903 cells nucleofected with siRNA to Akt3 or 10 \times 10^6 cells nucleofected with siPRAS40 (si1 and si2) were s.c. injected into nude mice. Six and 8 days later, tumors were removed from euthanized mice, flash frozen in liquid nitrogen, and pulverized and protein lysates were collected by the addition of 600 to 800 \mu L of protein lysis buffer [50 mmol/L Tris-HCl (pH 7.5) containing 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 50 mmol/L sodium fluoride, 10 mmol/L sodium \beta-glycerol phosphate, 5 mmol/L sodium pyrophosphate, 1 mmol/L activated sodium orthovanadate, protease inhibitor cocktail from Sigma, 0.1% (v/v) 2-mercaptoethanol]. Protein lysates were quantitated using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) followed by Western blotting to measure levels of Akt3 protein in tumors. For mechanistic studies, 5 \times 10^6 UACC 903 cells nucleofected with control siRNA, si-C-Raf, si-Akt2, or si-Akt3 or 20 \times 10^6 cells nucleofected with siRNA to PRAS40 (si1 or si2) were injected into nude mice to generate tumors of the same size developing at parallel time points. Size- and time-matched tumors were harvested 4 days later to assess changes in cell proliferation and apoptosis. Apoptosis and cell proliferation rates were measured in formalin-fixed, paraffin-embedded tumor sections using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) TMR Red Apoptosis kit from Roche (Mannheim, Germany) or purified mouse anti-human Ki-67 from PharMingen (San Diego, CA), respectively (4, 9). A minimum of six different tumors with four to six fields per tumor was analyzed, and results were represented as the average ± SE.

**Statistical analysis.** Statistical analysis was undertaken using the one-way ANOVA followed by the Dunnett’s post hoc test. Correlation assessment of \(p\)-Akt and pPRAS40 levels in protein lysates extracted from patient melanomas was undertaken using the Pearson’s correlation analysis. Results were considered significant at a \(P\) value of <0.05.

**Results.**

**Levels of pPRAS40 increase during melanoma tumor progression regulated by Akt3 signaling.** Akt controls the activity of many substrate proteins through phosphorylation, which in turn regulate multiple cellular processes (12, 16, 36, 37). To test the hypothesis that PRAS40 is the Akt3 substrate promoting melanoma cell survival, Akt activity and levels of pPRAS40 were initially measured using a model containing low-passage cell lines derived from human melanomas in different stages of melanoma tumor progression. Specifically, melanocytes were compared with low-passage cell lines established from primary human melanoma tumors at the radial (WM35 and WM3211) and vertical (WM115, WM98.1, and WM278) stages of growth as well as in a cell line derived from a metastatic melanoma (UACC 903). As reported previously, increased Akt3 expression was observed in more advanced tumors leading to increased phosphorylated (active) Akt (Fig. 1A; ref. 4). Melanocytes had low levels of pAkt and pPRAS40, one of the two radial growth phase (WM3211) and all three of the vertical growth phase as well as the metastatic cell line had elevated pAkt and pPRAS40 (Fig. 1A). Presence of increased pPRAS40 during the radial growth phase suggested that Akt and PRAS40 activity increased during this early stage in melanoma tumor progression (Fig. 1A). Increased PRAS40 expression was also found in four of the five cell lines having elevated pAkt. Thus, elevated Akt activity was accompanied by increased pPRAS40 in this in vitro melanoma tumor progression model.

