Identification and Characterization of Receptors Specific for Human Pancreatic Secretory Trypsin Inhibitor

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Summary

Specific binding sites for human pancreatic secretory trypsin inhibitor (PSTI) on 3T3 Swiss albino cells were studied using radioiodinated recombinant PSTI. Some ion species, pH, and temperature significantly influenced the binding of ^{125}I-PSTI. Kinetic studies showed that the binding of ^{125}I-PSTI to 3T3 Swiss albino cells reached the maximum level within 120 min at 4°C, with a slow dissociation rate. The half-maximal inhibition (ID_{50}) of ^{125}I-PSTI binding by unlabeled PSTI occurred at 1.0×10^{-10} M. On Scatchard analysis of the competitive binding data, linear plots indicated a single class of receptors with high affinity ($K_d = 5.3 \times 10^{-10}$ M) on 3T3 Swiss albino cells, the number of receptors being 5,400 per cell. Treatment of surface-bound radiolabeled PSTI with a chemical crosslinker (disuccinimidyl suberate) led to the identification of a membrane polypeptide of M_r 140,000 to which PSTI was crosslinked. The formation was inhibited by an excess amount of unlabeled PSTI in a dose-dependent manner. The binding of ^{125}I-PSTI to 3T3 Swiss albino cells was competitively inhibited by unlabeled PSTI but not by other peptide hormones, such as epidermal growth factor (EGF), bovine fibroblast growth factor, insulin-like growth factor, transforming growth factor α, platelet-derived growth factor, and tumor necrosis factor, indicating the presence of receptors specific for PSTI. Various protease inhibitors had no or only a little effect, and mercaptoethanol and dithiothreitol strongly decreased the binding of ^{125}I-PSTI. Incubation at 37°C resulted in rapid internalization of cell-bound ^{125}I-PSTI, followed by the appearance of trichloroacetic acid–soluble ^{125}I-radioactivity in the culture medium, due to degradation of internalized PSTI. In addition, PSTI stimulated [^{3}H]thymidine incorporation into DNA on 3T3 Swiss albino cells in a dose-dependent manner. The combined addition of PSTI and EGF stimulated [^{3}H]thymidine incorporation to an extent greater than that seen with either agent alone. These results indicated that the biological effect of PSTI was mediated by high affinity plasma membrane receptors, which were not a cell-surface proteinase(s). Specific binding of ^{125}I-PSTI was noted with the following cells: WI-38, 3T3 Swiss albino, HUVE, BDC-1, and H4-II-E-C3.

Pancreatic secretory trypsin inhibitor (PSTI),[1] first isolated by Kazal et al. (1), is a specific trypsin inhibitor in pancreatic juice. The physiological role of PSTI has been considered to be prevention of trypsin-catalyzed premature activation of zymogens in the pancreas and pancreatic duct (2). Recently, we demonstrated that various cancer tissues and most adenocarcinoma-derived cell lines expressed PSTI, and that the structure of PSTI cDNA expressed in neoplastic tissues was completely identical with that of pancreatic PSTI cDNA (3). Furthermore, we reported that serum PSTI was frequently

elevated in patients with various malignancies (4, 5) and that the change in serum PSTI was significantly correlated with those in acute-phase reactants in serum (6, 7).

Previously, Hunt et al. (8) and Scheving (9) reported that the amino acid sequence of PSTI was similar to that of epidermal growth factor (EGF). PSTI consists of 56 amino acids with a molecular mass of 6,242 daltons. EGF is a polypeptide hormone comprising 53 amino acids, and stimulates the growth of a variety of cell types in culture as well as the growth and differentiation of certain tissues in vivo (10, 11). The effects of EGF were shown to be mediated by a specific binding protein for EGF on the cell surface (12) and this EGF-binding protein was reported to be capable of autodigestion (13), and EGF also showed trypsin inhibitory activity (14).

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[1] *Abbreviations used in this paper:* DME/F-12, Dulbecco's modified Eagle's medium and Ham's F-12; EGF, epidermal growth factor; PN, protease-nexin; PSTI, pancreatic secretory trypsin inhibitor; Th, thrombin.

Recently, we showed the high homology between the sequences of human PSTI mRNA and murine EGF mRNA (15). The sequence homology of PSTI and EGF suggested the possibility that the functions of PSTI and EGF also resemble each other. In 1985, we demonstrated that human PSTI stimulated [³H]thymidine incorporation into DNA of human fibroblasts at a concentration present in human serum (16).

McKeehan et al. reported in 1986 (14) that the first 25 NH₂-terminal amino acid residues of the endothelial cell growth factor, purified from the medium of a human hepatic cancer cell line, were identical with those of human PSTI. The endothelial cell growth factor they reported had a molecular mass of 6,500 daltons, which is similar to that of PSTI, and its amino acid composition was also very similar to that of PSTI. In the same year, Fukuoka et al. (17) reported that the 6,500-dalton peptide purified from rat pancreatic juice stimulated DNA synthesis in 3T3 Swiss albino cells and the cell number was also increased after 24–48 h of incubation with 10–100 ng/ml of the peptide. This peptide was considered to be a cholecystokinin-releasing factor and to stimulate pancreatic enzyme secretion in response to food intake. Later, the amino acid sequence of the peptide was determined (18), and it was found to be identical to that of rat PSTI-I we purified (19). Also, we preliminarily reported that PSTI bound specifically to various cultured cells, the binding sites for PSTI being distinct from the receptors for EGF (20). These results suggested the possibility that, as in the cases of other polypeptide hormones, the growth stimulatory action of PSTI was mediated by a specific plasma membrane receptor protein. Thus, the identification and characterization of this protein were carried out using 3T3 Swiss albino cells to clarify the biological function of PSTI.

