The Prolyl Peptidases PRCP/PREP Regulate IRS-1 Stability Critical for Rapamycin-induced Feedback Activation of PI3K and AKT

Received for publication, January 14, 2014, and in revised form, June 2, 2014, Published, JBC Papers in Press, June 16, 2014, DOI 10.1074/jbc.M114.550038

Lei Duan‡1, Guoguang Ying‡5, Brian Danzer‡, Ricardo E. Perez¶, Zia Shariat-Madar¶, Victor V. Levenson¶2, and Carl G. Maki¶3

From the §Department of Anatomy and Cell Biology, Rush University Medical Center, Chicago, Illinois 60612, the ‡Laboratory of Cancer Cell Biology, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, China, the ¶Department of Pharmacology, University of Mississippi, University, Mississippi 38677, and †US Biomarkers, Inc., Buffalo Grove, Illinois 60089-6726

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR) pathway conveys signals from receptor tyrosine kinases (RTKs) to regulate cell metabolism, proliferation, survival, and motility. Previously we found that prolylcarboxypeptidase (PRCP) regulates proliferation and survival in breast cancer cells. In this study, we found that PRCP and the related family member prolylendopeptidase (PREP) are essential for proliferation and survival of pancreatic cancer cells. Depletion/inhibition of PRCP and PREP-induced serine phosphorylation and degradation of IRS-1, leading to inactivation of the cellular PI3K and AKT. Notably, depletion/inhibition of PRCP/PREP destabilized IRS-1 in the cells treated with rapamycin, blocking the feedback activation PI3K/AKT. Consequently, inhibition of PRCP/PREP enhanced rapamycin-induced cytotoxicity. Thus, we have identified PRCP and PREP as a stabilizer of IRS-1 which is critical for PI3K/AKT/mTOR signaling in pancreatic cancer cells.

Results: PRCP and PREP regulate IRS-1 stability and PI3K/AKT activation in pancreatic cancer cells. Depletion/inhibition of PRCP/PREP suppresses rapamycin-induced activation of PI3K/AKT with consequent additive/synergistic cytotoxicity.

Conclusion: PRCP and PREP regulate IRS-1 stability and PI3K/AKT activation in pancreatic cancer.

Significance: Prolyl peptidases are potential therapeutic targets in pancreatic cancer.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR) pathway conveys signals from receptor tyrosine kinases (RTKs) to regulate cell metabolism, proliferation, survival, and motility of cancer cells (1–3). Insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) phosphorylate insulin receptor substrates (IRS) on tyrosines that recruit the PI3K p85 subunit to the cell membrane and subsequently activate the PI3K/AKT/mTOR signaling cascade (4, 5). Aberrantly activated PI3K/AKT/mTOR pathway is a frequent feature in most cancers, making it a potential therapeutic target (1, 3). Rapamycin, an inhibitor of mTORC1, was among the first inhibitors of the PI3K/AKT/mTOR pathway used for cancer treatment (2, 3). Unfortunately, rapamycin has shown a very modest effect in randomized clinical trials, most likely due to the feedback loop that activates IRS-1 and PI3K/AKT (6–8).

Pancreatic adenocarcinoma is one of the most lethal cancers because of its late detection and resistance to conventional chemotherapy (9–11). Current targeted therapeutics have very limited effect on pancreatic cancer patients (9, 12–14). Past research has linked insulin and IGF signaling to the tumorigenesis and progression of pancreatic cancer (15, 16). The PI3K/AKT/mTOR pathway is downstream of IR/IGF-1R and is associated with development and clinical aggressiveness of pancreatic cancer (17–20).

Previously, we identified prolylcarboxypeptidase (PRCP) in a genetic screen for factors that can promote tamoxifen resistance in the MCF7 breast cancer cell line (21). In that study, overexpression of PRCP promoted MCF7 proliferation and survival in the presence of tamoxifen, while knockdown or inhibition of PRCP had the opposite effect. PRCP is a serine peptidase of the prolyl peptidase family (22) that selectively cleaves the C-terminal proline peptide bond in its substrates. Another family member, prolyl endopeptidase (PREP), has a high degree of sequence similarity, and is phylogenetically close to PRCP (22). PREP cleaves known PRCP substrates angiotensin (Ang) II/III and α-melanocyte stimulating hormone (MSH) (23, 24), as well as neurotensin (NTS), cholecystokinin, and gastrin-releasing peptides (25). These peptides are ligands for a number of G-protein coupled receptors (GPCR) such as angiotensin receptors and neurotensin receptors which can cross talk with IR/IGF-1R, and play a critical role in pancreatic cancer development in animal models (26, 27). Based on these results, we hypothesize that PRCP and PREP regulate cell signaling in pan-
Prolyl Peptidases Regulate IRS-1 and PI3K/AKT

Cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 30 μg/ml of fungin and 10 μg/ml of penicillin, 10 g/ml of kanamycin, 10 μg/ml of streptomycin, 1% non-essential amino acids, 2 mM glutamine, 10% FBS, and 1% penicillin/streptomycin. The cells were harvested 72 h after transfection.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—Goat anti-PRCP antibody has been described (28). Mouse polyclonal anti-PRCP antibody was from Abcam, Inc (Cambridge, MA). Goat anti-human PREP antibody was from R&D Systems (Minneapolis, MN). Mouse anti-β-actin antibody, rabbit anti-IRS-1 antibody, rabbit anti-IGF-1R antibody, and protein G Plus-agarose were from Santa Cruz Biotechnology. Rabbit anti-phospho-AKT (S473), rabbit anti-phospho-IRS-1 (S636/639, S307), rabbit anti-phospho-p70S6K (T389), and p70S6K were from Millipore (Billerica, MA). MTT was from Sigma-Aldrich. Z-Pro-Proinal (ZPP) was from Biomol, Inc (Plymouth Meeting, PA). Z-Gly-Pro-Pro-AMC was from Bachem (Torrance, CA).