**Overexpression of WT or active Akt3 increases levels of pPRAS40 in melanoma cells.** To show that Akt3 activity directly regulated levels of pPRAS40 in melanoma cells, HA-tagged Akt3 constructs expressing WT or active Akt3 were nucleofected into WM35 cells to mimic increased Akt3 expression (activity) observed in more aggressive melanoma cells (Fig. 1A). WM35 cells have very low endogenous levels of pAkt and pPRAS40, making this cell line ideal for ectopic Akt3 expression studies (Fig. 1A and B; ref. 4). Two constructs expressing Akt3 with different levels of activity were used: myr-Akt3 that is highly active or mutant E40K-Akt3 having only slightly elevated Akt3 activity that is within the normal physiologic range present in cells.6 Blots probed with HA or Akt3 showed equal expression of the ectopically expressed protein (Fig. 1B). Compared with catalytically inactive (dead) Akt3 protein expression, WT or active Akt3 constructs led to increased pAkt. Furthermore, amount of pPRAS40 correlated significantly with level of Akt3 activity in the cells (Fig. 1B). Expression of myr-Akt3 led to highest levels of pPRAS40 followed by E40K-Akt3 and lastly by WT Akt3 that had the lowest levels. Signaling specificity to the PI3K signaling cascade was shown by comparing changes in pAkt and pPRAS40 to activity or expression of proteins in the MAPK signaling cascade. No changes were detected in levels of phosphorylated ERK1/2 (pERK1/2) or expression of cyclin D1 or p27. Thus, increased Akt3 expression in cultured melanoma cells elevated cellular Akt activity, leading to higher levels of PRAS40 phosphorylation.

**Increased Akt activity in tumors from melanoma patients is accompanied by a corresponding increase in pPRAS40.** Because the foregoing experiments identified PRAS40 as a substrate protein phosphorylated by Akt3, studies next focused on establishing whether PRAS40 phosphorylation paralleled increased Akt activity observed in tumors from melanoma patients. A collection of 30 flash-frozen metastatic melanomas removed from patients was examined by Western blotting to measure the relative intensity of pAkt and pPRAS40 to determine whether phosphorylation levels paralleled one another. Prior studies using this tumor panel showed increased Akt3 expression or activity compared with

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6 M. Cheung et al. Cooperation between mutant (V600E) B-Raf and Akt3 promote melanoma development, submitted for publication.
Levels of pPRAS40 increase during melanoma tumor progression regulated by Akt3 pathway signaling. A, expression and phosphorylation of Akt3 and PRAS40 increase during melanoma tumor progression with highest levels occurring in advanced-stage tumors. Compared with melanocytes, low-passage melanoma cell lines established from primary tumors at the radial (RGP; WM35 and WM3211), vertical (VGP; WM115, WM98.1, and WM278), and metastatic (MM; UACC 903) stages of tumor progression had significantly higher levels of pAkt and pPRAS40. More advanced tumors also had increased PRAS40 expression. ERK2 served as a control for protein loading. B, PRAS40 is a direct substrate of Akt3 in melanomas. Western blotting (WB) shows that overexpression of WT or active forms of Akt3 (E40K-Akt3 and myr-Akt3) in WM35 cells leads to increased levels of pAkt and pPRAS40 compared with inactive Akt3 (Dead-Akt3) or vector controls. Western blots probed with anti-HA or anti-Akt3 antibody indicate equal ectopic protein expression from constructs. WT or active forms of Akt3 lead to increased pAkt and pPRAS40. E40K-Akt3 has Akt3 activity at levels within the normal physiologic range of cells. Levels of total PRAS40 were unaffected, as too were amounts of pERK1/2, cyclin D1, and p27 in cells. ERK2 served as a control for protein loading. C, increasing pAkt levels in tumors from human melanoma patients are accompanied by a corresponding increase in amount of pPRAS40. Protein lysates derived from 30 metastatic melanoma patients analyzed for pAkt and pPRAS40 levels by Western blotting showed increased amounts of pPRAS40 accompanying increasing pAkt levels. Values are quantitative averages of pAkt and pPRAS40 normalized to a-enolase (protein loading control) from three independent experiments. Three tumors (arrows) had elevated pAkt but low pPRAS40, whereas eight tumors with low pAkt had significant pPRAS40 levels.
cell line was determined empirically to identify two time points at which Akt signaling was significantly stimulated. Western blot analysis showed that exposure of UACC 903 cells to LY-294002 or API-2 but not to U0126, a MAPK pathway inhibitor (data not shown), reduced levels of pAkt and pPRAS40 in both cell lines (Fig. 2A and B). Interestingly, inhibition of PI3K with LY-294002 caused a decrease in Akt3 expression and increased levels of PRAS40 protein (Fig. 2A). However, because this change was not observed following Akt inhibition using API-2 (Fig. 2B), it was considered as a pharmacologic phenomenon related to LY-294002 and not investigated further. Thus, pharmacologic inhibition of upstream PI3K or Akt3 activity in melanoma cells reduced levels of pPRAS40.

