Targeting GIPC/Synectin in Pancreatic Cancer Inhibits Tumor Growth

Michael H. Muders,1 Pawan K. Vohra,3 Shamit K. Dutta,1 Enfeng Wang,1 Yasuhiro Ikeda,1 Ling Wang,1 D. Gomika Udugamasooriya,4 Adnan Memic,4 Chamila N. Rupashinghe,2,4 Gustavo B. Baretton,5 Daniela E. Aust,5 Silke Langer,5 Kaustubh Datta,1 Michael Simons,3,6 Mark R. Spaller,2,4 and Debabrata Mukhopadhyay1

Abstract

Purpose: Various studies have shown the importance of the GAIP interacting protein, COOH-terminus (GIPC, also known as Synectin) as a central adaptor molecule in different signaling pathways and as an important mediator of receptor stability. GIPC/Synectin is associated with different growth-promoting receptors such as insulin-like growth factor receptor I (IGF-IR) and integrins. These interactions were mediated through its PDZ domain. GIPC/Synectin has been shown to be overexpressed in pancreatic and breast cancer. The goal of this study was to show the importance of GIPC/Synectin in pancreatic cancer growth and to evaluate a possible therapeutic strategy by using a GIPC-PDZ domain inhibitor. Furthermore, the effect of targeting GIPC on the IGF-I receptor as one of its associated receptors was tested.

Experimental Design: The in vivo effects of GIPC/Synectin knockdown were studied after lentiviral transduction of luciferase-expressing pancreatic cancer cells with short hairpin RNA against GIPC/Synectin. Additionally, a GIPC-PDZ–targeting peptide was designed. This peptide was tested for its influence on pancreatic cancer growth in vitro and in vivo.

Results: Knockdown of GIPC/Synectin led to a significant inhibition of pancreatic adenocarcinoma growth in an orthotopic mouse model. Additionally, a cell-permeable GIPC-PDZ inhibitor was able to block tumor growth significantly without showing toxicity in a mouse model. Targeting GIPC was accompanied by a significant reduction in IGF-IR expression in pancreatic cancer cells.

Conclusions: Our findings show that targeting GIPC/Synectin and its PDZ domain inhibits pancreatic carcinoma growth and is a potential strategy for therapeutic intervention of pancreatic cancer.

Ductal adenocarcinoma is the most common malignancy of the pancreas. Despite even the most aggressive therapies, the 5-year survival rate for patients diagnosed with pancreatic cancer is <4% (1). This clearly indicates that novel approaches to the management of patients with pancreatic cancer are urgently needed. The establishment of new targets in pancreatic cancer treatment is an important step toward longer survival and better prognosis. One of these new targets could be GAIP interacting protein, COOH-terminus (GIPC, also known as Synectin), a protein shown to be overexpressed in pancreatic and breast cancer (2, 3).

GIPC/Synectin was originally identified as a binding partner of the regulator of G protein signaling (RGS) protein GAIP (RGS 19), a GTPase activating protein for heterotrimeric G proteins (4). Recently, different studies have suggested an important role of GIPC/Synectin in the biology of normal and malignant cells (2, 4–20). Interestingly, we have also shown that GIPC/Synectin is important for insulin-like growth factor receptor I (IGF-IR) stability in pancreatic cancer cell lines (2).

In this study, we show that knockdown of GIPC/Synectin reduces tumor growth after orthotopic transplantation of different pancreatic cancer cell lines in nude mice. In conjunction with these efforts, we developed a small peptide that blocks the PDZ domain of GIPC. This led to decreased tumor growth of different pancreatic cancer cell lines in vitro.
Translational Relevance

The 5-year survival rate in patients with ductal adenocarcinoma of the pancreas is 4%. Accordingly, new targets for the treatment of this deadly disease are urgently needed. In this study, we show that targeting GAIP interacting protein COOH-terminal (GIPC, also known as Synectin) and its PDZ-domain reduces pancreatic cancer growth significantly in vitro and in vivo. Additionally, the blockage of GIPC/Synectin was accompanied by a reduction of insulin-like growth factor receptor I protein levels. In summary, the use of a GIPC-PDZ domain inhibitor may be a viable option in the treatment of pancreatic adenocarcinoma in future.