Materials and Methods

Cell Lines and Medium. 3T3 Swiss albino cells (mouse fibroblast), WI-38 (human lung fibroblast), MIAPaCa-2 (human pancreatic cancer), CAPAN-1 (human pancreatic adenocarcinoma), and H4-II-E-C3 (rat hepatoma) were obtained from the American Type Culture Collection (Rockville, MD). HuH-6 (human hepatoblastoma) was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). BDC-1 (human bile duct carcinoma) and GAC-1 (gastric carcinoma) were established in our laboratory. Human umbilical vein endothelial cells (Endocell) were obtained from Sanko Junyaku Co. (Tokyo, Japan) and maintained in E-GM-UV (Kurabo Co., Osaka, Japan). All other cell lines were maintained in a 1:1 mixture of DME and Ham's F-12 (DME/F-12) (Sigma Chemical Co., St. Louis, MO), supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 µg/ml). For the binding studies, 2 × 10⁵ cells were seeded into 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY). The cultures were continued until the monolayers became confluent (3–4 d after seeding). Cells were propagated in a high humidity incubator at 37°C under an atmosphere of 5% CO₂ and 95% air. The culture medium was routinely changed every third day. Cell lines that grow adherently were harvested by scraping without prior trypsin treatment to avoid the possible loss of PSTI receptors due to proteolytic degradation. In some cases brief EDTA treatment was needed to facilitate detachment. The

cell number was determined with a hemocytometer and cell viability as determined by the trypan blue exclusion test was over 90%.

Growth Factors and Reagents. EGF (human urine), fibroblast growth factor (b-FGF; bovine brain pituitary extract), insulin-like growth factor-I (IGF-I; human recombinant) and platelet-derived growth factor (PDGF; porcine platelet) were obtained from Toyobo Co. (Osaka, Japan). Human recombinant TGF-α was obtained from Cosmo Co. (Tokyo, Japan). Human recombinant TNF was obtained from Suntory Co. (Kyoto, Japan). Soybean trypsin inhibitor (SBTI), PMSF, iodoacetate, EDTA, o-phenanthroline, 2-ME, DTT, disuccinimidyl suberate (DSS), lactoperoxidase, and BSA (7.5% solution) were obtained from Sigma Chemical Co. (St. Louis, MO). Carrier-free Na-¹²⁵I was obtained from Amersham Japan (Tokyo, Japan). [³H]Thymidine (20 Ci/mmol) was from New England Nuclear (Boston, MA). Highly purified aprotinin was kindly supplied by Bayer Co. (Munich, FRG). Penicillin, streptomycin, sodium bicarbonate (7.5% solution), and PBS tablets (Dulbecco's formula) were obtained from Flow Laboratories (Irvine, Scotland). An electrophoresis calibration kit for molecular weight proteins was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Other chemicals used in this study were of analytical grade.

Radioactive Iodination of Human PSTI. Recombinant PSTI was prepared by expressing a cDNA for PSTI (15), and was supplied by Shionogi Pharmaceutical Co. (Osaka, Japan). PSTI was radioiodinated with carrier-free Na-¹²⁵I by the lactoperoxidase method (21) with some modifications. Briefly, 5 µg of recombinant PSTI was placed in a conical microvial and then dissolved in 20 µl of 0.05 M sodium bicarbonate. Tris-HCl buffer (20 µl each of 0.05 M and 0.5 M, pH 7.4), 1 mCi of carrier-free Na-¹²⁵I, and 5 µg of lactoperoxidase were added to the iodination vial. The iodination reaction was initiated by the addition of 5 µl of H₂O₂ diluted 1:75,000. After 2 min, the reaction mixture was applied on a Sephadex G-25 column (PD-10 column; Pharmacia Fine Chemicals, Uppsala, Sweden), which was equilibrated and eluted with 0.05 M Tris-HCl, pH 7.4. ¹²⁵I-PSTI obtained from this column was diluted with DME/F-12 containing 0.2% (wt/vol) BSA and 0.025 M Hepes, pH 7.4 (binding medium), filter-sterilized, and then stored at 0°C. More than 90% of the radiolabeled peptide was precipitated by 10% TCA. Radioactivity was measured for 1 min with an automatic gamma counter (Micromedic 4/600; Rohm and Haas Co., Horsham, PA), with a counting efficiency of 73%. PSTI was radiolabeled to a specific activity in the range of 70–100 µCi/µg.

¹²⁵I-PSTI Binding Assay. Binding studies were performed with confluent monolayers of 3T3 Swiss albino cells in 24-well plastic plates (Becton Dickinson and Co., Lincoln Park, NJ), as previously described (22) with some modifications. Cells were seeded at the density of 1 × 10⁵ cells/well in 1 ml of culture medium and then grown to confluency. The binding reaction was carried out in 1 ml of binding medium. Kinetic studies on binding at 4, 25, and 37°C were first performed. For all subsequent experiments the steady-state binding conditions of 2 h at 4°C were used for further characterization of the receptors. The cells were incubated in 1 ml of binding medium with ¹²⁵I-PSTI (25,000 cpm) and for binding inhibition studies the indicated amounts of unlabeled peptides were added to duplicate or triplicate cultures. Specific binding was defined as the difference between total binding and nonspecific binding in the presence of a 200-fold excess amount of unlabeled peptides, and was always <30% of the total cell bound radioactivity. After incubation for 2 h at 4°C the plates were drained and then flooded two times with ice-cold 0.05 M Tris-HCl buffer, pH 7.4, to remove any unbound peptides. The cells were then dissolved in 1 ml of 0.5 N NaOH for 20 min at room temperature and the amount

of radioactivity was determined. For association kinetic experiments, incubation wells containing either ^{125}I-PSTI alone or ^{125}I-PSTI and excess unlabeled PSTI were prepared. At the start of the reaction, the preparation was added to the wells. At various times, supernatants were removed. Bound and free ^{125}I-PSTI were separated as described above. For dissociation kinetic experiments, cells were incubated in the presence of 1 ml of binding medium containing ^{125}I-PSTI at 4°C for 2 h. After discarding the binding medium, the cells were washed with ice-cold 0.05 M Tris-HCl buffer, pH 7.4, and then added to either 1 ml of binding medium alone or 1 ml of binding medium containing 3.3×10^{-6} M unlabeled PSTI. Each experimental point represented the average of duplicate or triplicate determinations. Binding data obtained under steady-state conditions were analyzed using sums of simple Michaelis-Menten terms as described (23). Kinetic data were analyzed using functions that are sums of exponential terms, as described elsewhere (24). Inhibition data were analyzed using an equation for competitive inhibition between two ligands for one type of site (25). Scatchard analysis (26) was performed to calculate the dissociation constant and the number of binding sites per cell.

