Potato Carboxypeptidase Inhibitor, a T-knot Protein, Is an Epidermal Growth Factor Antagonist That Inhibits Tumor Cell Growth*

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Epidermal growth factor (EGF) and its receptor (EGFR) are involved in many aspects of the development of carcinomas, including tumor cell growth, vascularization, invasiveness, and metastasis. Because EGF has been found to be overexpressed in many tumors of epithelial origin, it is a potential target for antitumor therapy. Here we report the potato carboxypeptidase inhibitor (PCI), a 39-amino acid protease inhibitor with three disulfide bridges, is an antagonist of human EGF. It competed with EGF for binding to EGFR and inhibited EGFR activation and cell proliferation induced by this growth factor. PCI suppressed the growth of several human pancreatic adenocarcinoma cell lines, both in vitro and in nude mice. PCI has a special disulfide scaffold called a T-knot that is also present in several growth factors including EGF and transforming growth factor α. PCI shows structural similarities with these factors, a fact that can explain the antagonistic effect of the former. This is the first reported example of an antagonistic analogue of human EGF.

In multicellular organisms peptide factors regulate a variety of cell functions and processes including cell proliferation (1). Epidermal growth factor (EGF) is a competence peptide factor that can induce the cells to advance into the G1 phase and is required for differentiation of epidermal tissues (1, 2). It is produced by many normal tissues and is present in serum. Both EGF and transforming growth factor α (TGF-α), a growth factor highly homologous to EGF, bind to EGF receptor (EGFR or ErbB-1) (3, 4) and produce similar biological responses.

Binding of EGF to EGFR induces receptor dimerization (5) and leads to receptor activation and tyrosine transphosphorylation (6). Ligand-receptor complexes are quickly internalized via coated pits and either recycled or subjected to lysosomal degradation (7).

Most human cancers arise in the epithelial component of organs including the skin, breast, lung, and gastrointestinal and genitourinary tracts. Alterations in growth factor signaling pathways during epithelial neoplasia are common and therefore may be important in the development and maintenance of the neoplastic phenotype (8). EGF, TGF-α, and their receptor, EGFR, seem to play a particularly prominent role in epithelial neoplasia (9, 10), and they have been implicated in processes such as tumor cell growth, vascularization, invasiveness, and metastasis (8, 11–13). In many tumors of epithelial origin (carcinomas), EGFR has been found to be overexpressed, and in some cases an autocrine loop involving TGF-α has been reported (2, 14). The importance of an autocrine loop activation involving TGF-α was first described in pancreatic cancer (15), which has a very poor prognosis. EGFR activation is involved in the genesis and progression of pancreatic neoplasia (16, 17). The increased levels of EGF, TGF-α, and EGFR produced by pancreatic tumors may provide tumor cells with a distinct growth advantage that contributes to the clinical aggressiveness of this malignancy.

Given the importance of EGFR in carcinomas, disruption of the activation of EGFR appears to be an excellent target for cancer therapy (2, 13, 18). Cancer cells seem to have lost the normal redundancy in signal transduction pathways and so are preferentially vulnerable to signal interceptors. EGFR activation can be disrupted in several ways, including with EGF antagonists (19), with tyrosine kinase inhibitors (13), and with antibodies directed against the EGFR (20). These three strategies have had various success ratios. Development of EGF/TGF-α antagonists by using short synthetic fragments of both growth factors has not been successful (19). The tyrosine kinase inhibitors have been shown to inhibit the development of tumors in animal models but have toxic side effects (13). In contrast, the use of antibodies seems to have some efficiency as antitumor treatment and it is being tested in clinical trials.

In the study presented here, we show that potato carboxypeptidase inhibitor (PCI), a proteinaceous protease inhibitor, is an antagonist of human EGF with antitumor properties. Some protease inhibitors have been reported to serve as cancer chemopreventive agents, because they can substantially suppress radiation- and chemical-induced malignant transformation in vitro and have strong anticarcinogenic activity in vivo.