In vivo this GIPC-PDZ targeting peptide suppressed pancreatic cancer growth in a mouse model. Furthermore, we tested the influence of targeting GIPC/Synectin on one of its associated growth factor receptors, IGF-IR. As expected, the octapeptide led to decreased association between IGF-IR and GIPC/Synectin in different pancreatic cancer cell lines. This, in turn, reduced IGF-IR protein levels in the cells. In essence, this study represents a first step towards developing a novel therapeutic for pancreatic adenocarcinoma treatment using a PDZ-inhibitor.

Materials and Methods

Cell culture, cell infection, and immunofluorescence. MIA-PaCa2, Panc1, and AsPC1 were purchased from the American Type Culture Collection. To generate the lentivectors, 293T cells were cotransfected with gag-pol expression plasmid pCMVΔ8.91, VSV.G envelope expression plasmid pMD-G, and vector plasmid pLKO.1 encoding cDNAs for GIPC/Synectin knockdown cells, protein lysates were analyzed by immunoblot for the localization of FITC-labeled octapeptide (PSQSSSEA) from GAIP, and vector plasmid pLKO.1 encoding cDNAs for GIPC/Synectin and IGF-IR. Control cells were transduced with an empty green fluorescent protein vector. RNA interference in cell culture was done as described (2). The last eight residues (PSQSSSEA) of the COOH-terminal (GIPC, also known as Synectin) and its PDZ-domain reduces pancreatic cancer growth significantly in vitro and in vivo. Additionally, the blockage of GIPC/Synectin was accompanied by a reduction of insulin-like growth factor receptor I protein levels. In summary, the use of a GIPC-PDZ domain inhibitor may be a viable option in the treatment of pancreatic adenocarcinoma in future.

Preparation of T7 phage displaying GIPC-PDZ. Double-strand DNA encoding the PDZ domain of GIPC/Synectin was double-digested with EcoRI and HindIII, and the DNA (0.8 μl of a 45 nmol/L 7.7 ng/μl solution) was used in the ligation reaction with the T7 Select 10-3 vector arms (Novagen, 0.5 μL) along with 10× ligase buffer (0.25 μL), ATP (100 mmol/L 0.25 μL, final concentration), DTT (100 mmol/L 0.25 μL), and T4 DNA ligase (0.5 μL 1 unit/μL). The sample was incubated at 16°C for 16 h. The T7 phage packaging reaction was done by adding packaging extract (Novagen, 12.5 μL, or half the manufacturer’s recommended amount) to the ligation reaction sample (2.5 μL) and allowing it to incubate for 2 h at room temperature. The reaction was stopped by the addition of a sterile Luria-Bertani Broth (LB) medium (135 μL). A plaque assay was done to determine the number of recombinant phage generated. Escherichia coli BTL5403 was grown at 37°C with shaking in LB/Amp medium (10 mL) until optical density (OD) at 600 nanometers (600 nm) reached 0.8. Top agarose (10 mL) was melted and equilibrated to this temperature. A series of dilutions was prepared prior to plating the phage. The first dilution was generated by adding packaged phage (10 μL) to LB/Amp medium (990 mL) for a total of 100-fold dilution (10^2). Subsequent dilutions were prepared by adding the previous dilution (100 μL) to fresh LB/Amp medium (900 μL); generally, 10^3 to 10^5 dilutions were plated. In this case, the 10^8 and 10^9 dilutions were plated by adding 400 μL of E. coli stock with the phage dilution sample (100 μL) and melted top agarose (3 mL) to a prewarmed LB or LB/Amp agar plate. The plates were inverted upon hardening and allowed to incubate at 37°C for 3 to 4 h (or at room temperature if incubated overnight). Plaques generated were counted to determine the titer and the number of recombinants generated. Eight plaques from the 10^8 dilution sample were selected and used to infect fresh E. coli stock (1 mL) as above. The samples were incubated at 37°C with shaking until lysis was observed. A phage lysis sample (1.5 μL) for each was subjected to PCR amplification (using the Novagen primers), purified (Qiagen PCR Removal Kit), and the DNA submitted for sequencing; the presence of the inserted sequence for the GIPC/Synectin-PDZ domain was confirmed for all samples.