Cells— Panc-1, PK-9 (a generous gift from Dr. H. Arafat), and Capan-1 (a generous gift from Dr. S. Batra) pancreatic cancer cell lines were maintained in Dulbecco’s Modified Eagle’s medium, 2 mM glutamine, 1 mM non-essential amino acids, 10 units/ml of penicillin, 10 μg/ml of streptomycin (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 30 μg/ml of fungin and 10 μg/ml of plasmocin (InvivoGen, San Diego, CA).

Lentiviral Infection—The lentiviral pLKO-PRCP shRNA have previously described (21). The lentiviral pLKO-PRCP shRNA (puromycin resistant) were purchased from OpenBiosystems (clone ID TRCN0000050198 for PREP shRNA#1, TRCN0000050199 for PREP shRNA#2). To generate hygromycin-resistant pLKO-PRCP shRNA, a fragment of DNA that encompasses the PREP shRNA sequence was cut from the pLKO-PRCP shRNA plasmid using the restriction sites KpnI and BamH1 and ligated to the pLKO-hygro vector digested with the same restriction enzymes. The lentiviral packaging and envelope vectors psPAX2 and pMD2G (Addgene plasmid 12260 and 12259 deposited by Dr. Didier Trono) and the pLKO-control shRNA (Addgene plasmid 1864 deposited by Dr. David M. Sabatini) (2) were obtained from Addgene plasmid repository. Lentiviral supernatants for the expression of shRNAs were generated from 293FT cells, and psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259) packaging and envelope vectors according to the OpenBiosystems protocol. Pancreatic cancer cells were infected and selected with puromycin and/or hygromycin for 10 to 14 days to establish polyclonal lines.

Plasmid DNA, Mutagenesis, and Transfection—Full-length human PRCP (isoform A) gene was amplified from the described PFB-PRCP (21) construct and subcloned into pMScv vector using XhoI and EcoRI restriction sites. Enzyme-dead PRCP mutant was generated by substitution of Ser-179 and His-455 of the catalytic triad with alanine as described (29) by PCR using the QuickChange Site-directed Mutagenesis kit (Agilent Technologies). The primer sequence for S179A is ATTGCCATAGGAGGCGCTATAGGTGCC-ATGC and GCATGCCACCATTAGCGCCTCCTATGGC-AAT, and for H455A it is CGGAGATCTAAGTTGGCGGGCCCTCTCTAGAGAT and ATCTCAGAGGGGCCCGGCTCCCCATAGATCTCCG.

For transient expression of wild type and mutant PRCP, 10⁶ Panc-1 cells were plated in 60-mm dishes for 24 h and then transfected with 5 μg of plasmid DNA using Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer’s instructions. The cells were harvested 72 h after transfection.

RNA Isolation and Real-time Quantitative PCR Analysis—Total RNA was prepared using RNAqueous-4PCR Kit (Ambion, TX); the first cDNA strand was synthesized using RETROscript kit (Ambion, TX). The manufacturers’ protocols were followed in each case. The primers for qPCR were from the Primer Bank. The primers for PRCP (NM_0054040) are TCTACACTGGTAATGAAAGGGGAC and CCCCACATGAA- CCCCGTGTTA. The primers for PREP (NM_002726) are GAGACCGCCGTACAGAT and CCAGGCGTAAGG-GTCACAAA. The primers for IRS-1 are CTGCAACACCTGTGCTAAGG and GTGCTAGTCGTAAGTC. The primers for GAPDH (NM_002046) are CAGTGAAGATGTAGCA- ACAAGCTT and AGTCCTTTCCAGATACAAAGT. SYBR green PCR kit (Applied Biosystems) was used according to the manufacturer’s instructions. AB7500 system (in 9600 emulation mode) was used as follows: activation at 95°C; 2 min, 40 cycles of denaturation at 95°C; 15 s and annealing/extension at 60°C; 60 s, followed by melt analysis ramping from 60°C to 95°C. The amplification efficiency was determined using a dynamic range of 5 log₁₀ concentrations (100, 10, 1, 0.1, and 0.01) of cDNA of control cells. The standard curves were established by log₁₀ cDNA concentrations against the log₁₀ Ct values. The relative expression of each gene was calculated from average Ct values of triplicates using the standard curve equation and normalized against the GAPDH gene.

Picogreen Staining of Cellular DNA—The cells underwent three rounds of standard freeze and thaw followed staining with Picogreen (1:200 dilution in TE (pH 8.0) buffer) as described (21). The fluorescence intensity of Picogreen was determined using a BioTek Mx microplate reader with an excitation of 480 nm and an emission of 520 nm. The cell number in each well was calculated with a standard cell titration curve of Picogreen-stained cell lysates.

Clonogenic Assay—250 cells were plated in a 60-mm plate and allowed to recover overnight. The cells were then treated with vehicle or drugs for 3 days. The cells were allowed to recover for 2 weeks, fixed with alcohol, and stained with crystal violet (2% w/v) or methylene blue (1% in 50% ethanol). Colonies were counted.

Analysis of Cell Viability and Apoptosis—Cells grown in 96-well plates were incubated in phenol red free culture medium containing 1.2 mM MT T (100 μl/well) at 37°C for 4 hours as described (21). The cells were then lysed by addition of 100 μl of 10% SDS/0.01 M HCl to each well and incubation at 37°C for 12 h. The plates were read for absorbance at 570 nm by a microplate reader. To determine apoptosis, cells grown in 6-well plates were trypsinized, and fixed in 25% ethanol overnight, and stained with propidium iodide (25 μg/ml, Calbiochem). Apoptosis was determined by flow cytome-
Prolyl Peptidases Regulate IRS-1 and PI3K/AKT

Depletion/Inhibition of PRCP and PREP Suppresses AKT Phosphorylation and PI3K Activity—Next we analyzed activation of mitogen and survival signaling pathways in Panc-1 cells with or without depletion of PRCP and PREP. We focused on the PI3K/AKT pathway because aberrant activation of this pathway is strongly linked with pancreatic cancer cell survival and proliferation (17–20). AKT is activated by phosphorylation cells when treated with ZPP, suggesting inhibition of PRCP and PREP is toxic to pancreatic cancer cells.