Affinity Crosslinking of ^{125}I-PSTI to 3T3 Swiss Albino Cells. Crosslinking experiments were performed with essentially the method described earlier (27). 3T3 Swiss albino cells were grown to confluence in 24-well plastic plates in DME/F-12 containing 10% (vol/wt) FCS. 1–2 d before ligand binding, the cell culture medium was removed and replaced with prewarmed DME/F-12 containing 0.5% FCS. The cells were incubated in 1 ml of binding medium containing ^{125}I-PSTI (25,000 cpm), and the binding reaction was terminated by washing the monolayer twice with binding medium and once with 0.05 M PBS, pH 7.5. The cells were incubated for 20 min at 4°C in the presence of 1 ml of PBS containing 0.3 mM DSS. The DSS solution was prepared as a 0.03 M stock in DMSO immediately before a crosslinking study. At the end of this incubation, the reaction was terminated by the addition of 20 μl of 2 M Tris-HCl, pH 8.0. After discarding the buffer, the cells were suspended in 100 μl of 0.05 M Tris-HCl, pH 7.5, containing 0.001 M EDTA, 1×10^{-4} M PMSF, 0.2 M NaCl, and 1.0% (wt/vol) Triton X-100 (extraction buffer). The cells were incubated in the extraction buffer for 20 min at 4°C. Insoluble materials were removed by centrifugation at 15,000 g for 10 min at 4°C. The supernatant was analyzed immediately by electrophoresis. The competition study of ^{125}I-PSTI binding with other growth factors, such as EGF, b-FGF, IGF-I, and TGF-α, was examined in a similar manner. All growth factors were used in the PSTI crosslinking experiment as competitors at the concentration of 1.0×10^{-6} M.

SDS-PAGE. SDS-PAGE was performed essentially as described by Laemmli (28). Briefly, all samples were boiled for 3 min in SDS sample buffer (0.06 M Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, and 5% 2-ME) and then subjected to electrophoresis on SDS-PAGE PLATE 4/20 gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan). The molecular weights were determined using the following standards; myosin (200,000), β-galactosidase (116,250), phosphorylase b (92,500), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000). After electrophoresis, the gels were stained with Coomassie blue (0.25% in 25% isopropanol and 10% acetic acid), dried, and then exposed to Kodak X-omat AR film (Eastman Kodak, Rochester, NY), with an intensifying screen, at −70°C for 4–7 d.

Internalization and Degradation of Cell Bound PSTI. The internalization and degradation of PSTI were estimated by a procedure similar to that described by Costlow and Hample (29). A confluent monolayer of 3T3 Swiss albino cells was incubated with ^{125}I-PSTI at 4°C for 2 h on a 24-well plastic plate. The cells were washed

three times with ice-cold DME/F-12 containing 10% FCS, and then shifted to 37°C with prewarmed binding medium and further incubated at 37°C. At the times indicated, the culture medium was harvested, and the cells were washed once with ice-cold PBS and then incubated for 5 min at 4°C with 1 ml of 0.05 M glycine-HCl buffer, pH 3.0, containing 0.15 M NaCl. After removal of the glycine buffer, the cells were washed twice with ice-cold binding medium and then solubilized in 0.5 N NaOH. The radioactivity found in the glycine buffer represented surface-bound PSTI, and that found in solubilized cells represented the internalized PSTI. To quantitate the degradation of internalized PSTI in cells, TCA was added to a final concentration of 10% (vol/wt), to culture medium collected before determination of cell-bound PSTI. The soluble counts were determined after removal of the precipitate by centrifugation at 3,000 g for 20 min.

[³H]Thymidine Incorporation Assay. Cells were plated into 24-well plastic plates at 1×10^5 cells/well and then allowed to grow to confluence in 1 ml of DME/F-12 containing 10% FCS. The confluent cultures were then shifted to serum-free DME/F-12 containing 0.1% BSA for 48 h before the assay. The cells were incubated with PSTI and/or EGF for 24 h and then [³H]thymidine (0.2 μCi/ml) incorporation was allowed for 2 h. The culture was washed twice with ice-cold PBS, and precipitated with ice-cold 10% TCA. The precipitate was solubilized with 500 μl of 1 N NaOH and then placed in a liquid scintillation vial (Wheaton Scientific, Milliville, NJ). An aliquot of the solubilized materials was neutralized by the addition of 100 μl of 6 N HCl. [³H]Thymidine incorporated into TCA-insoluble fractions containing 9 ml of AQUASOL-2 (New England Nuclear, Boston, MA) was assayed with a liquid scintillation counter (Mark III Liquid Scintillation System, model 6880; Searle Analytic, Des Plaines, IL).

Results

Characteristics of Binding of ^{125}I-PSTI on 3T3 Swiss Albino Cells. As our preliminary experiments revealed that 3T3 Swiss albino cells bound the highest amount of ^{125}I-PSTI (20), the optimal conditions for cell binding of ^{125}I-PSTI were initially determined using 3T3 Swiss albino cells as model cells. Specific binding of ^{125}I-PSTI to 3T3 Swiss albino cells was time and temperature dependent (Fig. 1). At 37°C, a steady state was rapidly attained within 30 min, whereas at 4°C the rate of association was much slower. However, the binding at 4°C increased over 2 h, after which time the specific binding was higher than that obtained for 30 min at 37°C. On continued incubation for 4 h at 37°C, the amount of radioactivity that remained bound to the cells decreased to one-third the maximal value. Considering this result, the standard experimental condition of 120 min of incubation at 4°C was used throughout the study.