ELISA protocol. Neutrinid plates (Pierce) were coated in duplicate with 100 μL of 10 μg/mL solutions of NH2-terminal biotinylated peptides (PSQSSSEA; from GAIP), KKETEV and KKEAV (two sequences that bind PDZ domain of PSD-95; ref. 21), and allowed to incubate for 1 to 2 h at 37°C (or overnight at 4°C) with mild shaking. The unbound peptides were washed away three times with PBS 0.5% Tween-20 (TBST) buffer. Blocking buffer was prepared by diluting bovine serum albumin (10 mg/mL) in PBS stock 2-fold in ddH2O (for a 5% bovine serum albumin final concentration) and free biotin (1 μmol/L). Alternatively, SuperBlock in PBS buffer (Pierce) with free peptides were monitored by bioluminescence. This experiment was stopped 14 d postimplantation. For another group of mice, GIPC/Synectin-negative (n = 5) and control MIAPaCa2 cells (n = 5) were implanted s.c. into the right flank of nude mice for detection of IGF-IR using immunoblot. After 55 d the mice were euthanized, and tumor tissue was evaluated for IGF-IR and GIPC/Synectin expression by immunoblot analysis. Selected tumors were evaluated with standard histology using H&E staining and for proliferation using Ki67 staining.

Peptide design. GIPC is a PDZ domain–containing protein that interacts specifically with the COOH terminus of RGS-GAIP (4), a GTPase-activating protein for Ga13 (4), a subunit located on clathrin-coated vesicles. The last eight residues (PSQSSSEA) of the COOH-terminal sequence of GAIP were selected for preparation of linear peptide ligands. In order to enhance cell permeability, myristolation was done on the NH2 terminus, where it would not interfere with the critical COOH-terminal binding epitope. An analog in which FITC was incorporated was prepared for visualization experiments. A third peptide ligand with a corresponding sequence, prepared with NH2-terminal biotinylation, was used to conduct pull-down assays to prove the in vitro binding of this peptide to GIPC/Synectin via its single PDZ domain. As a control peptide, an octapeptide with the following sequence was used: Myr-ADSTLREK.

Cell culture, cell infection, and immunofluorescence. MIA-PaCa2, Panc1, and AsPC1 were purchased from the American Type Culture Collection. To generate the lentivectors, 293T cells were cotransfected with gag-pol expression plasmid pCMVΔ8.91, VSV.G envelope expression plasmid pMD-G, and vector plasmid pLKO.1 encoding cDNAs for GIPC/Synectin shRNA in pLKO.1 was purchased from Open Biosystems. Supernatant was collected 48 h later and frozen at -80°C. MIA PaCa2 or AsPC1 cells were then infected with a multiplicity of infection of 8 overnight at 37°C, and they were injected after infection. To ensure the efficiency of GIPC/Synectin knockdown cells, protein lysates were analyzed by immunoblot for GIPC/Synectin and IGF-IR. Control cells were transduced with an empty green fluorescent protein vector. RNA interference in cell culture was done as described (2). The localization of FITC-labeled octapeptides was analyzed by fluorescence microscopy using a microscope (Zeiss Axiovert 100M; Carl Zeiss Inc.). After excitation by 488 nm light, the 520-nm emission was measured using special filters.