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‡ The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PCI, potato carboxypeptidase inhibitor; TGF-α, transforming growth factor α; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RITC, rhodamine B isothiocyanate.
(21–23). However, the mechanisms responsible for this activity are unknown.

PCI is a 39-amino acid protein naturally occurring in potatoes that can form complexes with several metallo-carboxypeptidases, inhibiting them in a strong competitive way with a Ki in the nanomolar range (24). We have developed a procedure to obtain the inhibitor in a recombinant form in Escherichia coli (25–27). Its structure is known in aqueous solution (28) and in crystal complex with carboxypeptidase A (29). The 27-residue globular core of PCI is stabilized by three disulfide bridges. Residues 35–39 form a C-terminal tail that docks on the carboxypeptidase A active center. PCI contains a small cysteine-rich module, called a T-knot scaffold, that is shared by several different protein families, including the EGF family (30–32).

We report here that PCI is an antagonistic analogue of human EGF, the first one described. PCI bound to EGF and inhibited its activation by EGF. The structural similarities of PCI with this and other growth factors probably account for its properties as EGF antagonist. In addition, PCI inhibited the growth of human pancreatic cell lines and tumors transplanted in nude mice. Our results indicate that these antitumor properties are probably a result of the EGF antagonistic activity of PCI.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant EGF was purchased from R & D Systems (Abingdon, UK), 125I-labeled EGF from ICN (Costa Mesa, CA), human recombinant insulin was from Boehringer Mannheim (Mannheim, Germany), monoclonal antibody against EGF (clone EGFRI) was from Amersham Pharmacia Biotech (Little Chafton, UK), recombinant anti-phosphotyrosine antibody (RC20) was from Transduction Laboratories (Lexington, KY), protein molecular weight standards were from Bio-Rad (Hercules, CA), and the EZAU reduction assay kit was from Biomedica Corp. (Vienna, Austria). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO).

PCI was obtained as a recombinant protein. The construction of a synthetic gene for PCI, its expression in E. coli, and a procedure to detect, quantify, and purify recombinant PCI secreted into the culture medium have been previously reported (25–27). Its expression in E. coli (St. Louis, MO).

Routine Cell Culture—Capan-1, Panc-1, A431, and H1T were obtained from the American Type Culture Collection (Rockville, MD). Capan-1, A431, and H1T cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, and 20 μg/ml gentamicin unless otherwise indicated. Panc-1 cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and 20 μg/ml gentamicin.

Cell Proliferation Assays and Growth Curves—The inhibitory effects of PCI on cell growth were determined with a proliferative assay. To measure proliferation in medium with serum, Capan-1, Panc-1, and HIT tumor cells were seeded at a density of 5 × 103/well in 96-well plates in medium containing 10% FBS. PCI was immediately added at concentrations of 0.1–200 μg/ml. The cells were fed every 4 days with medium containing fresh PCI. Control cells were grown without PCI. After 24 days, the cells were trypsinized, seeded at a density of 2 × 103/well in 96-well plates, and treated with 0, 1, or 50 μg/ml PCI. Control cells were grown in PCI-free medium. Every 3 days, 8 replicate wells for each treatment were submitted to the EZAU reduction assay to estimate the number of cells.

Tumor Transplantation Experiments—For injection into nude mice, Capan-1 and A431 cells were trypsinized and resuspended in DME, 6-week-old female Cd1 nude mice were each injected subcutaneously in the dorsal area with 1 × 106 cells in 0.1 ml of DME, 12 days after the injection, when the tumors had reached at least 2 mm in diameter, treatment was started. The tumors were injected daily with 11, 60, or 120 μg of PCI. PCI control mice were treated with PBS alone. Each group was composed of five mice. The tumors were measured weekly. After 32 days, the animals were killed by CO2 asphyxiation. Their tumor volumes were determined by using the formula [length x width2]/2.