To further test the role of PRCP and PREP in pancreatic cancer cells, we knocked-down each protein singly or in combination in each of the cell lines by infection with shRNA-encoding lentiviruses (scrambled-shRNA virus served as control). Proliferation of the control and knockdown (KD) cells was then assayed. Immunoblots confirmed PRCP and PREP were depleted in the single or double-knockdown (DKD) cells (e.g. Panc-1 cells, Fig. 2A). Depletion of PRCP did not affect PREP expression, and vice versa (Fig. 2A). All the cell lines with depletion of PREP showed complete loss of the cellular prolylendopeptidase activity analyzed using the fluorogenic substrate Gly-Pro-AMC (data not shown), functionally confirming that the cellular PREP is efficiently depleted by the PREP shRNA. Panc-1 cells depleted of either PRCP or PREP had a slight decrease in proliferation (Fig. 2B). However, depletion of both PRCP and PREP resulted in a complete or near-complete block in proliferation (Fig. 2B). Similarly, knockdown of either PRCP or PREP in PK-9 and Capan-1 cells caused a slight reduction in proliferation, while simultaneous knockdown of both PRCP and PREP significantly decreased proliferation (Fig. 2B). Transient (72 h) treatment with ZPP also reduced colony formation in Panc-1 cells (Fig. 2C). The results suggest PRCP and PREP are required for proliferation and survival in pancreatic cancer cell lines. Finally, we wished to determine if the reduction in cell viability upon PRCP/PREP inhibition was associated with apoptosis. To this end, Panc-1 cells were treated with different doses of ZPP for 3 days and analyzed for apoptosis by propidium iodide (PI) staining and FACS analysis for the subdiploid cell population (32). The results showed that ZPP induced apoptosis of Panc-1 cells in a dose-dependent manner (Fig. 2D).
at serine 473 (Ser-473). We found that AKT phosphorylation at Ser-473 was slightly reduced in Panc-1, PK9, and Capan-1 cells with single knockdown of PRCP but not PREP, and was significantly reduced by depletion/knockdown of both PRCP and PREP (Fig. 3A). On the other hand, transient overexpression of wild-type PRCP, but not the enzyme-dead mutant PRCP (29), increased phosphorylation of AKT (Fig. 3B). Inhibition of PRCP and PREP by ZPP also caused a decrease in AKT phosphorylation in all the three cell lines (Fig. 3C). These findings indicate inhibition and/or knockdown of PRCP/PREP reduces levels of activated AKT in these cells. However, inhibition of AKT by the specific inhibitor MK2206 showed only modest reduction in Panc-1 cell viability by MTT assay (Fig. 3D and E), and MK2206 (10 μM) did not induce apoptosis in Panc-1 cells (data not shown). These findings suggest reduced cell viability by PRCP/PREP knockdown or inhibition cannot be explained by AKT inhibition alone.

AKT activation is dependent on PI3K activity. To test if PRCP/PREP regulates PI3K, whole cell lysates of Panc-1 cells depleted of PRCP/PREP or treated with ZPP and controls were immunoprecipitated with anti-p85 (PI3K active subunit) antibodies. Whole cell lysates of Panc-1 cells treated with the chemical PI3K inhibitor LY294002 were also immunoprecipitated as a control. The resulting immunoprecipitates were then analyzed for PI3K activity. Immunoblots showed comparable levels of p85 were immunoprecipitated under each

FIGURE 2. Depletion or inhibition of PRCP and PREP reduces pancreatic cancer cell proliferation and viability. A, whole cell lysates of Panc-1 cells with expression of scrambled shRNA (control), PRCP shRNA #1 or #2 (PRCP KD1 or KD2), PREP shRNA #1 or #2 (PREP KD1 or KD2), and expression of both PRCP shRNA #1 and PREP shRNA #1 (DKD) were immunoblotted for PRCP, PREP, and β-actin. B, Panc-1 (left), PK-9 (middle), and Capan-1 (right) cells with or without depletion of PRCP and/or PREP were grown in 96-well plates and harvested at the indicated days. Cellular DNA was stained with Picogreen, and average fluorescence signal from octuplicate was normalized to that harvested at day 1 and presented as a graph with standard deviation indicated (representative of three independent experiments). There are significant differences between control cells and the cells with depletion of PRCP and/or PREP (all p values are less than 0.005). C, Panc-1 cells in triplicate were treated with the indicated doses of ZPP for 3 days and recovered for 2 weeks. The average numbers of colonies formed on the plates were presented as a graph with standard deviation indicated (representative of three independent experiments). There are significant differences between ZPP (50 μM and above, p < 0.01)-treated and vehicle (DMSO)-treated cells. D, Panc-1 cells in triplicate were treated with indicated doses of ZPP for 3 days. The cells were stained with PI and analyzed for the subdiploid population. The percentage of the subdiploid cells were presented as a graph with standard deviation indicated (representative of three independent experiments). There are significant differences between ZPP (50 μM and above, p < 0.01)-treated and vehicle (DMSO)-treated cells.
condition (Fig. 4, upper). The results showed that PI3K activity was significantly lower ($p < 0.01$) in the cells depleted of PRCP/PREP or treated with ZPP, and was completely inhibited in the cells treated with LY294002 (Fig. 4A, lower). To test if inhibition of PI3K affects cell viability, Panc-1, PK-9, and Capan-1 cells were treated with different doses of LY294002 for 4 days and subsequently analyzed by MTT assay. The results showed a dose-dependent reduction in viable cells in all the three pancreatic cancer cell lines treated with LY294002 for 4 days and subsequently analyzed by MTT assay (Fig. 4B).