In vivo GIPC/Synectin knockdown experiments and noninvasive imaging of tumor burden. All procedures involving animals were approved by and conducted according to guidelines of the Institutional Animal Care and Use Committee of the Mayo Foundation. For these experiments we used female nude mice (age 10 wk). 10 × 10^6 control and GIPC/Synectin-negative MIAPaCa2 (n = 10 treatment, n = 10 control), resuspended in 50 μL of sterile PBS, were injected directly into the pancreas. To monitor tumor burden, the mice were imaged using the IVIS 200 Bioluminescence Imaging System (Xenogen Corp.). For imaging, 150 mg/kg D-luciferin (Xenogen) was applied i.p. 10 min before scanning. Thirty-nine days after injection, the mice were euthanized. The final tumor volume was measured and calculated using the formula 1/2 x a x b^2, where a is the longest tumor axis and b is the shortest tumor axis. Procedures were adapted accordingly for the treatment of GIPC-expressing (n = 10) and GIPC shRNA–expressing AsPC1 (n = 10) pancreatic cancer cells. Not all tumors in this group
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Results

Lentiviral GIPC/Synectin shRNA transduction inhibits tumor cell growth in vivo. To evaluate the effect of GIPC/Synectin in the in vivo tumor growth of pancreatic cancer, we injected GIPC shRNA—expressing MIA-PaCa2 tumor cells orthotopically into the pancreas of 20 nude mice. The viability of these cells was carefully checked prior to transplant. Green Fluorescent Protein tagged vectors were used to ensure a high transduction efficiency (data not shown). GIPC/Synectin were evaluated by immunoblot, showing a clear reduction of GIPC/Synectin (Fig. 1A). In order to monitor tumor growth by bioluminiscence, all carcinoma cells were additionally transfected with a luciferase vector. Tumor development was monitored at different time points after application of n-luciferin (Fig. 1B). The experiment was stopped 39 days after tumor transplantation. Throughout the entire time course and by the end of the experiment, we found significantly suppressed primary tumor growth (P = 0.04; Wilcoxon test) in mice that received tumor cells expressing GIPC shRNA. At the end of the experiment, only 5 of 10 animals were tumor-bearing in the GIPC knockdown group, compared with 8 of 10 animals in the control group (Fig. 1C). No metastasis was found in either group (Fig. 1B). To address the heterogeneity of pancreatic cancer we implanted 1 × 10⁶ control and GIPC shRNA—expressing AsPC1 pancreatic cancer cells orthotopically into 20 nude mice. As expected, this experiment also showed significantly reduced tumor growth (P = 0.03; Mann-Whitney test) in the GIPC knockdown group compared with the control group (Fig. 1D).

A GIPC-PDZ binding octapeptide reduces cancer cell proliferation in vitro. To illustrate the use of GIPC/Synectin as a therapeutic target, we developed a peptide that binds to the PDZ domain of GIPC/Synectin as a target in pancreatic cancer. For evaluation, the Zeiss Axioplan 2 microscope was used. Green Fluorescent Protein tagged vectors were used to ensure a high transduction efficiency (data not shown). GIPC/Synectin were evaluated by immunoblot, showing a clear reduction of GIPC/Synectin (Fig. 1A). In order to monitor tumor growth by bioluminiscence, all carcinoma cells were additionally transfected with a luciferase vector. Tumor development was monitored at different time points after application of n-luciferin (Fig. 1B). The experiment was stopped 39 days after tumor transplantation. Throughout the entire time course and by the end of the experiment, we found significantly suppressed primary tumor growth (P = 0.04; Wilcoxon test) in mice that received tumor cells expressing GIPC shRNA. At the end of the experiment, only 5 of 10 animals were tumor-bearing in the GIPC knockdown group, compared with 8 of 10 animals in the control group (Fig. 1C). No metastasis was found in either group (Fig. 1B). To address the heterogeneity of pancreatic cancer we implanted 1 × 10⁶ control and GIPC shRNA—expressing AsPC1 pancreatic cancer cells orthotopically into 20 nude mice. As expected, this experiment also showed significantly reduced tumor growth (P = 0.03; Mann-Whitney test) in the GIPC knockdown group compared with the control group (Fig. 1D).
was tagged with FITC to visualize its localization by confocal microscopy and myristoylated to ensure that the peptide entered the cells, as shown in Fig. 2C. In a next step, we tested the influence of the designed peptide on the viability and proliferation of different human pancreatic carcinoma cells. Viability was inhibited in a dose-dependent fashion in a concentration range from 0 to 300 μmol/L as tested with a MTS assay in different pancreatic carcinoma cell lines (Fig. 3A). A thymidine incorporation assay clearly showed that this decrease in viability can be attributed to the reduction in proliferation (Fig. 3B). In Fig. 3C the time dependency of the peptide effect is shown.