**siRNA-mediated inhibition of Akt3 decreased levels of pPRAS40 in melanoma cells.** Because Akt3 is the predominant Akt isoform active in melanoma cells, we next examined whether siRNA-mediated inhibition of Akt3, but not Akt2, decreased pPRAS40 (4). Increasing concentrations of Akt3 or Akt2 siRNA (50–300 pmol) were nucleofected into melanoma cell lines (UACC 903 and WM115), and levels of pAkt and pPRAS40 were measured by quantification of Western blots (Fig. 2C). Levels of protein

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**Figure 2.** Pharmacologic or siRNA-mediated inhibition of Akt3 signaling reduced PRAS40 phosphorylation in melanoma cells. A, LY-294002, an inhibitor of PI3K, inhibits pAkt and pPRAS40 levels in UACC 903 cells. PI3K pathway activity was ensured by serum starving cells for 24 h followed by exposure to DMEM supplemented with 10% FBS ± 50 μmol/L LY-294002 for 15 or 30 min. Controls were untreated cells or cell exposed to vehicle (DMSO). Western blot analysis shows absence of pAkt and pPRAS40 in LY-294002–treated cells but not in DMSO or untreated cells. ERK2 served as a control for protein loading. B, Akt inhibitor API-2 reduces pAkt and pPRAS40 levels in melanoma cells. UACC 903 and WM115 cells were serum starved for 24 h followed by exposure to 100 μmol/L API-2 in serum-free medium for 48 h. Cells were then stimulated with DMEM containing 10% FBS for the indicated time (5–30 min), and protein lysates were harvested from cells for Western blot analysis. pAkt and pPRAS40 levels decreased significantly in API-2–treated cells but not in untreated or DMSO vehicle-treated cells. ERK2 served as a control for protein loading. C, siRNA-mediated knockdown of Akt3, but not Akt2, reduced pAkt and pPRAS40 levels in melanoma cells. Expression of Akt3 or Akt2 in UACC 903 and WM115 was inhibited using siRNA targeting each respective isoform. siRNA was introduced by nucleofection using an Amaxa Nucleofector. Increasing concentrations of each respective siRNA were used (50, 100, 150, 200, 250, and 300 pmol), resulting in a dose-dependent protein inhibition. To control for amount of siRNA introduced into cells, scrambled siRNA (siScram.) was added to give a total of 300 pmol. Addition of 300 pmol of scrambled siRNA had no significant effect on levels of pAkt or pPRAS40. Four days after nucleofection, protein lysates were harvested and analyzed by Western blotting. Protein band intensity for Akt3, Akt2, pAkt, PRAS40, or pPRAS40 was measured and normalized against α-enolase for protein loading. Knocking down Akt3, but not Akt2, protein expression reduced levels of pAkt and pPRAS40.
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expression or phosphorylated protein in each lane were normalized against an α-enolase loading control. Akt2 knockdown served as a control because it is activated or amplified in several cancer types but not in melanoma (38–41). Increasing concentrations of siRNA against Akt3 or Akt2 led to a concentration-dependent decrease in expression of each respective protein (Fig. 2C). In contrast to Akt2 reduction, decreased Akt3 expression caused a dose-dependent decrease in both pAkt and pPRAS40 in UACC 903 and WM115 cells. No change was detected in level of total PRAS40 following knockdown of expression of either protein (Fig. 2C). Thus, siRNA-mediated inhibition of Akt3, but not Akt2, reduced levels of pPRAS40 in melanoma cells, showing that Akt3 regulates PRAS40 phosphorylation in melanoma cells.