Inhibition of PI3K by LY294002 or another chemical PI3K inhibitor wortmannin reduced AKT phosphorylation (Fig. 4C), demonstrating AKT is downstream of PI3K. LY294002 and wortmannin also caused a dose-dependent increase in apoptosis in Panc-1 cells (Fig. 4D). The results indicate PI3K is important for the growth and survival of these cells, and that depletion or inhibition of PRCP/PREP may reduce pancreatic cancer cell viability and survival in part by the inhibition of PI3K.

**PRCP and PREP Regulate IRS-1 Protein Stability**—IRS proteins play an important role in activation of PI3K by recruiting the regulatory PI3K p85 subunit to the cell membrane (33). Lysates from Panc-1, PK-9, and Capan-1 cells with or without depletion of PRCP/PREP were tested for IRS-1, IR, and IGF-1R by immunoblotting. IRS-1 levels were decreased in the cells depleted of PRCP and PREP while IR and IGF-1R protein level was unchanged (Fig. 5A). In contrast, overexpression of wild type PRCP but not the enzyme-dead PRCP increased IRS-1 level (Fig. 5B). These results suggest PRCP/PREP regulates expression of IRS-1 and not IR or IGF-1R. The IRS-1 mRNA level was not changed in the cells with depletion of PRCP and PREP by quantitative PCR analysis (data not shown), suggesting the PRCP and PREP regulate IRS-1 at the protein level.

Serine phosphorylation of IRS-1 promotes its degradation (34), and we examined IRS-1 phosphorylation by immunoblotting. Specific phosphoserine antibodies for IRS-1 showed that phosphorylation of serine 636/639 and serine 307 was increased in the cells depleted of PRCP/PREP but not in the control Panc-1 cells (Fig. 5C). In contrast, levels of tyrosine phosphorylated IRS-1 were decreased in IRS-1 immunoprecipitates (Fig. 5C, lower), suggesting that PRCP/PREP regulates IRS-1 protein stability through modulating its phosphorylation.

**FIGURE 3. Inhibition/depletion of PRCP and PREP decreases AKT phosphorylation.** A, whole cell lysates of Panc-1, PK-9, and Capan-1 cells without (control) or with depletion of PRCP (PRCP KD), PREP (PREP KD), or both (DKD) were immunoblotted for phospho-AKT (Ser-437) and AKT. Panc-1 cells were transiently transfected with vector, wild type PRCP, and enzyme-dead PRCP for 72 h, whole cell lysates were immunoblotted for phospho-AKT (Ser-437), total AKT, PRCP, and β-actin. C, Panc-1, PK-9, and Capan-1 cells were treated with ZPP (400 μM) for 3 days. Whole cell lysates were immunoblotted for phospho-AKT (Ser-437) and total AKT. D, Panc-1 cells were treated with vehicle or MK2206 (10 μM) for 24 h. Whole cell lysates were immunoblotted for phospho-AKT (Ser-437) and AKT. E, Panc-1 cells were treated in octuplicate with vehicle or MK2206 (10 μM) for 3 days. MTT assay was performed, and average absorbance from octuplicate was normalized to vehicle-treated conditions and presented as a graph with standard deviation indicated. There is significant difference between vehicle-treated and MK2206-treated conditions ($p < 0.05$) (representative of three independent experiments).
consistent with decreased total IRS-1. The half-life of IRS-1 but not IGF-1R was shortened in the cells depleted of PRCP and PREP (Fig. 5, D and E), suggesting that the increased serine phosphorylation of IRS-1 induced by depletion of PRCP and PREP was accompanied by degradation of the IRS-1 protein.

Depletion or Inhibition of PRCP and PREP Blocks Rapamycin-induced Feedback Increase of IRS-1 and Activation of PI3K/AKT, and Enhances Rapamycin-induced Cytotoxicity—IRS-1-PI3K-AKT signaling activates mTOR, which then phosphorylates and activates the ribosomal protein S6 kinase (S6K1) to promote protein translation and growth (2, 35, 36). However, activated S6K inhibits AKT signaling by promoting the phosphorylation and degradation of IRS-1. mTOR has been clinically tested as a therapeutic target for pancreatic cancer. However, inhibition of mTOR (e.g. by rapamycin) causes feedback activation of AKT by enhancing IRS-1-mediated activation of PI3K and AKT, diminishing the therapeutic effect of mTOR inhibitors (6, 37). Our results suggest depletion or inhibition of PRCP/PREP promotes IRS-1 degradation. Therefore, we examined if depletion or inhibition of PRCP and PREP would block rapamycin-induced activation of IRS-1 and AKT and enhance the cytotoxic effect of rapamycin. Panc-1 cells with or without depletion of PRCP and PREP were treated with vehicle or rapamycin for 2 days. In control cells, rapamycin treatment increased IRS-1 protein levels and phosphorylation of AKT (Fig. 6 A). Depletion of both PRCP and PREP blocked the rapamycin-induced increase in IRS-1 protein and AKT phosphorylation (Fig. 6 A). Interestingly, phosphorylation at serine 307 induced by PRCP/PREP depletion was blocked by rapamycin, whereas phosphorylation at serine 636/639 of IRS-1 was relatively unaffected (Fig. 6 B). Levels of pS6K were largely unaffected in the cells depleted of PRCP and PREP, suggesting mTOR remained active. The half-life of IRS-1 protein in rapamycin-treated cells with depletion of PRCP and PREP was also shortened compared with rapamycin-treated control cells (Fig. 6, C and D) while mRNA levels remain similar (Fig. 6E). These results suggest depletion of PRCP and PREP induces both mTOR/S6K-sensitive (Ser-307) and insensitive (Ser-636/639) phosphorylation of IRS-1.