Cell Cycle Analysis—Logarithmically growing Capan-1 cells were treated with 50 μg/ml PCI for 12 days in a 75-cm2 flask. Adherent cells were collected by trypsinization and combined with cells floating in the medium. After being washed with PBS, the cells were resuspended in 2000 μl of 0.01% CAC was added. After 2 h of fixation, the cells were stained with propidium iodide (50 μg/ml) and RNase (20 μg/ml) was also added. Flow cytometry was performed by exciting the cells with a 488-nm laser (Becton Dickinson and Co., Rutherford, NJ).

PCI Uptake Assays were performed by measuring the PCI concentration in Capan-1 culture medium for 24 h. In three separate experiments, cells growing logarithmically in a 25-cm2 flask in DME supplemented with 10% FBS were treated with PCI at 10, 50, and 200 μg/ml. Samples of 20 μl of medium were taken every 30 min for 24 h. The concentration of PCI was determined by inhibition assays of carboxypeptidase A (24) and enzyme-linked immunosorbent assay using a rabbit polyclonal antibody raised against PCI. Medium without PCI was used as a reference.

Comparison of the Three-dimensional Structures of PCI and EGF—We developed a computer program (Knot-Match)$^2$ to superimpose the proteins by three-dimensional aligning of their disulfide bridges. The program clusters structures from Protein Data Bank proteins by means of a density search algorithm. Molecular graphics and simulations were performed with the Power Indigo 2 from Silicon Graphics. The structures of PCI and growth factors were visualized with the TURBO FRODO program (33). The conformation of loops was analyzed by the Arch-Type program (34).

Ligand Binding Assays—Capan-1 cells were seeded at a density of 1.25 × 105/well in 24-well plates in the presence of DME supplemented with 10% FBS. After 48 h, the medium was replaced with DMEM without FBS. 24 h later, the cells were washed twice with ice-cold binding buffer (DME supplemented with 20 mM HEPES, pH 7.5, and 0.3% (v/v) bovine serum albumin) and incubated for 4 h at 4 °C with binding buffer containing 35 pmol 125I-labeled EGF and various concentrations of EGF or PCI. The cells were then washed rapidly three times with ice-cold PBS with 0.1% bovine serum albumin and solubilized by incubating them for 30 min at room temperature with 1 N NaOH with 0.1% SDS. The radioactivity in the suspension was determined with a γ counter (LKB, Uppsalan, Sweden). Nonspecific binding was determined as the amount of radioactivity bound to cells incubated with a 100-fold molar excess of unlabeled EGF. The data were analyzed by computer fitting of one ligand with two binding sites.

Phosphorylation Analysis—Receptor phosphorylation analyses of serum-starved cells were performed as follows. 50% confluent serum-starved Capan-1 and A431 cells in 60-mm dishes were treated with various concentrations of PCI (in DME) for 10 min and imme-

$^2$ J. M. Mas, B. Oliva, C. Blanco-Aparicio, M. A. Molina, R. de Lorenzo, E. Queiro, and F. X. Avilés, submitted for publication.
diately stimulated with EGF (5 ng/ml for Capan-1 and 1 ng/ml for A431 cells, also in DMEM) for 10 min. The cells were then lysed, and the EGFR was immunoprecipitated from cell extracts using equal amounts of proteins of each sample with anti-EGFR antibody EGFRI. The kinase activity of the immunoprecipitated receptor was measured by incubating it with [γ-32P]ATP for 10 min (35). Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The bands were quantified with a beta counter. The level of tyrosine phosphorylation of the immunoprecipitated receptor was assessed by immunoblotting with anti-Tyr(P) antibody RC-20 (36). The blots were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) and then autoradiographed. The bands were quantified by densitometry.