siRNA-mediated inhibition of PRAS40 retards anchorage-independent growth by increasing apoptosis in melanoma cells. To investigate the functional role of PRAS40 in melanoma development, siRNA was used to inhibit protein expression in cells and effect on anchorage-independent growth and apoptosis was measured. Knockdown of Akt3 protein expression (activity) served as a control to show the consequence of shutting down the signaling cascade upstream of PRAS40. Following introduction of siRNA, cells were seeded in polyHEMA-coated 96-well plates, and 3 days later, anchorage-independent cell viability and apoptosis rates were measured. Western blot analysis confirmed knockdown of PRAS40 and Akt3 protein expression and showed corresponding increases in cleaved caspase-3 levels, suggesting an increase in levels of apoptosis (Fig. 3A). This possibility was further supported following detection of increased cellular apoptosis observed by measuring changes in levels of caspase-3/7 in cells in which siRNA had been used to decrease PRAS40 or Akt3 protein levels (P < 0.05, one-way ANOVA; Fig. 3B). Furthermore, anchorage-independent cell viability measured using the MTS assay decreased in a statistically significant (P < 0.05, one-way ANOVA) manner following reduction of PRAS40 or Akt3 protein levels (Fig. 3C). Similar results were observed when PRAS40 was knocked down using two different siRNA molecules compared with buffer only or scrambled siRNA controls. Mechanistically, cells, in which PRAS40 or Akt3 protein expression had been reduced, had significantly higher levels of cleaved caspase-3 and cellular apoptosis, suggesting that increased apoptosis was contributing to decreased anchorage-independent growth (Fig. 3A and B), which was subsequently confirmed in tumors developing in animals where PRAS40 has been targeted. Thus, reduction of PRAS40 or upstream Akt3 reduced anchorage-independent growth in a similar fashion by increasing levels of cellular apoptosis.

Inhibiting Akt3 signaling through PTEN expression or siRNA-mediated reduction of pPRAS40 increased apoptotic sensitivity of melanoma cells. One mechanism leading to deregulated Akt3 activity in melanomas involves decreased PTEN activity (through PTEN loss; ref. 9). To determine whether PTEN loss and consequently increased Akt3 activity also elevated levels of PRAS40 phosphorylation, PTEN expression as well as levels of pAkt and pPRAS40 were examined in a melanoma model in which PTEN protein was initially expressed from an introduced chromosome in melanoma cells. siRNA-mediated reduction of pPRAS40 increased apoptotic levels of cellular apoptosis.

Figure 3. siRNA-mediated inhibition of PRAS40 reduces anchorage-independent growth by increasing cellular apoptosis. A, siRNA-mediated knockdown of PRAS40 increases levels of cleaved caspase-3. Following siRNA-mediated knockdown of Akt3 or PRAS40, protein lysates were harvested from cells growing 5 d in polyHEMA-coated plates for Western blot analysis. Increased levels of cleaved caspase-3 (an apoptotic marker) were observed in cells in which PRAS40 or Akt3 protein expression had been reduced. ERK2 served as a control for protein loading. B, reducing PRAS40 expression increases apoptosis during anchorage-independent growth. siRNA (200 pmol) was nucleofected into UACC 903 cells, and following recovery, cells were plated into polyHEMA-coated 96-well plates. Three days later, amount of cellular apoptosis was measured by estimating caspase-3/7 levels using the Apo-ONE Homogeneous Caspase-3/7 Assay kit. Results were expressed as relative fluorescent units (RFU). Cells transfected with buffer only or a scrambled siRNA served as controls. Decreased levels of PRAS40 led to a significant ~25% increase in cellular apoptosis (P < 0.05, one-way ANOVA). C, selective knockdown of Akt3 and PRAS40 reduced anchorage-independent growth of melanoma cell. siRNA (200 pmol) was used to knock down Akt3 and PRAS40 protein levels in melanoma cell line UACC 903, which were then seeded (2 × 10^3 per well in 100 μL medium) into polyHEMA-coated 96-well plates. Three days later, anchorage-independent cell proliferation was measured using the MTS assay. A significant decrease of proliferating cells was observed (P < 0.05, one-way ANOVA) following knockdown of Akt3 (42%) or PRAS40 (21–29%). Cells transfected with universal buffer or scrambled siRNA served as controls.
significantly higher levels of pAkt and pPRAS40. Thus, decreased PTEN expression (activity) increased Akt3 activity with a corresponding increase in pPRAS40 (Fig. 4A). These results suggest that PRAS40 lies downstream of PTEN and Akt3 in the signaling pathway, making it an important Akt3 substrate for melanomas.