FIGURE 4. Inhibition/depletion of PRCP and PREP reduces PI3K activity. Inhibition of PI3K induces cytotoxicity. A, equal amount of cell lysates of control Panc-1 cells and PRCP/PREP-knockdown cells (DKD) as well as cells treated with DMSO, ZPP (200 μM), or LY294002 (50 μM) for 2 days were immunoprecipitated with anti-p85 antibodies and analyzed for PI3K activity in triplicate and the immunoprecipitates were subsequently immunoblotted for p85 (top). The average PI3K activity was presented as a graph with standard deviation indicated (bottom). There are significant differences between control cells and DKD cells (p < 0.01) and between DMSO-treated cells and ZPP treated cells (p < 0.01) and LY294002-treated cells (p < 0.001) (representative of three independent experiments) B, Panc-1, PK-9, and Capan-1 cells were treated with different doses of LY294002 for 4 days. The cells were analyzed with MTT assay, and the average relative absorbance was presented as a graph with standard deviation indicated. C, Panc-1 cells were treated with vehicle, LY294002 (50 μM), or wortmannin (1 μM) for 24 h. Whole cell lysates were immunoblotted for phospho-AKT (S473) and AKT. D, Panc-1 cells were treated with different doses of wortmannin or LY294002 for 4 days. The cells were stained with PI and analyzed for the subdiploid population. The percentage of the subdiploid cells were presented as a graph with standard deviation indicated. There are significant differences (p < 0.01) between vehicle-treated cells and the cells treated with wortmannin (0.5 μM and 1 μM) or LY294002 (20 μM and 50 μM) (representative of three independent experiments).
phosphorylation of IRS-1, and subsequent IRS-1 degradation. Consistent with depletion of PRCP and PREP, ZPP also blocked the increase in IRS-1 levels and phosphorylation of AKT induced by rapamycin, while only modestly reducing levels of pS6K (Fig. 6F).

To test if PRCP and PREP regulate rapamycin-induced activation of PI3K, Panc-1 cells with or without depletion of PRCP and PREP were treated with vehicle or rapamycin for 2 days and whole cell lysates immunoprecipitated with anti-IRS-1 or anti-p85 (PI3K active subunit) antibodies. The resulting immunoprecipitates were then analyzed for PI3K by in vitro kinase activity. The results showed that in control cells, the IRS-1- and p85-associated PI3K activity was increased in cells treated with rapamycin (Fig. 7, A and B), consistent with rapamycin causing feedback activation of IRS-1-PI3K. In the cells with depletion of PRCP and PREP, both the basal and rapamycin-induced activation of PI3K was significantly lower than that in control cells (Fig. 7, A and B).

Because PRCP/PREP knockdown or inhibition reduced IRS-1 and pAKT levels in rapamycin-treated cells, we hypothesized that the combination of rapamycin and ZPP would show increased cytotoxicity (reduction in viable cells) by efficiently
blocking mTOR while inhibiting the feedback activation of AKT. To test this, Panc-1, PK9, and Capan-1 cells were treated with increasing doses of rapamycin in the absence or presence of ZPP for 4 days. Viability measurements (MTT assay) showed that rapamycin caused a dose-dependent reduction in viability in all three cell lines (Fig. 8, A, D, and G). Importantly, combination rapamycin plus ZPP caused a more pronounced decrease in viable cells in each cell line (Fig. 8, A, D, and G).

These results are consistent with the notion that PREP/PRCP inhibition can enhance the cytotoxic effect of rapamycin, most likely by inhibiting feedback activation of IRS-1 and AKT. ZPP also caused a dose-dependent reduction in viability in all three cell lines (Figs. 8, B, E, and H). When combined with rapamycin a much more pronounced reduction in viability was observed. Notably, the effects of combining ZPP plus rapamycin appeared more than additive, so we conducted synergy test by treating

FIGURE 6. Depletion/inhibition of PRCP/PRCP blocks rapamycin-induced increase in IRS-1 and phosphorylation of AKT. A, Panc-1 cells without (control) or with (DKD) depletion of PRCP/PRCP were treated with vehicle or rapamycin (10 nM) for 48 h. Whole cell lysates were immunoblotted for IRS-1, phospho-AKT (S473), AKT, and β-actin. B, whole cell lysates were also immunoblotted for IRS-1, phospho-IRS-1 (Ser-307 and Ser-636/639), phospho-p70S6K (Thr-389), p70S6K, and β-actin. C, cells were treated with rapamycin (10 nM) for 48 h and then CHX (20 μg/ml) and pulse chased for 4 h. Whole cell lysates were immunoblotted for IRS-1 and β-actin. D, IRS-1 protein signal was quantified by densitometry and average relative protein level from three independent experiments was presented as a graph with standard deviation indicated. E, mRNA was also extracted from the cells with or without CHX treatment for 4 h and analyzed for IRS-1 gene expression by quantitative PCR. F, Panc-1 cells were treated with rapamycin (10 nM) in the presence or absence of ZPP (400 μM) for 48 h. Whole cell lysates were immunoblotted for IRS-1, β-actin, phospho-AKT (Ser-473), and AKT.
Panc-1 and PK-9 cells with two doses of ZPP and rapamycin singly and combined and the dose effect was analyzed by CompuSyn software. The results showed that combination of ZPP and rapamycin has synergistic effect (CI < 1) on both Panc-1 (Fig. 8C) and PK-9 cells (Fig. 8F).

DISCUSSION

The PI3K/AKT pathway is perhaps the most aberrantly activated signaling pathway in human cancer cells affecting a wide spectrum of cellular functions such as metabolism, proliferation, motility, and survival (1–3). The PI3K/AKT pathway is activated by RTKs e.g. IR/IGF-1R which are involved in the development of different cancers including cancer of pancreas (5, 15, 26). AKT activates mTOR that further phosphorylates its downstream targets ribosomal protein p70 S6 kinase (S6K1) and initiation factor 4E-binding protein-1 (4E-BP1) to control transcription and translation (2, 35, 36). Notably, the mTOR pathway exerts a negative feedback on the upstream PI3K by down-regulating IRS and Grb10, important adaptor proteins for RTK signaling (6, 7, 38, 39). Inhibition of mTOR releases this negative feedback loop and results in increased levels of IRS-1 protein and activation of PI3K/AKT (6–8, 37), which may counteract the therapeutic effect of the mTOR inhibitor rapamycin on tumors.