For the receptor phosphorylation analyses of Capan-1 and A431 cells growing in DMEM plus 10% FBS, cells were treated with 10 ng/ml EGF or 50 μg/ml PCI. The kinase activity of the receptor was measured as described above.

Data Analysis—Means ± S.E. are depicted unless indicated otherwise. Student’s t test or analysis of variance for repeated measures was used for comparisons between data sets.

Covalent Cross-linking Experiments—The cross-linking experiments were carried out as previously reported in Ref. 47. Briefly, cells of A431 human epidermal carcinoma line were lysed and homogenized. Samples were mixed with different concentrations of PCI alone and PCI plus EGF. Cross-linking was initiated by addition of glutaraldehyde. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (5%), electrotransferred to polyvinylidene difluoride membranes, and immunostained with antibodies against EGFR (ErbB-1). The presence of PCI was corroborated by immunostaining with rabbit antibodies against PCI on the same membrane.

RESULTS

Inhibition of Proliferation of Pancreatic Tumor Cell Lines by PCI—To determine whether PCI could inhibit the growth of tumor cells, we tested its ability to affect the in vitro growth of two human pancreatic adenocarcinoma cell lines, Panc-1 and Capan-1, and the hamster insulinoma cell line HIT. Cells were cultured in medium supplemented with 10% fetal bovine serum. Concentrations of PCI greater than 10 μg/ml significantly inhibited growth, particularly for Capan-1 and HIT cells (Fig. 1A). The maximal effect was obtained at 50 μg/ml PCI; higher concentrations did not have stronger effects.

We subsequently obtained the growth curve of Capan-1 cells in the presence of 1 and 10 μg/ml PCI. There was a clear decrease in the growth rate of the cells in the presence of 10 μg/ml PCI (Fig. 1B). No significant differences were observed in the cells treated with 1 μg/ml PCI compared with controls.

Further studies were undertaken to assess the effect of protracted treatment with PCI on the proliferation of Capan-1 cells. The growth rate of the cells that had been pretreated with PCI for 3 weeks was significantly lower than that of Capan-1 control cells (Fig. 1C). The inhibitory effect was observed even when the pretreated cells were grown without PCI. These experiments demonstrated that PCI had a long lasting inhibitory effect on the growth of Capan-1 cells that was maintained even after PCI was removed from the culture medium.

Inhibition by PCI of Capan-1 Tumor Growth in Nude Mice—We next determined whether PCI could also reduce the growth of solid tumors obtained by subcutaneous injection of Capan-1 cells into nude mice (Fig. 2). There was a significant reduction of the growth of the tumor transplantsations at the three PCI doses tested. No toxic side effects were observed in any of the treated animals. Histological examination of the tumors showed no appreciable morphological differences between tumors from treated and control animals (data not shown), suggesting that the decreased size of tumors in treated animals was not due to cytotoxic effects or massive cellular death. That PCI could inhibit the growth of human pancreatic tumor cells is particularly important because the prognosis for pancreatic cancer is very poor and there are no effective treatments (37).

Effects of PCI on Cell Cycle Traversal—To further characterize the effects of PCI on tumor cell growth, analyses of cell cycle phase distribution were performed with Capan-1 cells. Flow cytometry did not reveal any cell cycle changes in Capan-1 cells treated with 50 μg/ml PCI for up to 7 days compared with controls. No significant increase in the percentage of apoptotic cells was observed by flow cytometry, direct counting of apoptotic cells after nucleus staining, or agarose electrophoresis of nuclear DNA. However, after 12 days of treatment with PCI, a significant increase in the percentage of apoptotic cells (the sub-G0 population) was observed by flow cytometry (being the mean ± S.D. of 6.9 ± 0.9% for control and 10.5 ± 1.5% for

![Fig. 1. Effects of PCI on the growth of pancreatic adenocarcinoma tumor cell lines.](image-url)
treated cells). A small increase (65.2 ± 0.9% to 68.5 ± 2.9%) in the percentage of cells in the G0/G1 phase was also detected. These findings suggested that the increase in the percentage of apoptotic cells in presence of PCI could be one of the mechanisms responsible for the inhibitory effect of this protein on tumor cell growth.