Fig. 2 shows the dissociation of ^{125}I-PSTI from its binding sites on 3T3 Swiss albino cells. This experiment was performed as originally described by Demeyts et al. (30) to determine whether or not the PSTI receptor exhibited any cooperative properties. A complex kinetic pattern was revealed, characterized by fast and slowly dissociating components, similar to that observed for a number of receptor systems (31). The dissociation rate constants obtained in this experiment were $2.1 \pm 0.8 \times 10^{+2}$/min in medium alone and $1.7 \pm 0.9 \times 10^{-2}$ min^{-1} in the presence of excess unlabeled
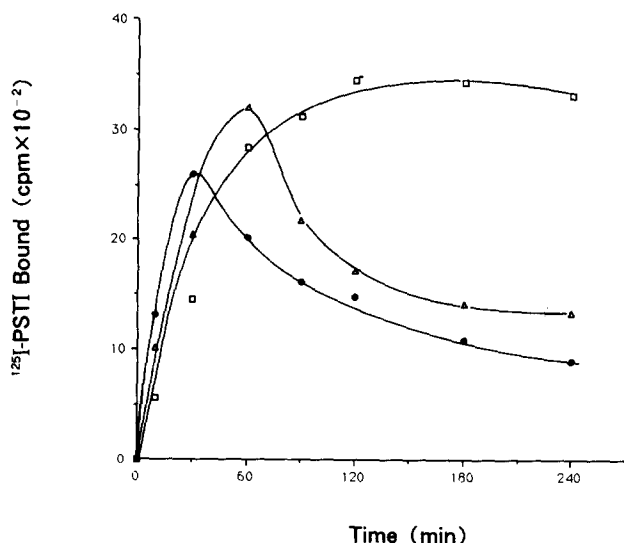
**Figures 1.** Kinetics of ^{125}I-PSTI binding at 4°C (□), 25°C (△), and 37°C (●) with 3T3 Swiss albino cells. For each point, 8 × 10⁵ cells were incubated in a final volume of 1.0 ml of binding medium at the indicated temperatures with 25,000 cpm ^{125}I-PSTI (2.0 × 10⁻⁹ M) in the presence or absence of a 1,000-fold excess of unlabeled PSTI. Specific binding was calculated as described under Materials and Methods. Each value represents the mean for duplicate determinations.

PSTI, while in both cases the rate constant of the slow component was $<10^{-2}$/min. A comparison of the dissociation of ^{125}I-PSTI from the cells in medium alone, when only a fraction of the receptors are occupied, with that in the presence of 3.3 × 10⁻⁶ M unlabeled PSTI, when almost all the receptors are occupied, reveals that the rate of dissociation
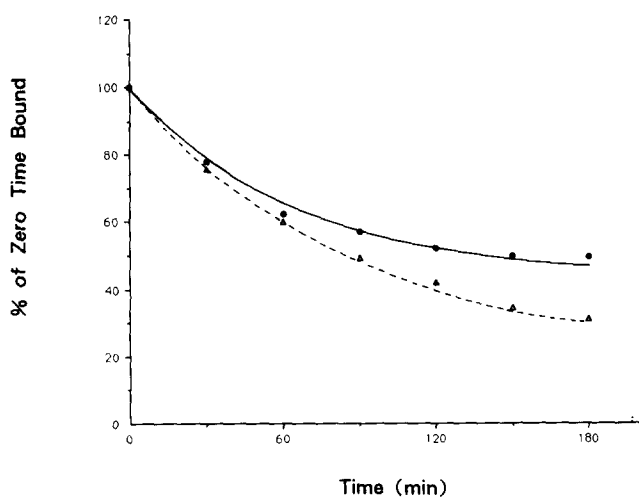
Figure 2. Dissociation of PSTI from its binding sites on 3T3 Swiss albino cells at 4°C. 8 × 10⁵ cells were preincubated with ^{125}I-PSTI (2.0 × 10⁻⁹ M) for 2 h at 4°C. After discarding the binding medium, the cells were incubated in either binding medium alone (●) or binding medium containing a 1,000-fold excess of unlabeled PSTI (△). The cells were maintained at 4°C and the bound radioactivity was measured at the indicated times. Each point represents the mean of duplicate measurements. Specific binding was calculated as described in Fig. 1.
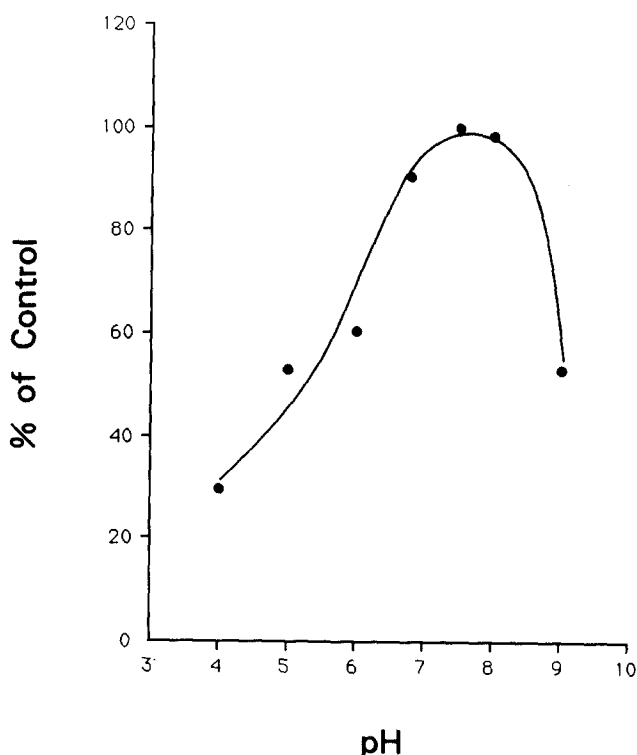
Figure 3. pH dependence of ^{125}I-PSTI binding to 3T3 Swiss albino cells. The pH of the incubation buffer (0.2 M Tris) was adjusted with 6 M HCl or 10 M NaOH as required. Each pH value is the final one in the incubation medium. Specific binding was calculated as described under Materials and Methods. Each point represents the mean of duplicate measurements.

of PSTI from its receptor is insensitive by occupancy of adjacent receptors. This suggests that PSTI receptors on 3T3 Swiss albino cells are noncooperative.