The GIPC-blocking octapeptide leads to a significant reduction of tumor growth in mice. To evaluate the in vivo efficiency of the peptide, we s.c. injected 1 × 10⁷ MIA-PaCa2 or 5 × 10⁶ AsPC1 pancreatic cancer cells into 20 nude or SCID mice and injected a total of 500 μg per mouse per day of the peptide into the developing tumor. All tumors were subjected to histologic evaluation (Fig. 4A). During the whole course of treatment the PDZ domain inhibitor did not show any toxicity in the rodents. No weight loss in one of the two groups was detected. At the end of treatment (22 days in the AsPC1 group or 27 days in the MIA-PaCa2 group), we sacrificed the mice and compared tumor growth between peptide-treated mice (n = 10) and the control group (n = 10) that received buffered saline solution mixed with DMSO. The PDZ inhibitor–treated mice had significantly smaller tumors than had the control group (P = 0.009 in the AsPC1 group; P = 0.01 in the MIA-PaCa2 group, Mann-Whitney test; Fig. 4A). The evaluation of the proliferation marker Ki67 showed a significantly lower proliferation activity in the treatment group compared with the control group in the

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**Fig. 1.** A, immunoblot for GIPC after transduction of MIA-PaCa2 pancreatic cancer cells with a lentivirus-expressing shRNA for GIPC. Compared with the control cells, which were transduced with an empty vector, the protein levels of GIPC are reduced. Western blots were scanned and cropped according to the molecular weight of GIPC. B, bioluminescence images after n-Luciferin application. GIPC/Synectin expression in MIA-PaCa2 cells was reduced by lentiviral shRNA, in the control group an empty Luc vector was used. C, Kaplan–Meier diagram showing the percentage of tumor-free mice in relation to the time after orthotopic tumor transplantation. Ten nude mice were transplanted orthotopically with GIPC-deficient MIA-PaCa2 cells; the other 10 nude mice were transplanted with MIA-PaCa2 which were transduced with an empty luciferase vector. The difference is significant (P = 0.04; univariate log rank test). Tumor take rates on day 19 posttransplantation: control, 5 of 10; GIPC shRNA, 0 of 10; on day 24 posttransplantation: control, 8 of 10; GIPC shRNA, 3 of 10; on day 39 posttransplantation (end of experiment): control, 8 of 10; GIPC shRNA, 4 of 10. D, box plots of the tumor volume after implantation of AsPC1 cells in the pancreas of 20 nude mice. Ten mice received AsPC1 cells that were treated with shRNA and 10 mice were transplanted with AsPC1 cells that were transduced with an empty Luc vector. After 14 d the mice were sacrificed and tumor volume was measured. The group that received GIPC-deficient AsPC1 cells had significantly smaller tumor volume than did the control group. Mean in the treatment group (GIPC shRNA) 402 mm³ (95% confidence interval (95% CI), 137-380 mm³); mean in the control group (empty vector), 498 mm³ (95% CI, 302-501 mm³); P = 0.03 (treatment versus control; Wilcoxon test).
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AsPC1 \( (P = 0.02; \text{t-test}; \text{Fig. 4B}) \) and the MIAPaCa2 group \( (P = 0.013; \text{t-test}; \text{Fig. 4C}) \).