Internalization of PCI by Capan-1 and Panc-1 Cells—The results so far obtained led us to examine whether PCI was taken up by tumor cells. Using fluorescent labeling of PCI, we demonstrated that PCI was easily internalized by Capan-1 and Panc-1 cells. RITC-conjugated PCI was observed in the cytoplasm of the cells as early as 30 min after its addition to the culture medium of logarithmically growing cells. The fluorescence was located mainly around the nucleus (Fig. 3A).

To better characterize the kinetics of the internalization of PCI by the cells, we measured the PCI concentration in the medium every 30 min for 24 h after its addition. Three separate experiments were performed using three different PCI initial concentrations. In all cases, PCI underwent several cycles of disappearance and reappearance (Fig. 3B). 24 h after its addition, PCI was purified from the medium and subjected to mass spectrometry analysis, which showed that the molecular mass of PCI remained unaltered. This finding was not surprising, because PCI has been reported to be very resistant to proteolytical degradation (38).

Computer Comparison of the Three-dimensional Structures of PCI and EGF—PCI and several mammalian growth factors, ω-toxins, and other proteins share a cystine-knot scaffold, the so-called T-knot (30–32). To gain insight into the possible mechanisms responsible for the growth inhibitory effects of PCI, we compared the three-dimensional structures of PCI and other T-knot proteins by means of Knot-Match program.2 The program yielded several groups. One of these groups contained
dorsal area with 13. Female Cd1 nude mice were injected subcutaneously in the dorsal area with 1 × 10^7 cells. Treatment was started 12 days after the injection. The tumors were injected daily with various doses of PCI dissolved in PBS. Control mice were treated with PBS alone. Each group was composed of five mice. The data are expressed as the final volume of the tumors (Vf) relative to their initial volume (Vi) and are the means ± S.E. The symbols above the bars denote significant difference from control values: *, p < 0.05; #, p < 0.025; &, p < 0.01; **, p < 0.001.

![Fig. 2](image.png)

**Fig. 2.** Suppression by PCI of Capan-1 tumor growth in nude mice. Female Cd1 nude mice were injected subcutaneously in the dorsal area with 1 × 10^7 cells. Treatment was started 12 days after the injection. The tumors were injected daily with various doses of PCI dissolved in PBS. Control mice were treated with PBS alone. Each group was composed of five mice. The data are expressed as the final volume of the tumors (Vf) relative to their initial volume (Vi) and are the means ± S.E. The symbols above the bars denote significant difference from control values: #, p < 0.1; +, p < 0.025; &, p < 0.01; **, p < 0.001.

Proteins were superimposed, based on disulfide bridges topology. Among them are Leu296, Tyr37, Arg43, and Leu47, which correspond to Ala31, Trp22, Lys10, and Val38 of PCI (Fig. 4).

The structural and conservative positional similarities between PCI and human EGF and TGF-α suggested that PCI could act as an EGF/TGF-α antagonist, competing with these growth factors for binding to EGFR and thus inhibiting its activation. This could be the mechanism for the suppressive effect of PCI on tumor cell growth, because EGFR stimulation by either EGF or TGF-α seems to be required for proliferation by a variety of tumor cell lines and carcinomas (13, 39, 40). We used several approaches to test the hypothesis that PCI is an EGFR antagonist.

**Suppression by PCI of the Stimulatory Effect of EGF on the Growth of Capan-1 Cells—**We performed experiments with serum-starved Capan-1 cells in presence of PCI, EGF, insulin, or PCI and growth factor simultaneously. Both growth factors had a stimulatory effect on cell growth that was stronger in the case of EGF. The presence of PCI completely abolished the EGF stimulation of cell proliferation but had no effect in the case of insulin-induced cell growth (Fig. 5).