Fig. 3 shows the binding dependence on pH, from 3.0 to 9.0. The optimal pH for specific binding was 7.4–8.0.

Table 1 shows the effects of various ions in the incubation buffer. Ca^{3+} and Mg^{2+} decreased the binding of ^{125}I-PSTI in a dose-dependent manner and had additive effects, while the other divalent ions tested had less effect on the binding. On the other hand, monovalent cations such as Na^+ and K^+ had no effect at all on the binding at the concentration of 0.1 M.

Specificity of PSTI Binding. Fig. 4 shows the inhibition of binding of ^{125}I-PSTI to 3T3 Swiss albino cells by unlabeled PSTI. Binding of ^{125}I-PSTI to the cells was competitively inhibited by increasing concentrations of unlabeled PSTI, from 1.0 × 10⁻¹¹ M to 3.0 × 10⁻⁸ M. The half-maximal inhibition (ID⁵⁰) of the PSTI binding occurred at 1.0 × 10⁻¹⁰ M.

Fig. 5 shows a Scatchard plot of the binding data presented in Fig. 4. Scatchard analysis suggested that 3T3 Swiss albino cells had a single binding site with an apparent dissociation constant (K^d) of 5.3 × 10⁻¹⁰ M, with the estimated number of binding sites for PSTI being 5,400 per cell.

Table 1. *Effects of Various Ion Species on ^{125}I-PSTI Binding*

| Ion	Concentration	Specific binding of ^{125}I-PSTI
	mM	*%*
Control	—	100*
Na$^+$	100	102
	10	100
K$^+$	100	97
	10	99
Ca^{2+}	10	49
	5	50
	2	89
Mg^{2+}	10	63
	5	82
	2	98
Mn^{2+}	10	101
	1	100
Ni^{2+}	10	100
	1	102
Zn^{2+}	10	90
	1	99
Ca^{2+} + Mg^{2+}	10 + 10	26
	5 + 5	38
	2 + 2	83

The effects of various cation species on ^{125}I-PSTI binding to 3T3 Swiss albino cells were examined. Cells were incubated with 25,000 cpm ^{125}I-PSTI for 2 h at 4°C in the presence of various cation species.
* Values are expressed as percentages of the control level.
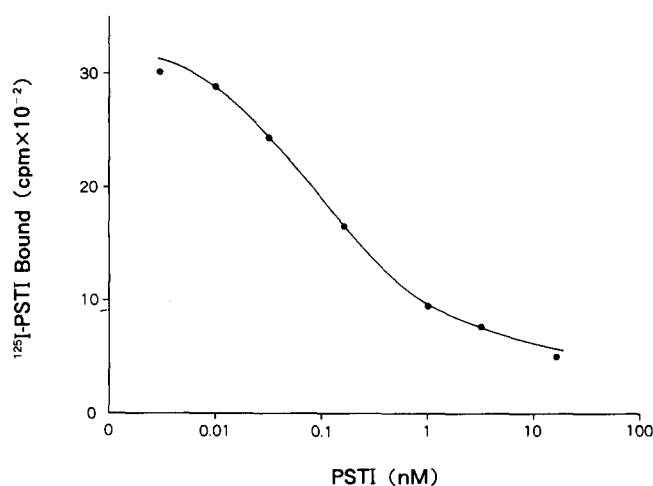
Figure 4. Inhibition of ^{125}I-PSTI binding to 3T3 Swiss albino cells by unlabeled PSTI. Cells were incubated with 25,000 cpm ^{125}I-PSTI for 2 h at 4°C in the presence of increasing concentrations of unlabeled PSTI. The binding data were analyzed as described under Materials and Methods. Each point represents the mean of duplicate measurements.
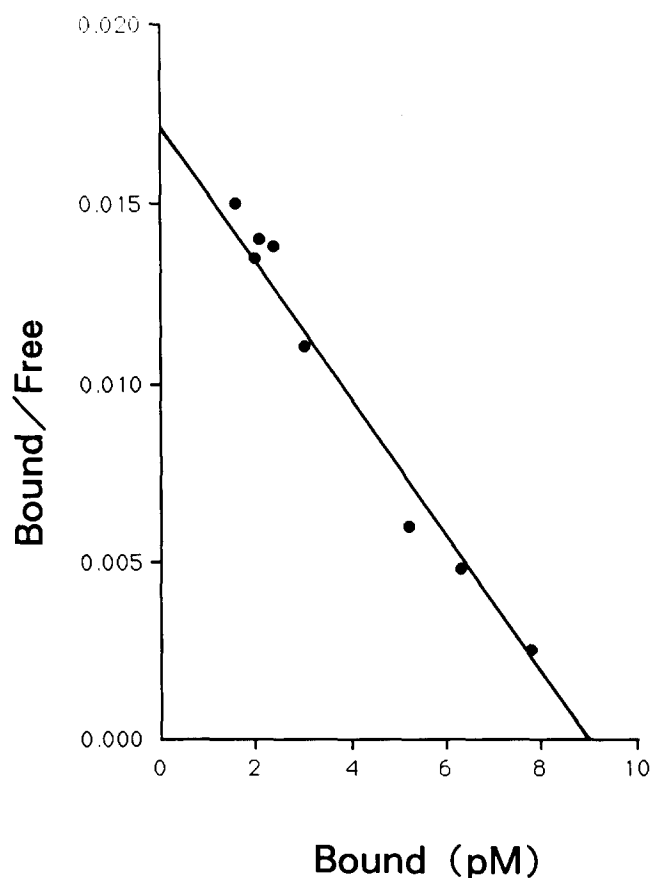
Figure 5. Scatchard plot of binding data presented in Fig. 4.