IRS-1 is an adaptor protein critical for cellular signaling downstream of IR/IGF-1R. IRS-1 is recruited to and tyrosine-phosphorylated by the activated IR/IGF-1R. The phosphorylated tyrosines of IRS-1 interact with the PI3K p85 subunit and recruit PI3K to cell membrane for activation (6–8, 37), which may counteract the therapeutic effect of the mTOR inhibitor rapamycin on tumors.

Depletion of PRCP and PREP reduces rapamycin-induced increase of cellular PI3K activity.

A. Depletion of PRCP and PREP reduces rapamycin-induced increase of cellular PI3K activity. A and B, Panc-1 cells without (control) or with (DKD1) depletion of PRCP/PREP were treated with vehicle or rapamycin (10 nM) for 3 days. Equal amount of cell lysates were immunoprecipitated with anti-IRS-1 antibodies (A) or anti-p85 antibodies (B). The immunoprecipitates were analyzed for PI3K activity. Average PI3K activity from triplicate was normalized to that in vehicle-treated control cells and presented as a graph with standard deviation indicated (upper panel). The immunoprecipitates were immunoblotted for IRS-1 or p85 (lower panel).

B. Depletion of PRCP and PREP reduces rapamycin-induced increase of cellular PI3K activity.

FIGURE 7. Depletion of PRCP and PREP reduces rapamycin-induced increase of cellular PI3K activity. A and B, Panc-1 cells without (control) or with (DKD1) depletion of PRCP/PREP were treated with vehicle or rapamycin (10 nM) for 3 days. Equal amount of cell lysates were immunoprecipitated with anti-IRS-1 antibodies (A) or anti-p85 antibodies (B). The immunoprecipitates were analyzed for PI3K activity. Average PI3K activity from triplicate was normalized to that in vehicle-treated control cells and presented as a graph with standard deviation indicated (upper panel). The immunoprecipitates were immunoblotted for IRS-1 or p85 (lower panel).
of PRCP/PREP led to loss of IRS-1 protein by degradation, which blocked rapamycin-mediated activation of PI3K/AKT. In control cells, rapamycin increased IRS-1 protein level and AKT phosphorylation (Fig. 5), which is consistent with previous observations on release of mTOR-mediated feedback inhibition of PI3K/AKT by rapamycin (6). Upon depletion or inhibition of PRCP and PREP, rapamycin failed to increase IRS-1 expression, most likely due to heightened serine phosphorylation and IRS-1 degradation in the PRCP/PREP deleted cells (Fig. 6). It is noteworthy that depletion of PRCP and PREP induced rapamycin-sensitive and insensitive phosphorylation of IRS-1. While phosphorylation of serine 307 induced by depletion/inhibition of PRCP/PREP was blocked by rapamycin, phosphorylation of serine 636/639 was largely unchanged. Previous study shows that serine 636/639 can be phosphorylated by S6K (43). In the current study, rapamycin did not inhibit phosphorylation of serine 636/639 induced by depletion of PRCP and PREP, suggesting that besides S6K, PRCP and PREP may regulate additional kinases for the phosphorylation of serine 636/639. Notably, although depletion of PRCP and PREP induced phosphorylation of serine 307, it did not increase levels of activated S6K (p-p70S6K). Since serine 307 is phosphorylated by S6K downstream of mTOR, the results suggest that PRCP and PREP may inhibit S6K activity toward IRS-1 via an

FIGURE 8. Inhibition of PRCP/PREP in combination with rapamycin leads to additive/synergistic cytotoxicity. A, D, G, Panc-1, PK-9, and Capan-1 cells were plated in 96-well plates. The cells in octuplicate were treated with different doses of rapamycin in the presence of vehicle or ZPP (25 μM for Panc-1 and PK-9, 50 μM for Capan-1) for 4 days. B, E, and H, cells were treated with different doses of ZPP in the presence of vehicle or rapamycin (0.25 nM) for 4 days. The cells were analyzed with MTT assay and the average relative absorbance was presented as graphs with standard deviation indicated. C and F, Panc-1 cells and PK9 cells were treated with two indicated doses of ZPP and rapamycin singly or combined. The dose effect was analyzed by CompuSyn software for drug synergy and presented as curves. All of the CI values are smaller than one in both Panc-1 and PK-9 cells indicating synergistic effects (representative of three independent experiments).
The proposed model for the regulation of the IRS-1–PI3K–mTOR pathway by PRCP and PREP. The IRS-1–PI3K–mTOR pathway forms a signaling loop and regulates cell proliferation and survival. IRS-1 is negatively regulated by S6K and unspecified kinases by currently unknown mechanisms on serine phosphorylation of IRS-1, preventing degradation of IRS-1. Inhibition of PRCP and PREP may enable S6K and unspecified kinases to phosphorylate IRS-1 and facilitate its degradation, leading to down-regulation of PI3K activity. Rapamycin inhibits mTOR and S6K, leading to feedback stabilization and activation of IRS-1/PI3K/AKT in the presence of PRCP and PREP. When PRCP and PREP are depleted or inhibited, unspecified kinases are able to phosphorylate and degrade IRS-1 regardless of rapamycin. Therefore, rapamycin-mediated stabilization and activation of IRS-1 and PI3K is blocked by depletion/inhibition of PRCP and PREP, leading to enhanced cytotoxicity.

unknown mechanism, or may activate a phosphatase responsible for dephosphorylation of serine 307. The function of PRCP and PREP on IRS-1 appears critical for cell viability as rapamycin-induced cytotoxicity was enhanced by ZPP (Fig. 8). IRS-1 can regulate a number of downstream effectors such as PI3K/AKT, ERK, and MAP kinase. Since inhibition of PI3K was also toxic to Panc-1 cells, it is likely that PRCP and PREP regulate cell proliferation and survival in part through IRS-1-mediated activation of PI3K. We propose that PRCP and PREP inhibit the function of S6K and potentially other serine kinases on the phosphorylation and degradation of IRS-1. By doing so, PRCP and PREP promote the PI3K-AKT-mTOR pathway for cell proliferation and survival (Fig. 9).