Targeting the PDZ domain of GIPC alters IGF-IR levels. Previously, we showed that GIPC/Synectin is associated with IGF-IR. This interaction has been proven to be important for the IGF-I receptor stability and to be mediated by the PDZ domain.

To test whether the peptide blocks the association between IGF-IR and GIPC/Synectin, we transfected MIA-PaCa2 cells with a FLAG-tagged wild-type GIPC/Synectin plasmid, and blotted against IGF-IR after immunoprecipitation for FLAG. After treatment with the peptide and blockage of the proteasomal degradation of IGF-IR to ensure similar IGF-IR protein levels, the association between IGF-IR and GIPC/Synectin was significantly reduced (Fig. 5A). These results proved that the small peptide was effective in inhibiting the association between IGF-IR and GIPC/Synectin. As a second step, we evaluated the effects of the inhibitor treatment on IGF-IR expression in more detail. In our previous study, we showed that RNA interference for GIPC/Synectin also suppressed protein levels of IGF-IR. This effect could be reversed by proteosomal inhibition, suggesting that GIPC/Synectin affects protein stability of IGF-IR. Accordingly, we examined IGF-IR expression by Western blot after GIPC-PDZ inhibitor treatment. The immunoblots clearly showed decreased protein levels of IGF-IR in MIAPaCa2 and AsPC1 pancreatic cancer cells (Fig. 5B). Proteasome inhibitor treatment prevents the IGF-IR protein reduction (Fig. 5C). Resembling the reduction of IGF-IR protein levels in GIPC shRNA–expressing MIAPaCa2 pancreatic cancer cells \textit{in vivo} (Fig. 6A), treatment with the GIPC-PDZ–blocking peptide also led to a significant reduction of
IGF-IR protein levels in both experimental groups, the AsPC1 (Fig. 6B) and the MIAPaCa2 group (Fig. 6C).

**Discussion**

The main focus of this study was to examine the *in vivo* effect on tumor growth after GIPC/Synectin inhibition and its effect on one of the associated growth factor receptors, IGF-IR. We used two approaches: (*a*) *ex vivo* transduction of pancreatic cancer cells with lentiviral shRNA followed by orthotopic transplantation; and (*b*) blocking GIPC/Synectin with a PDZ inhibitor by intratumoral injections into an established tumor.

Here, we have shown that knocking down GIPC/Synectin, a protein highly expressed in pancreatic adenocarcinoma (2), inhibits pancreatic cancer growth in an orthotopic mouse model. This supports the data of our prior study that described a prominent inhibition of pancreatic cancer cell proliferation after GIPC/Synectin down-regulation *in vitro* (2). The high expression of GIPC/Synectin in pancreatic adenocarcinoma and the important function for the proliferation of pancreatic cancer cells make it a target for therapeutic intervention. Accordingly, we developed an octapeptide that can block the