To determine whether altered levels of PRAS40 phosphorylation mediated by PTEN loss and/or increased Akt3 activity led to altered apoptotic sensitivity of melanoma cells, levels of cleaved caspase-3 were measured following exposure of cells to the apoptosis-inducing agent staurosporine (Fig. 4B). Elevated levels of cleaved caspase-3 indicated higher levels of apoptosis were observed in melanoma cells expressing PTEN (36A, 29A, and 37A), which had low levels of pAkt and pPRAS40 compared with 36A revertant cell lines or parental UACC 903 cells lacking the protein (Fig. 4B). Interestingly, staurosporine also reduced levels of pPRAS40 in all cells. Therefore, because cells containing PTEN had lower initial levels of pPRAS40, treatment with 1 μmol/L staurosporine for 4 h led to undetectable levels of pPRAS40. In contrast, pPRAS40 could be detected albeit at lower levels following staurosporine treatment of cells lacking PTEN protein, which had significantly higher initial levels of pAkt3 and pPRAS40 (Fig. 4B). No significant change was observed in levels of total PRAS40 protein.

To directly show that decreased pPRAS40 would increase apoptotic sensitivity of melanoma cells, siRNA was used to reduce expression of PRAS40 or Akt3 followed by exposure to 0.4 μmol/L staurosporine for 4 h (Fig. 4C). Compared with untransfected cells or cells nucleofected with control scrambled siRNA, reduced Akt3 or PRAS40 protein expression similarly increased cleaved caspase-3 levels in both UACC 903 and WM115 cells following staurosporine exposure (Fig. 4C). Collectively, these results indicate that increased PRAS40 phosphorylation, associated with PTEN loss and/or Akt3 activation, functions to decrease sensitivity of melanoma cells to apoptotic stimuli, thereby promoting chemoresistance to these agents.

siRNA-mediated inhibition of PRAS40 decreased the tumorigenic potential of melanoma cells. The role played by PRAS40 in melanoma tumorigenesis is currently unknown. To identify its functional role in melanoma tumorigenesis, siRNA was used to inhibit PRAS40 in UACC 903 cells s.c. injected into mice. Two different PRAS40 siRNAs were independently introduced into UACC 903 cells via nucleofection, resulting in transfection efficiencies of >90%, which effectively knocked down protein expression. siRNA against Akt3 was used to knock down protein expression, which was then used as a control. Decreased PRAS40 protein levels 48 h after nucleofection are shown in Fig. 5A. In contrast to protein knockdown observed following treatment of cells with siRNA against PRAS40 or Akt3, control scrambled siRNA or siRNA to C-Raf did not alter expression of either of these proteins. siRNA-mediated knockdown of PRAS40 persisted for >10 days in culture (Fig. 5B), which is similar to prior reports for...
duration of siRNA-mediated protein knockdown using the nucleo-
fection approach (4, 35, 42). Following s.c. injection of cells into
mice, size of developing tumors was measured on alternate days up
to 17.5 days following nucleofection (Fig. 5C). A reduction in
tumorigenic potential was observed in UACC 903 cells in which
PRAS40 expression had been decreased similar to that occurring
following Akt3 protein knockdown (Fig. 5C). In contrast, siRNA-
mediated inhibition of C-Raf, a scrambled siRNA, or buffer controls
did not alter tumor development. Tumors removed from animals
7.5 and 9.5 days after nucleofection of siRNA into the cells showed
decreased PRAS40 protein expression compared with a tumor
formed from cells nucleofected with a scrambled siRNA control
(Fig. 5D). Furthermore, decreased PRAS40 protein expression led to
a corresponding reduction in pPRAS40 protein present in tumors.
Thus, inhibition of PRAS40 or upstream Akt3 in melanoma cells
similarly reduced the tumorigenic potential of melanoma cells.