The specificity of ^{125}I-PSTI binding was investigated by examining the capacity of other growth factors and cytokines in comparison to unlabeled PSTI to compete with ^{125}I-PSTI binding. Only PSTI could compete with ^{125}I-PSTI binding, the other growth factors not doing so (Fig. 6). This suggested, at least for 3T3 Swiss albino cells, that the receptor to which PSTI binds was specific for this peptide.

Affinity Crosslinking of ^{125}I-PSTI to 3T3 Swiss Albino Cells. To study the structure of the binding site for ^{125}I-PSTI on 3T3 Swiss albino cells, ^{125}I-PSTI was chemically crosslinked to cell monolayers through the homofunctional reagent, DSS. When 0.3 mM DSS was used as a crosslinker, ^{125}I-PSTI was found to be associated with a macromolecular complex of M_r 140,000 (Fig. 7). Excess unlabeled PSTI effectively competed with ^{125}I-PSTI binding in a dose-dependent manner. However, excess unlabeled human EGF, b-FGF, and IGF-I did not compete with ^{125}I-PSTI for binding or crosslinking to the apparent M_r 140,000 species.

Internalization and Degradation of Bound ^{125}I-PSTI. Internalization and subsequent degradation are common consequences of peptide hormone–receptor interaction (32). As shown for other protein ligands, elution of cell surface–bound PSTI was markedly pH dependent. Cell-bound ^{125}I-PSTI became resistant to release with pH 3.0 buffer after 1 h at 37°C, which indicated that receptor-bound PSTI had been internalized by the cells. The early increase in intracellular radio-
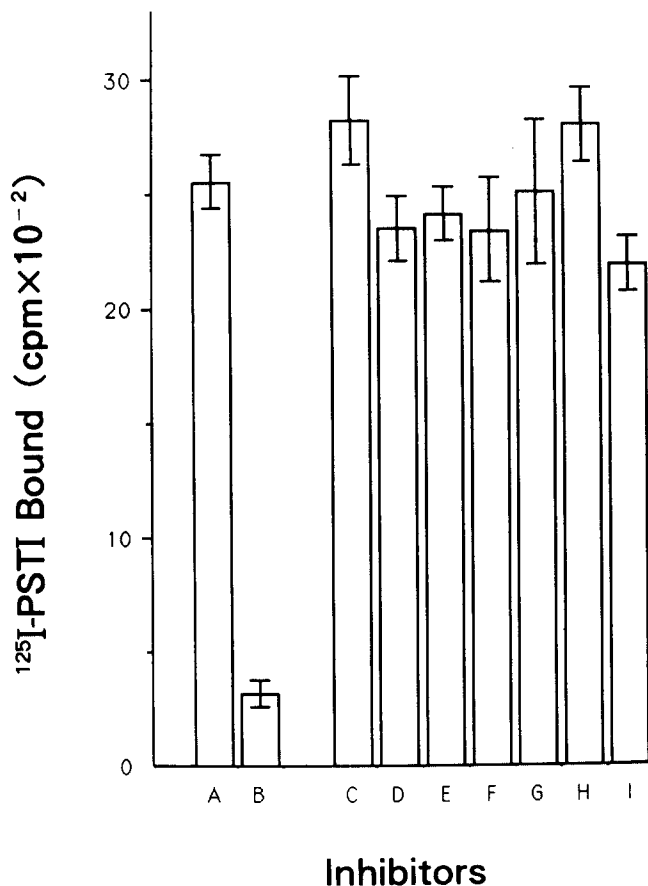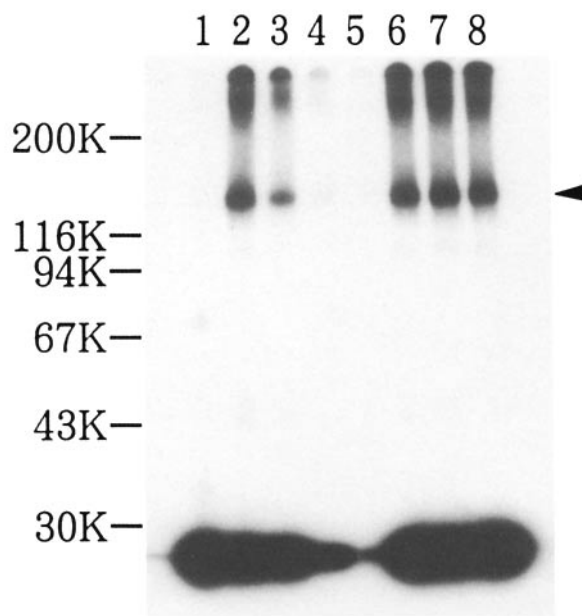
Figure 6. Specificity of ^{125}I-PSTI binding to 3T3 Swiss albino cells. Cells (8 × 10^5 cells/well) were incubated with ^{125}I-PSTI (2.0 × 10^{-9} M) and the following unlabeled peptides, at the concentrations indicated: (A) none; (B) recombinant human PSTI, 3.3 × 10^{-6} M; (C) recombinant human EGF, 3.3 × 10^{-6} M; (D) recombinant human TGFα, 3.6 × 10^{-6} M; (E) bovine b-FGF, 1.5 × 10^{-6} M; (F) porcine platelet PDGF, 1.2 × 10^{-6} M; (G) recombinant human IGF-1, 2.6 × 10^{-6} M; (H) recombinant human TNF, 1.2 × 10^{-6} M; and (I) aprotinin, 3.3 × 10^{-6}. Incubation was performed for 2 h at 4°C and binding was assayed as described under Materials and Methods. Values are means ± SD for three experiments.