**PCI Competition with EGF for Binding to EGFR—**Binding experiments using the Capan-1 cell line were performed. The binding of 125I-labeled EGF was measured in the presence of increasing concentrations of EGF (Fig. 6A). The data revealed that Capan-1 had high and low affinity receptors for EGF. For the former, the IC50 for EGF was 0.6 pm. We then measured the binding of 125I-EGF in presence of increasing concentrations of PCI (Fig. 6B). PCI competed with 125I-EGF for binding to the high and low affinity receptors. The IC50 of the high affinity receptors for PCI was 100 pm.

**PCI Inhibition of EGFR Kinase Activity and Transphosphorylation Induced by EGF—**We next investigated whether PCI could inhibit the activation of the receptor induced by EGF in the EGFR-overexpressing vulvar carcinoma cell line A431 (41).
and in Capan-1. In experiments using serum-starved A431 cells, we found that there was a low level of EGFR kinase activity. Addition of EGF caused a rapid increase in this activity (detectable after 10 min), and PCI was found to be an effective inhibitor of this activation. Preincubation of the cells with 50 μg/ml PCI for 10 min completely suppressed any detectable activation of EGFR by EGF (Fig. 7A). Similar results were obtained in Capan-1 cells, in which 50 μg/ml PCI strongly reduced the activation of EGFR by EGF (Fig. 7B).

We also examined the effect of PCI on EGFR activation in Capan-1 and A431 cells growing in medium with 10% FBS, without added EGF. These cells showed a significant level of EGFR activation that was not affected by addition of EGF for 10 min (data not shown). When PCI was added for 10 min, the kinase activity of the receptor was significantly reduced about

20% (Fig. 8A), probably as a result of the PCI-induced blockage of EGFR activation by growth factors either present in the FBS or produced by the cells. When PCI was maintained in the culture medium for 6 days, the reduction in the kinase activity was stronger; in this case the possibility that PCI down-regulates EGFR cannot be ruled out (Fig. 8B).

Covalent Cross-linking Experiments—Cross-linking assays showed that PCI interacts with EGFR and that this interaction could be reverted by EGF (not shown). PCI only cross-reacts with monomers of EGFR, thus indicating that it is inhibiting the dimerization capacity of EGF. PCI seems to act as an

FIG. 4. Superimposition of the three-dimensional structures of PCI and EGF by using the Knot-Match program. Upper left, ribbon representation of superimposed PCI (yellow) and EGF (white) backbones. Right, Van der Waals representation of the three-dimensional structures of PCI and EGF. The side chains of the residues that coincide after superimposition of the disulfide bridges of the two proteins are color-coded: brown, hydrophobic; cyan, basic; green, polar; purple, aromatic. Center, Van der Waals representation of the superimposition of the three-dimensional structures of PCI (yellow) and EGF (white) using the same color code as in the representations on the right. The table shows the residues that coincide after the superimposition.

FIG. 5. Suppression by PCI of the stimulatory effect of EGF on the growth of serum-starved Capan-1 cells. Cells were treated during 72 h with EGF (10 ng/ml), insulin (5 μg/ml), PCI (50 μg/ml), EGF and PCI, or EGF and insulin. The values shown are the means ± S.E. from 8 replicate wells for each treatment. Significant difference from control values are indicated: †, p < 0.05; *, p < 0.001. The results shown are a representative of three different experiments.

FIG. 6. Displacement of 125I-labeled EGF from Capan-1 cells by unlabeled EGF (A) and PCI (B). Monolayers of serum-starved cells were incubated at 4 °C with 350 pM of 125I-labeled EGF and various concentrations of EGF and PCI. Cell-bound radioactivity was determined following 4 h of incubation and extensive washing. Nonspecific binding was determined in the presence of an excess of the unlabeled factor and was subtracted from the total amount of cell-bound radioactivity. The specifically bound percentage of 125I-labeled EGF (B) was used to calculate log [(B/100 – B)], which is plotted against the logarithm of the concentrations of EGF and PCI. The plots were also used to calculate the IC50.
antagonist analogue of EGF preventing the dimerization process.