PRAS40 regulates tumor development by mediating cellular
apoptosis in the tumor environment. Because inhibition of
PRAS40 significantly reduced tumor development, subsequent
studies focused on identifying the mechanism by which inhibition
occurred. Rates of tumor cell apoptosis and cell proliferation were
compared in size- and time-matched tumors following siRNA-
mediated inhibition of PRAS40 compared with control cells
nucleofected with buffer, siRNA against C-Raf, or Akt2. Magnitude
of apoptosis assessed by TUNEL and proliferation measured using
Ki-67 immunohistochemistry were examined (Fig. 6A and B).
UACC 903 cells nucleofected with siRNA to PRAS40 or Akt3 had 15-
to 20-fold more TUNEL-positive cells compared with control cells
treated with buffer only, siRNA to Akt2, or C-Raf (P < 0.05, one-way
ANOVA; Fig. 6A). In contrast, no statistically significant difference
was observed in rates of cellular proliferation in nonapoptotic
tumor areas, which remained between 6% and 9% (P > 0.05, one-
way ANOVA; Fig. 6B). Thus, targeting PRAS40 or upstream Akt3
similarly inhibited melanoma tumor development by increasing
apoptosis levels but not by altering the proliferation rates in
melanoma tumors.

Figure 5. siRNA-mediated inhibition of PRAS40 or upstream Akt3 retards melanoma tumor development in nude mice. A, siRNA targeting PRAS40 or Akt3 reduced
protein expression in cultured melanoma cells. Amaxa nucleofection was used to introduce siRNA into UACC 903 cells. Western blot analysis of protein lysates
harvested 2 d after nucleofection showed significant reduction of protein levels. ERK2 served as a control for protein loading. Controls were untransfected cells,
cells nucleofected with buffer, scrambled siRNA, or siRNA against C-Raf. B, siRNA-mediated knockdown of PRAS40 protein persists beyond 10 d in cultured
cells. One million UACC 903 cells were nucleofected with 100 pmol of siPRAS40 #1 or siPRAS40 #2 and replated in culture dishes, and protein lysates were harvested
2, 4, 6, 8, and 10 d later for Western blot analysis to determine PRAS40 protein levels in cells. Decreased PRAS40 protein levels were observed up to 10 d after
nucleofection into cells. ERK2 served as a control for protein loading on Western blots, whereas scrambled siRNA served as a control for RNA interference specificity.
C, inhibition of PRAS40 or upstream Akt3 significantly reduced melanoma tumor development. siRNA against PRAS40, upstream Akt3, C-Raf, or scrambled
siRNA was introduced into UACC 903 (white arrow), and 36 h later, cells were s.c. injected into nude mice (black arrow). Inhibition of PRAS40 or upstream Akt3 led to a
significant delay in tumor development compared with controls. Control cells were nucleofected with buffer only, a scrambled siRNA, or siRNA against C-Raf.
Points, mean of a minimum of 12 injection sites in six mice from two separate experiments; bars, SE. D, siRNA targeting PRAS40 reduced protein expression in
developing melanoma tumors. UACC 903 cells in which PRAS40 had been targeted using siRNA were s.c. injected into nude mice, and tumors were removed
6 or 8 d later. This is equivalent to 7.5 or 9.5 d after nucleofection of siRNA in the cells. Western blot analysis of tumor protein lysates showed significant reduction in
expression of PRAS40. Untransfected cells or cells nucleofected with scrambled siRNA were used as controls. ERK2 served as a protein loading control.
Discussion