Figure 7. Characterization of PSTI receptor by affinity crosslinking. Cells (1 × 10^7 cells/ml) were incubated with ^{125}I-PSTI in the absence of unlabeled PSTI (lane 2), or in the presence of increasing concentrations of unlabeled PSTI (1.0 × 10^{-10}, 1.0 × 10^{-9} M, and 1.0 × 10^{-8} M in lanes 3, 4, and 5, respectively), ^{125}I-PSTI only (25,000 cpm; lane 1), EGF (1.0 × 10^{-6} M; lane 6), b-FGF (1.0 × 10^{-6} M; lane 7), or IGF-I (1.0 × 10^{-6} M; lane 8). ^{125}I-PSTI was crosslinked to the cells with 0.3 mM DSS and then analyzed by 4/20 gradient SDS PAGE. The arrow indicates the PSTI-receptor complex of M_r 140,000. The autoradiogram was obtained as described under Materials and Methods.

activity was followed by a gradual decrease. ^{125}I radioactivity was first detected in the medium after 1 or 2 h and continued to increase until 6 h. These results suggested that internalization and subsequent degradation of ^{125}I-PSTI occurred after binding to specific receptors at 37°C (Fig. 8).

Effects of Various Agents on Specific Binding of ^{125}I-PSTI. The effects of various protease inhibitors and chemical reagents on ^{125}I-PSTI binding were investigated (Table 2). Treatment of 3T3 Swiss albino cells with o-phenanthroline resulted in a little loss of binding activity, whereas other protease inhibitors had no or only a little effect on the binding activity of ^{125}I-PSTI. 2-ME and DTT strongly decreased the binding activity toward ^{125}I-PSTI.

Stimulation of DNA Synthesis by PSTI. The time course of DNA synthesis, monitored as the incorporation of labeled thymidine, following the addition of PSTI to confluent and quiescent 3T3 Swiss albino cells is shown in Fig. 9. Under these experimental conditions, an increased rate of DNA synthesis was detectable after 12 h incubation in the presence of 100 ng/ml PSTI. The maximal stimulation was observed at ∼30 h. The stimulatory effects of increasing concentrations of PSTI and EGF on DNA synthesis are presented in Table 3. [^{3}H]Thymidine incorporation was dependent on the concentration of PSTI. This pattern of thymidine incorporation stimulation by PSTI was similar to those observed for EGF. Moreover, the combined addition of PSTI and EGF stimulated [^{3}H]thymidine incorporation to an extent greater than that seen with either agent alone.

Specific Binding of ^{125}I-PSTI to Various Cultured Cells. The capacity of a variety of cell lines to bind ^{125}I-PSTI was examined (Table 4). No appreciable specific binding of ^{125}I-PSTI was detected with the following cultured cells: MIA PaCa-2, CAPAN-1, HuH-6, and GAC-1. Specific binding of ^{125}I-PSTI was noted with the following cells: WI-38, 3T3 Swiss albino, HUVE, BDC-1, and H4-II-E-C3. These results indicated that some, but not all, cultured cells derived from a number of species (human, mouse, and rat) were capable of binding human PSTI.

Discussion

The present study demonstrated rapid, specific, and reversible binding of radioactive iodinated human PSTI to 3T3 Swiss

1138   Human Pancreatic Secretory Trypsin Inhibitor Receptor
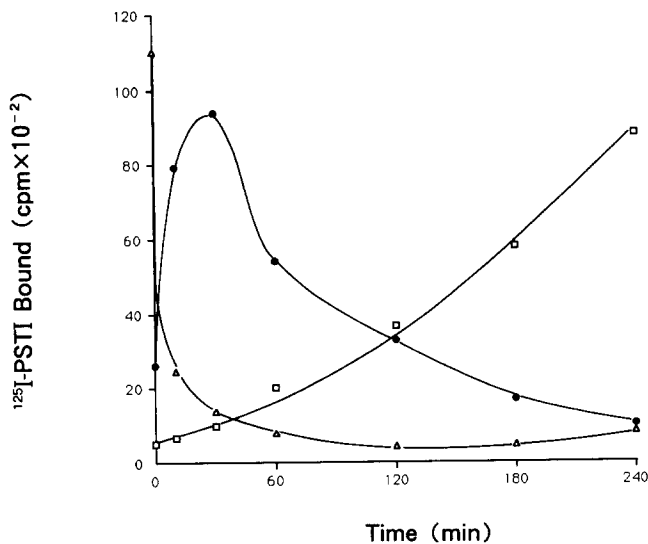
Figure 8. Internalization and degradation of ^{125}I-PSTI bound to 3T3 Swiss albino cells. Surface bound (△), internalized (●), and degraded (□) ^{125}I-PSTI. ^{125}I-PSTI was determined as described under Materials and Methods. Each point represents the mean of duplicate measurements.

Table 2. *Effects of Various Agents on Specific Binding of ^{125}I-PSTI*

Treatment	Concentration	Percent of control*
None		100
Soybean trypsin inhibitor	50 μg/ml	98
PMSF	5×10^{-3} M	89
Leupeptin	5×10^{-3} M	90
Iodoacetate	5×10^{-3} M	96
p-Chloromercuribenzoate	5×10^{-3} M	76
EDTA	5×10^{-3} M	91
o-Phenanthroline	5×10^{-3} M	86
Mercaptoethanol	1×10^{-3} M	50
	5×10^{-3} M	34
Dithiothreitol	1×10^{-3} M	32
	5×10^{-3} M	20

Cells (2 × 10⁵/ml) were preincubated with various protease inhibitors or chemical reagents for 10 min at room temperature. The treated cells were then centrifuged at 15,000 *g* for 10 min and the pellets were resuspended in the binding buffer. The binding activity was determined after this resuspension. Cells that had not been exposed to reagents or enzymes but incubated in the same manner and centrifuged for the same period were used as a control.

* Values are expressed as percentages of the control level and means of duplicate determinations.

albino cells. The maximal binding was observed at 4°C, indicating that the binding site was stable under the conditions used. On the other hand, incubation at 4°C resulted in minimal label degradation of ^{125}I-PSTI, which permitted high specific binding (Fig. 1).

Divalent cations are known to cause specific binding to other polypeptide hormone receptors (33, 34). The present study showed that monovalent cations had no effect at physiological concentrations on the specific binding of ^{125}I-PSTI, whereas Ca^{2+} and Mg^{2+} decreased the specific binding (Table 1). Divalent cations such as Ca^{2+} and Mg^{2+} could alter the conformation of PSTI and/or the binding site or might alter the electrostatic interaction between them. Treatment with 2-ME and DTT resulted in a significant loss of binding activity (Table 2), indicating that intrachain disulfide bonds might be of some importance for the binding of PSTI to cells.