DISCUSSION

Much effort is currently being devoted to finding new molecules that target signal transduction pathways (13), including antagonists that bind to growth factor receptors without activating them (8, 19, 20). Such antagonists are of both theoretical and clinical interest, because they can lead to a better understanding of the interactions responsible for the binding of growth factors to their receptors and can be used as new antitumor drugs.

The EGFR is one of the most studied growth factor receptors due to its importance in the development and functionality of epidermal tissues as well as carcinomas, where it is frequently overexpressed. Both EGF and TGF-α bind to this receptor, whose activation initiates a cascade of biological processes (42) and is required for proliferation in many cell types and cancer cells. Despite its importance, no antagonist for human EGFR has been reported so far. An inhibitory ligand of the mammalian EGFR has been recently described (43), and monoclonal antibodies against human EGFR have been produced (44). PCI is, however, the first reported antagonistic analogue of human EGF that is able to bind to EGFR without eliciting the activation of the receptor. In addition, PCI can suppress tumor cell growth, probably as a result of diminished EGFR activation.

The results of the binding experiments presented here demonstrated that PCI competed with EGF for binding to EGFR. The affinity of the receptor for EGF was higher than for PCI, as was expected. The internalization of PCI by the cells observed by using fluorescent labeling was perhaps a consequence of the endocytosis of the EGFR once it was bound to PCI. Some indirect evidence supports this hypothesis. First, after internalization PCI was located around the nucleus, as is EGF (45). Second, PCI underwent several cycles of appearance and disappearance from the culture medium, which might have been produced by the recycling and lysosomal degradation of the receptor (7). This reappearance in the culture medium of a dissociated recycled ligand has also been observed in the case of EGF (46). Third, PCI was not altered by the cells, which rules out the possibility that PCI binds to a membrane metallo-carboxypeptidase, given that the last residue of the inhibitor is quickly cleaved when it binds to these kinds of enzymes (24).

PCI not only bound to EGFR, but it also inhibited the activation of the receptor induced by EGF. The experiments performed in serum-starved Capan-1 and A431 cells demonstrated that PCI binding did not significantly activate EGFR and that the inhibitor blocked the EGF-induced increase in the kinase activity of the receptor induced by EGF. The experiments performed in serum-starved Capan-1 and A431 cells demonstrated that PCI binding did not significantly activate EGFR and that the inhibitor blocked the EGF-induced increase in the kinase activity of the receptor. In these conditions, the addition of PCI not only bound to EGFR, but it also inhibited the activation of the receptor induced by EGF. PCI not only bound to EGFR, but it also inhibited the activation of the receptor induced by EGF. By contrast, in cells growing in presence of FBS, a significant level of EGFR activation was observed, probably as a result of the presence of growth factors in serum capable of activating the receptor. In these conditions, the addition of PCI reduced the kinase activity of the receptor in a significant way, suggesting that it competed with the growth factors present in serum for binding to EGFR (Fig. 8A). In all the previous experiments, the cells were incubated with PCI only for 10 min. When the inhibitor was maintained for 6 days in the culture medium of A431 cells growing with serum, the reduction in the kinase activity of the receptor was stronger, perhaps because of the PCI-induced down-regulation of EGFR (Fig. 8B).
computer-based analysis showed that PCI had clear structural similarities to EGF that can explain its antagonistic activity. Both PCI and EGF are small proteins with three disulfide bridges arranged in a special scaffold called the T-knot. We developed a computer program to superimpose proteins by structural alignment of the disulfide bridges. When applied to PCI and growth factors such as EGF, TGF-α, and heregulin, it revealed that two loops of the inhibitor and part of its C-terminal tail superimposed onto the corresponding growth factor loops. It also revealed that some residues of EGF involved in receptor binding (19) fit in the space with residues of PCI with similar physico-chemical properties (Fig. 4).