Deregulation of Akt3 pathway signaling occurs in ~70% of melanomas functioning to promote tumorigenesis by deregulating apoptosis (4). Validating the role of any downstream Akt3 substrate in apoptosis deregulation, therefore, requires genetic and pharmacologic inhibition to validate its role in this process (5, 12, 43, 44). In this study, PRAS40 is shown to be an Akt3 substrate that deregulates apoptosis to promote melanoma development and chemoresistance to apoptosis-inducing agents. Thus, this is the first report to document a functional role for PRAS40 in melanoma development.

Figure 6. Inhibition of PRAS40 increases cellular apoptosis without altering proliferation in the tumor environment. A, increased apoptosis occurring following siRNA-mediated inhibition of PRAS40 decreased melanoma tumor development. TUNEL-positive cells present in temporally and spatially matched tumors 4 d after s.c. injection in mice were quantified to measure tumor apoptosis levels. A 15- to 20-fold increase in TUNEL-positive cells occurred following siRNA-mediated inhibition of PRAS40 or Akt3 but not Akt2, C-Raf, or buffer transfected controls. Columns, mean from six different tumors with four to six fields counted per tumor; bars, SE. B, no significant change was detected in tumor cell proliferation following inhibition of PRAS40 or upstream Akt3. Ki-67–positive cells present in temporally and spatially matched tumors 4 d after s.c. injection in mice were quantified to assess levels of proliferation. No significant difference in proliferation rates was detected. Cells nucleofected with buffer or siRNAs against C-Raf or Akt2 were used as controls. Columns, mean from six different tumors with four to six fields counted per tumor; bars, SE.

Demonstration of PRAS40 as an Akt3 substrate in melanoma was established using siRNA and pharmacologic inhibition. Pharmacologically inhibiting Akt3 using API-2 or upstream PI3K using LY-294002 reduced levels of pPRAS40 in melanoma cells. In contrast, a MEK inhibitor, U0126, had no effect on levels of pPRAS40, indicating signaling pathway specificity. Other groups have similarly reported decreased phosphorylation of PRAS40 following exposure to PI3K inhibitor wortmannin or LY-294002 but not when using MAPK inhibitor PD98059 or U0126 (25, 26, 28, 29). Direct inhibition of Akt3 using siRNA specifically targeting this protein decreased levels of pPRAS40 similar to pharmacologic inhibition. In contrast, targeting Akt2 using siRNA had no discernible effect on pPRAS40 levels, which further showed involvement in the Akt3 signaling cascade. Thus, PRAS40 is a substrate downstream of Akt3 in melanomas.

Regulation of pPRAS40 levels by Akt3 during melanoma development was established using a cultured melanoma tumor progression model as well as in advanced melanomas removed from patients. Levels of pPRAS40 increased during melanoma tumor progression with lowest amounts detected in melanocytes and highest levels occurring in advanced-stage melanomas. This increase paralleled elevated Akt3 activity observed in more aggressive melanomas and could be observed both in cultured cell line progression models and in tumors taken directly from melanoma patients. The majority of melanomas from patients with high levels of phosphorylated (active) Akt have correspondingly higher levels of pPRAS40. The few patient melanomas that do not have elevated pPRAS40 likely have other yet to be identified genetic defects activating the signaling cascade downstream of PRAS40. Other groups have also reported elevated levels of pPRAS40 in cell lines established from breast and lung tumors compared with control tissue, further supporting an important role for PRAS40 in cancer development (26).