Our results suggest that inhibitors of PRCP and PREP may be potentially tested for treatment of pancreatic cancer. ZPP has an IC50 of 10 nM for cellular PREP (data not shown) while an IC50 of 10 μM for recombinant PRCP (30), indicating ZPP is more selective for PREP. The significant cytotoxic effect of ZPP on pancreatic cancer cells can only be achieved at concentrations above 10 μM, implicating that inhibition of both PREP and PRCP are required for cytotoxicity by the inhibitor. Future study will test if a PRCP specific inhibitor can also induce cytotoxicity or whether combination of a PRCP inhibitor with ZPP can effectively kill pancreatic cancer cells.

Pancreatic adenocarcinoma is an aggressive cancer with poor long-term survival. The PI3K/AKT/mTOR pathway plays a critical role in progression of pancreatic cancer. mTOR inhibitors have failed in clinical pancreatic cancer trials, most likely due to feedback activation of the IRS-1/PI3K/AKT pathway. In the current study, depletion or inhibition of PRCP/PREP blocked feedback activation of IRS-1/PI3K/AKT and enhanced cytotoxicity in mTOR inhibitor (rapamycin)-treated pancreatic cancer cells. Identification of prolyl peptidases as critical regulators of the IRS-1/PI3K-AKT-mTOR signaling loop in this study may help design more effective therapies for pancreatic cancer and other cancers dependent on this pathway.

Acknowledgments—We thank Dr. Hwya Arafat (Thomas Jefferson University) for providing Panc-1 and PK9. We are grateful for Dr. Surinder Batra (University of Nebraska Medical Center) for providing Capan-1 cells. We thank Dr. Trono Didier (EPFL School of Life Sciences) for providing the lentiviral packaging and enveloping vectors psPAX2 and pMD2.G.

REFERENCES
1. Vivanco, I., and Sawyers, C. L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat. Rev. Cancer 2, 489–501
2. Sorkin, A., and Fauquet, C. (2006) Phosphatidylinositol-3-kinase and its substrates. Nat. Rev. Mol. Cell Biol. 7, 733–747
3. Cortez, D., and Kastan, M. B. (2004) The PI3K/AKT/mTOR pathway as drug target in human cancer. Nat. Rev. Mol. Cell Biol. 5, 866–876
4. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. Cell 100, 57–70
5. Vousden, K. H. (2004) Translating the p53 pathway. Nat. Rev. Cancer 4, 841–852
6. Easton, J. B., Kurmasheva, R. T., and Houghton, P. J. (2006) IRS-1: auditing phosphoinositide 3-kinase pathways in pancreatic cancer–from molecular signalling to clinical trials. Nat. Rev. Cancer 6, 135–146
7. Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P. M., Olefsky, J. M., and Kobayashi, M. (2000) A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol. Endocrinol. 14, 783–794
8. O’Reilly, K. E., Rojo, F., She, Q. B., Solit, D., Mills, G. B., Smith, D., Lane, H., Hofmann, F., Hicklin, D. J., Ludwig, D. L., Baselga, J., and Rosen, N. (2006) mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. 66, 1500–1508
9. Kauffman, A., and Lemoine, N. R. (2009) Pancreatic cancer: molecular targeted therapies. Mt. Sinai J. Med. 77, 606–619
10. Wilkowski, R., Wolf, M., and Heinemann, V. (2008) Primary advanced unresectable pancreatic cancer. Recent Results Cancer Res. 177, 79–93
11. Hidalgo, M. (2010) Pancreatic cancer. N. Engl. J. Med. 362, 1605–1617
12. Philip, P. A. (2011) Development of targeted therapies for pancreatic cancer. Lancet Oncol. 12, 206–207
13. Wong, H. H., and Lemoine, N. R. (2009) Pancreatic cancer: molecular pathogenesis and new therapeutic targets. Nat. Rev. Gastroenterol. Hepatol. 6, 412–422
14. Borja-Cacho, D., Jensen, E. H., Saluja, A. K., Buchsbaum, D. J., and Vickers, S. M. (2008) Molecular targeted therapies for pancreatic cancer. Am. J. Surg. 196, 430–441
15. Rieder, S., Michalski, C. W., Friess, H., and Kleeff, J. (2011) Insulin-like growth factor signaling as a therapeutic target in pancreatic cancer. Anticancer Agents Med. Chem. 11, 427–433
16. Bao, B., Wang, Z., Li, Y., Kong, D., Ali, S., Banerjee, S., Ahmad, A., and Sarkar, F. H. (2011) The complexities of obesity and diabetes with the development and progression of pancreatic cancer. Biochim. Biophys. Acta 1815, 135–146
17. Falasch, M., Selvaggi, F., Buus, R., Sulpizio, S., and Edling, C. E. (2011) Targeting phosphoinositide 3-kinase pathways in pancreatic cancer—from molecular signalling to clinical trials. Anticancer Agents Med. Chem. 11, 455–463
18. Yamamoto, S., Tomita, Y., Hoshida, Y., Morooka, T., Nagano, H., Dono, K., Umeshita, K., Sakon, M., Ishikawa, O., Ohigashi, H., Nakamori, S,
Monden, M., and Aozasa, K. (2004) Prognostic significance of activated Akt expression in pancreatic ductal adenocarcinoma. Clin. Cancer Res. 10, 2846–2850