The inhibitory effect of PCI on the growth of human pancreatic adenocarcinoma cell lines was demonstrated in a variety of conditions. In the case of Capan-1 cells growing in medium with serum, the effect of PCI was apparent after 7–10 days of culture (Fig. 1) and was only observed when the PCI concentration was higher than 10 μg/mL. These findings correlate with the results obtained from cell cycle analyses, which indicated that after 12 days of treatment with PCI, the percentage of apoptotic cells significantly increased over the control values. A small increase in the number of cells in the G0–G1 phase was also observed. These results are in agreement with those obtained by Wu et al. (44) using a monoclonal antibody against EGF that blocks EGF binding and inhibits the proliferation of many tumor cell lines. They found that in some lines this antibody induced G0 arrest or apoptosis. The effects of PCI on cell cycle traversal could explain the inhibition of cell growth induced by this protein.

The results obtained in the proliferation experiments using serum-starved Capan-1 cells indicate that the antiproliferative effect of PCI is probably due to the fact that it is an EGF antagonist. When EGF was added to serum-starved Capan-1 cells, it stimulated cell proliferation, but this effect was abolished if PCI was administered simultaneously to the cells. This result is in perfect agreement with those obtained when studying insulin EGF-1 cell growth was not affected by the presence of PCI. The inhibitor is therefore capable of specifically suppressing the EGF proliferative effect on serum-starved Capan-1 cells. In cells growing in medium with FBS, PCI probably competes with the growth factors present in serum capable of binding to EGFR, as EGFR activation experiments indicate, and blocks their proliferative effect. This offers an explanation for the inhibitory effect of PCI on cells growing with serum.

When Capan-1 cells were grown in presence of PCI for at least 3 weeks and then transferred into fresh medium, the growth rate of this cells even without PCI was significantly lower than that of control cells (Fig. 1C). If protracted treatment with PCI down-regulates EGFR, as some of our results seem to suggest (Fig. 8B), the lower amounts of EGFR could be responsible for the reduction in the growth rate of the treated cells.

Some protease inhibitors have been reported to have anticarcinogenic properties (22), but the mechanisms responsible for these properties are unclear. We offer an explanation for the tumor growth suppressive activity of a protease inhibitor, PCI, showing that it acts as a growth factor antagonist. The effect of PCI on tumor growth seems attributable to its special topology and not to its protease-inhibitory activity. Several protease inhibitors with cancer-chemopreventive properties have a T-knot scaffold, and our work suggests that they may also act as growth factor antagonists.

Several properties of PCI make it a good candidate for a therapeutic agent. First, it was able to inhibit the development of human adenocarcinoma tumors transplanted into nude mice without inducing any observable toxic side effects. This fact is particularly interesting because there are not effective treatments available for pancreatic cancer. Second, PCI is a small protein very resistant to denaturation and proteolytic degradation. And third, PCI had a long lasting inhibitory effect on the in vitro growth of pancreatic adenocarcinoma cell lines that was maintained even when it was removed from the culture medium. In addition, we have obtained a transgenic mice that develop insulinomas, and preliminary results indicate that PCI also reduces the growth of these tumors and increases the survival time of the animals.3

In summary, we have described the antitumor properties of PCI, a small protein with three disulfide bridges arranged in a T-knot, and we have demonstrated that it is the first antagonistic analogue of human EGF described. PCI is of both theoretical and clinical interest and opens the possibility of engineering PCI-like EGF antagonists with improved properties. At present, we are testing whether PCI can also inhibit the growth of other carcinoma cell lines expressing EGFR. That the most common cancers (lung, prostate, breast, and colon) are of epithelial origin gives an additional clinical interest to this approach.

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