The importance of Akt3 deregulation for regulating pPRAS40 levels during melanoma development was shown using an ectopic expression model in which Akt3 was expressed in melanoma cells having very low endogenous levels of Akt3 activity and pPRAS40. Ectopically expressed Akt3 increased activity, leading to a proportional and corresponding increase in pPRAS40 levels. Increased Akt3 activity at physiologic levels following overexpression of normal WT or active E40K-Akt3 producing activity within the normal physiologic range led to higher pPRAS40 levels. Similar to these results, induction of a conditionally active myristoylated Akt1 in NIH3T3 cells led to increased phosphorylation of ectopically expressed PRAS40 (25). Thus, increasing Akt3 activity occurring during melanoma tumor progression leads to increased pPRAS40 levels.

The role played by PRAS40 in melanoma development was shown using siRNA-mediated inhibition to measure effect on the tumorigenic potential of melanoma cells. Two different siRNA molecules targeting PRAS40 at different sites reduced tumor development to levels similar to those occurring when Akt3 was targeted using siRNA. siRNA effectively reduced protein expression in cultured cells and significantly delayed tumor development in animals. Mechanism leading to tumor inhibition was established by comparison of apoptosis and proliferation in temporally and spatially matched tumors. Although PRAS40 inhibition did not affect proliferation, it did increase rates of apoptosis in the tumor by 15- to 20-fold compared with control cells in which siRNA was used to target Akt2 or C-Raf. Targeting PRAS40 also led to a ~30% decrease in anchorage-independent growth, which seemed to be
caused by increased levels of apoptosis, mediated through cleaved caspase-3. These observations were not surprising because we previously showed that Akt3 played a significant role in regulating apoptosis in melanomas (4). pPRAS40 has been previously linked to caspase-3-mediated apoptosis through studies investigating neuronal apoptotic cell death after transient focal cerebral ischemia (28, 45). No colocalization of pPRAS40 and caspase-3 could be detected in cells either undergoing apoptosis or progressing toward apoptosis, which indicated that pPRAS40 inhibits cellular apoptosis by preventing caspase-3 cleavage (28, 45). The mechanism and signaling cascade downstream of pPRAS40 regulating cellular apoptosis remains to be fully elucidated. However, PRAS40 is an Akt3 substrate deregulating apoptosis during melanoma tumor development by mediating levels of cleaved caspase-3.

Lack of therapeutic effectiveness in melanomas is due, in part, to deregulation of apoptosis (1, 5, 36). Because most chemotherapeutics function by triggering the apoptotic pathway, apoptotic deregulation in melanoma cells plays an important role in promoting chemoresistance to these agents (4, 36, 46, 47). The identity of PRAS40 as an Akt3 substrate promoting apoptotic deregulation and chemoresistance in melanoma cells is important for unraveling effective therapeutic strategies for melanoma patients. Here, cell-based models in which pPRAS40 levels were decreased, by PTEN expression or siRNA-mediated protein inhibition, were exposed to the apoptosis-inducing agent staurosporine, which is frequently used to establish cellular sensitivity to apoptosis-inducing agents in melanomas (9, 48). The presence of PTEN in melanoma cells significantly decreased pPRAS40 levels and sensitized cell to staurosporine-mediated apoptosis. Likewise, decreasing pPRAS40 protein levels using siRNA similarly increased rates of staurosporine-mediated apoptosis. The antiapoptotic activity of pPRAS40 mediated by NGF has been reported to play a neuroprotective role by triggering the apoptotic cell death occurring after transient focal cerebral ischemia (28, 45, 49, 50). Thus, increased PRAS40 phosphorylation promotes melanoma cell survival in the tumor environment as well as development of chemoresistance.

In conclusion, we identified PRAS40 as an Akt3 substrate deregulating apoptosis to promote melanoma tumorigenesis and chemoresistance. Targeting PRAS40 reduced the tumorigenic potential of melanoma cells and increased sensitivity to agents triggering apoptosis. In contrast, presence of high levels of pPRAS40 provided melanoma cells with selective survival advantage in the tumor environment and resistance to apoptosis-inducing agents. Thus, this study provides a mechanistic-based underpinning for therapeutically targeting PRAS40 to inhibit melanoma development by restoring normal apoptotic control and sensitizing cells to apoptosis-inducing chemotherapeutic agents.

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References


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