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**Fig. 4.** *A*, representative histology picture of untreated MIAPaCa2 pancreatic cancer cells after s.c. transplantation into mice. The tumor was explanted on day 27. At this time some tumors showed necrosis which a reactive inflammatory infiltrated. Box plots, volume of AsPC1 and MIAPaCa2 tumors after s.c. implantation and intratumoral treatment with PSQSSSEA at day 22 (AsPC1 group, end of the treatment) and at day 27 (MIAPaCa2 group, end of treatment). In the AsPC1 group the treatment was started with 10 SCID mice. In the control group (AsPC1) one mouse died after injection. The treatment in the AsPC1 group was started when the tumor was established and reached the following volumes: 201 mm³ average tumor size in the control group (95% CI: 138-285 mm³) and 154 mm³ average tumor size in the treatment group (95% CI: 122-186 mm³). On treatment day 22 (AsPC1), the peptide-treated mice had significantly smaller tumors than did the control group (P = 0.009, Mann-Whitney test; average tumor size in the control group, 378 mm³ (95% CI: 214-542 mm³); average tumor size in the treatment group, 218 mm³ (95% CI: 125-249 mm³)). In the MIAPaCa2 group 10 nude mice were treated and 10 nude mice served as a control. Compared with the AsPC1 group we started with larger tumor volumes with the treatment and treated 27 d instead of 22 d. At the starting point the tumors in the MIAPaCa2 group reached the following volumes: 289 mm³ average tumor size in the control group (95% CI: 112-584 mm³) and 231 mm³ average tumor size in the treatment group (95% CI: 149-462 mm³). On treatment day 27 (MIAPaCa2), the peptide-treated mice had significantly smaller tumors than did the control group (P = 0.01, Mann-Whitney test; average tumor size in the control group, 4,036 mm³ (95% CI: 2,275-6,239 mm³); average tumor size in the treatment group, 661 mm³ (95% CI: 228-2,528 mm³)). *B*, evaluation of the proliferation rate in the tumor tissue of mice that were injected with AsPC1 pancreatic cancer cells. The proliferation rate was evaluated by staining for Ki-67. Nuclear staining per 1,000 cells was counted in representative tumors (n = 7). The difference between treated and control tumors is significant (P = 0.02; t-test). Microphotographs show staining for Ki-67 in selected AsPC1 tumors of the control and the treatment group (magnification ×400; Zeiss Axioplan 2; KS400 image capture software). C. Ki67 index in tumor tissue of mice that were implanted with MIAPaCa2 pancreatic cancer cells. The difference between treated tumors (n = 6) and control tumors (n = 7) is significant (P = 0.0129; t-test). Microphotographs show Ki67 staining of MIAPaCa2 tumors after peptide and control treatment. (magnification ×400; Zeiss Axioplan 2; KS400 image capture software).
function of GIPC/Synectin. This blocking peptide can significantly reduce tumor growth, and inhibits the proliferation of pancreatic cancer cells. This confirms the importance of GIPC/Synectin as a central protein in pancreatic cancer cells.

GIPC/Synectin has shown to be important for protein trafficking and receptor stability, which gives the blocking peptide PSQSSSEA a lot of opportunities to disrupt important cell functions: a role of GIPC/Synectin in cancer cell invasion and metastasis has been shown already (23). Also, GIPC/Synectin is associated with different integrins (24) and Syndecan-4 showing a role for GIPC/Synectin in cancer progression (5, 6). GIPC is also recruited by APPL to TrkA endosomes (18). TrkA phosphorylation plays a role in nerve growth factor–mediated growth of Mia PaCa2 (25). Moreover, a recent report suggested that APPL is important for the correct localization of Akt/PKB inside the cell (26). It will be important to evaluate the effect of the GIPC-PDZ-blocking peptide on these different pathways in detail. In our previous work we found that GIPC/Synectin is associated with IGF-IR and is important for IGF-IR stability (2, 27). Accordingly, we focused on the effect of the peptide treatment on IGF-IR, an important growth-promoting receptor in cancer cells.

Our previous results prove that GIPC/Synectin is important in IGF-IR protein expression. These functions were proven to be PDZ domain–dependent. In confirmation of these results we showed that blocking GIPC/Synectin with a cell-permeable GIPC-PDZ inhibitor reduced protein levels of IGF-IR significantly in vitro and in an animal model. To address the mechanism of IGF-IR reduction after peptide treatment, we inhibited the proteasomal pathway. It has already been shown that the proteasomal pathway plays a role in IGF-IR receptor turnover (28). Proteasomal inhibition following the application of PSQSSSEA recovers IGF-IR. These data suggest a protective function of the GIPC/Synectin–IGF-IR-interaction in maintaining IGF-IR levels.