19. Schlieman, M. G., Fahy, B. N., Ramsamooj, R., Beckett, L., and Bold, R. J. (2003) Incidence, mechanism and prognostic value of activated AKT in pancreas cancer. Br. J. Cancer 89, 2110–2115

20. Eser, S., Reiff, N., Messer, M., Seidler, B., Gottschalk, K., Dobler, M., Hieber, M., Arbeiter, A., Klein, S., Kong, B., Michalski, C. W., Schlitter, A. M., Esposito, I., Kind, A. J., Rad, L., Schnieke, A. E., Baccarini, M., Alessi, D. R., Rad, R., Schmid, R. M., Schneider, G., and Saur, D. (2013) Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer. Cancer Cell 23, 406–420

21. Duan, L., Motchoulski, N., Danzer, B., Davidovich, I., Shariat-Madar, Z., Rahimy, E., Mahdi, F., and Schmaier, A. H. (2005) Prolylcarboxypeptidase regulates autophagy and resistance to 4-hydroxytamoxifen-induced cytotoxicity in ER-positive breast cancer cells. J. Biol. Chem. 280, 2864–2878

22. Rosenblum, J. S., and Kozarich, J. W. (2003) Prolyl peptidases: a serine protease subfamily with high potential for drug discovery. Curr. Opin. Chem. Biol. 7, 496–504

23. García-Horsman, J. A., Männistö, P. T., and Venäläinen, J. I. (2007) On the protease subfamily with high potential for drug discovery. J. Biol. Chem. 282, 2864–2876

24. Garcia-Horsman, J. A., Mannistö, P. T., and Venäläinen, J. I. (2007) On the role of prolyl oligopeptidase in health and disease. Neuropeptides 41, 1–24

25. Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D., and Scharpè, S. (1995) Proline motifs in peptides and their biological processing. Faseb J. 9, 736–744

26. Rozengurt, E., Sinnett-Smith, J., and Kisfalvi, K. (2010) Crosstalk between receptor signaling systems: a novel target for the antidiabetic drug metformin in pancreatic cancer. Clin. Cancer Res. 16, 2505–2511

27. Evers, B. M. (2005) Gastrointestinal growth factors and neoplasia. Am. J. Surg. 190, 279–284

28. Schlieman, M. G., Fahy, B. N., Ramsamooj, R., Beckett, L., and Bold, R. J. (2003) Incidence, mechanism and prognostic value of activated AKT in pancreas cancer. Br. J. Cancer 89, 2110–2115

29. Adams, G. N., Stavrou, E. X., Fang, C., Merkulova, A., Alai, M. A., Naka-jima, K., Morooka, T., Merkulov, S., Larusch, G. A., Simon, D. I., Jain, M. K., and Schmaier, A. H. (2013) Prolylcarboxypeptidase promotes angiogenesis and vascular repair. Blood 122, 1522–1531

30. Shariat-Madar, Z., Mahdi, F., and Schmaier, A. H. (2004) Recombinant prolylcarboxypeptidase activates plasma prekallikrein. Blood 103, 4554–4561

31. Schulz, I., Zeitschel, U., Rudolph, T., Ruiz-Carrillo, D., Rahfeld, J. U., Gerhartz, B., Biv, V., Demuth, H. U., and Rossner, S. (2005) Subcellular localization suggests novel functions for prolyl endopeptidase in protein secretion. J. Neurochem. 94, 970–979

32. Darzynkiewicz, Z., Halicka, H. D., and Zhao, H. (2010) Analysis of cellular DNA content by flow and laser scanning cytometry. Adv. Exp. Med. Biol. 676, 137–147

33. Metz, H. E., and Houghton, A. M. (2011) Insulin receptor substrate regulation of phosphoinositide 3-kinase. Clin. Cancer Res. 17, 206–211

34. Pederson, T. M., Kramer, D. L., and Rondinone, C. M. (2001) Serine/threonine phosphorylation of IRS-1 triggers its degradation: possible regulation by tyrosine phosphorylation. Diabetes 50, 24–31

35. Laplante, M., and Sabatini, D. M. (2012) mTOR Signaling in Growth Control and Disease. Cell 149, 274–293

36. Mamane, Y., Petroulakis, E., LeBacquer, O., and Sonenberg, N. (2006) mTOR, translation initiation and cancer. Oncogene 25, 6416–6422

37. Javel, M. M., Shroff, R. T., Xiong, H., Varadachary, G. A., Fogelman, D., Reddy, S. A., Davis, D., Zhang, Y., Wolff, R. A., and Abbruzzese, J. L. (2010) Inhibition of the mammalian target of rapamycin (mTOR) in advanced pancreatic cancer: results of two phase II studies. BMC Cancer 10, 368

38. Hsu, P. P., Miao, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A., and Sabatini, D. M. (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. Science 332, 1317–1322

39. Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villén, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., and Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. Science 332, 1322–1326

40. Shepherd, P. R., Withers, D. J., and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem. J. 333, 471–490

41. Bergmann, U., Funatomi, H., Kornmann, M., Beger, H. G., and Korc, M. (1996) Increased expression of insulin receptor substrate-1 in pancreatic cancer. Clin. Cancer Res. 2, 970–979

42. Dearth, R. K., Cui, X., Kim, H. J., Kuiatse, I., Lawrence, N. A., Zhang, X., Divisova, J., Britton, O. L., Mohsin, S., Allred, D. C., Hadsell, D. L., and Lee, A. V. (2006) Mammary tumorigenesis and metastasis caused by overexpression of insulin receptor substrate 1 (IRS-1) or IRS-2. J. Biol. Chem. 281, 21044–21051

43. Sumner, E. A., and Houghton, A. M. (2011) Regulation of insulin receptor substrate by angiotensin II. Am. J. Physiol. Heart Circ. Physiol. 298, H2697–H2703

44. Adams, G. N., Stavrou, E. X., Fang, C., Merkulova, A., Alai, M. A., Nakajima, K., Moroko...