Because GIPC is an adaptor molecule for the binding to Myosin VI (29), it is very probable that knocking down GIPC/Synectin disturbs the transportation machineries of IGF-IR after binding its ligand. Alternatively, Varsano et al. have recently shown that SEA, part of the blocking octapeptide, is also a binding motif for APPL (18). APPL itself is associated with Rab5 (Ras related in brain 5) and is important for the GIPC/Synectin transport to the endosome. Therefore, blocking the SEA domain may inhibit binding of APPL to GIPC/Synectin. This, in turn, may inhibit the transport of GIPC/Synectin to the endosome and ultimately, to IGF-IR, leading to less GIPC/Synectin available for IGF-IR binding. This mechanism may also reduce the chance of an interaction between IGF-IR and GIPC/Synectin, which synergizes with the direct inhibition of the interaction by blocking the GIPC-PDZ domain.

There seems to be another important role of GIPC in IGF-IR function that is independent of transportation processes. Recently, a study in Xenopus showed that knocking out GIPC/Synectin inhibits eye development by disturbing IGF-IR signaling; this study, however, did not report any reduction of IGF-IR protein levels (30). Therefore, GIPC/Synectin may have another important function in connecting the tyrosine kinase receptor IGF-IR to G-protein signaling pathways (27). At this point, it is not possible to comment on the contribution of the different GIPC-associated proteins for the growth inhibition, which results after blocking GIPC. Accordingly, future studies should evaluate the importance of the GIPC associated molecules like IGF-IR, Neuropilin, TrkA, and APPL for the function of this octapeptide.

With their size of approximately 90 residues and their modular nature, PDZ domains are considered as typical protein

[Fig. 5. A, immunoblot against IGF-IR after immunoprecipitation for FLAG (IP: Flag IB: IGF-IR). Forty-eight hours before harvesting protein MIAPaCa2 cells were transiently transfected with Flag-tagged wild-type GIPC/Synectin. Cells were treated with 100 μmol/L of PSQSSSEA or the control peptide dissolved in DMSO. Proteasome inhibitor was used to sustain IGF-IR expression by inhibiting octapeptide-induced degradation. This immunoblot showed that the blocking peptide is effective in reducing the association between GIPC/Synectin and IGF-IR. The Western Blot for FLAG showed equal transfection efficiency. B, Western Blot analysis of cells treated with PSQSSSEA (200 μmol/L, 100 μmol/L, control peptide) overnight after incubation with 50 ng/mL recombinant human IGF-I in MIAPaCa2 and AsPC1 pancreatic cancer cells. The analysis showed a down-regulation of IGF-IR Western blots were scanned and cropped according to the molecular weight of IGF-IR. β-actin serves as a loading control. C, evaluation of IGF-IR protein levels by immunoblot after treatment with PSQSSSEA and recombinant human IGF-I in MIAPaCa2 pancreatic cancer cells. Before harvesting of the protein 25 μmol/L proteasome inhibitor was added for 2 h. Immunoblots were scanned and cropped according to the molecular weight of IGF-IR. β-actin serves as a loading control.]
interaction domains. PDZ domains are elements in a large mosaic of binding networks that crisscross the cell both temporally and spatially. This shows how complex the biological effect of a GIPC-PDZ blocker might be (31). To achieve specificity we focused on specific domains within the PDZ domain of GIPC that are responsible for the interaction with the COOH-terminal end of receptors and intracellular proteins. This approach allows greater specificity of the generated PDZ blocker. According to nature and importance of PDZ domains in cell interactions with other proteins are possible and might even enhance the effectiveness of the GIPC-PDZ blocker. To address this complexity of PDZ domain interaction network further studies are necessary to understand the cross talk with other cell signaling pathways.

In conclusion, this study has shown that targeting GIPC/Synectin with short interfering RNA or an inhibitory PDZ domain–targeting peptide substantially reduced pancreatic adenocarcinoma growth in vivo. In any case, this peptide now represents a lead compound that can be subjected to a variety of peptidomimetic or organic modifications. These may impart improved efficacy and bioavailability properties to the next generation of such PDZ domain–targeting inhibitors in future in vivo investigations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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