Scatchard analysis suggested that 3T3 Swiss albino cells had a single high affinity binding site for PSTI, with a K_d of 5.3 × 10⁻¹⁰ M, and that there were 5,400 sites per cell. In addition, Fig. 2 indicated that the binding was reversible and PSTI receptors were noncooperative. Thus, PSTI has high affinity and low capacity binding sites on 3T3 Swiss albino cells. In order to identify the high affinity PSTI binding domain present on the surface of 3T3 Swiss albino cells, experiments involving crosslinking to a polypeptide present on the cell surface associated with PSTI were performed. An intense band at a position corresponding to 140,000 daltons was observed on the SDS-PAGE auto-radiogram. As this band disappeared completely with excess unlabeled PSTI, we concluded that the 140,000 dalton band represented a PSTI–PSTI receptor complex. Human EGF, b-FGF, and IGF-I did not cause the disappearance of this band.
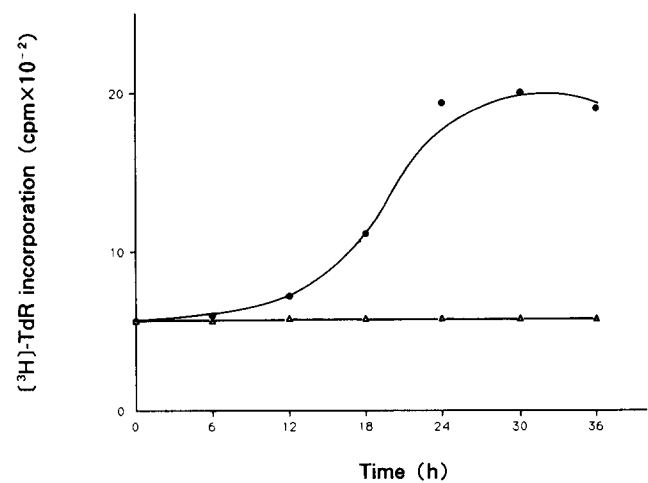
After internalization, intracellular degradation of PSTI could be demonstrated (Fig. 8). Our results indicated that receptor-mediated endocytosis and intracellular degradation occurred after PSTI binding to cell surface receptors. This phenomenon was also observed for a protease-nexin (PN), known as a serine

Figure 9. Time course of [³H]thymidine incorporation after stimulation of 3T3 Swiss albino cells. Confluent, quiescent cultures of cells were stimulated by the addition of 100 ng/ml PSTI (●). Nothing was added to control cultures (△).

Table 3. *Stimulation of 3T3 Swiss Albino Cells by PSTI and EGF*

Factors	Final concentration	[³H]Thymidine incorporation	
		cpm/well	Percent of control
None		412.2 ± 15.4*	100
PSTI	1 ng/ml	721.4 ± 110.2	180
	5 ng/ml	1,480.2 ± 131.5	360
	10 ng/ml	1,883.8 ± 163.5	460
EGF	1 ng/ml	1,051.0 ± 91.2	260
	5 ng/ml	1,897.2 ± 90.5	460
	10 ng/ml	2,412.8 ± 130.0	590
PSTI + EGF	5 + 5 ng/ml	3,241.4 ± 295.5	790
	10 + 10 ng/ml	4,057.2 ± 279.0	990
SBTI + EGF	5 µg/ml + 5 ng/ml	2,104.6 ± 154.5	510
FCS	5%	1,692.1 ± 142.5	410
	10%	2,455.9 ± 139.5	600

The stimulation of thymidine incorporation by PSTI and EGF was examined. Varying concentrations of PSTI with or without EGF were added to the cells. 24 h later, [³H]thymidine was added and the cells were labeled for 2 h.
* Values are means ± SD for three experiments.

Table 4. *Binding of ¹²⁵I-PSTI to Various Cultured Cells*

Cells	Total ¹²⁵I-PSTI added to the well	Bound ¹²⁵I-PSTI to 1 × 10⁶ cells		
		Total	Nonspecific	Specific
		cpm		
WI-38	23,300	2,510	210	2,300
3T3 Swiss albino	22,900	2,830	250	2,580
HUVE	23,900	1,620	340	1,280
MIA PaCa-2	24,400	640	450	190
CAPAN-1	24,700	830	620	210
BDC-1	23,900	920	200	720
HuH-6	23,800	1,550	620	930
GAC-1	24,000	1,260	1,080	180
H4-II-E-C3	25,100	1,600	540	1,060

The binding of ¹²⁵I-PSTI to various cultured cells was examined. The binding data were analyzed as described under Materials and Methods. Values are means for duplicate determinations.

protease inhibitor. Low et al. (35) reported that PN, a component released by normal human fibroblasts into the culture medium, formed covalent linkages with thrombin (Th). After the Th-PN complexes bound to fibroblast cells, they were rapidly internalized and degraded.

PSTI was originally isolated as a trypsin inhibitor, so it might be thought that the specific binding sites for PSTI are the cell-surface proteinase(s). Our present results demon-strated that pretreatment of 3T3 Swiss albino cells with various protease inhibitor had no effect on the specific binding of ¹²⁵I-PSTI (Table 2), and that aprotinin, a bovine pancreatic trypsin inhibitor, also did not compete with ¹²⁵I-PSTI binding. In a separate experiment, we observed that the addition of the excess amount of various protease inhibitors did not inhibit the binding of PSTI to 3T3 Swiss albino cells (data not shown). Therefore, the binding sites for PSTI can

be different from the cell-surface proteinase, though a possibility still remains. PSTI itself stimulated DNA incorporation into 3T3 Swiss albino cells in a dose-dependent manner, as shown in Table 3, and the pattern resembled that in the case of EGF. The combined addition of PSTI and EGF stimulated [^{3}H]thymidine incorporation to an extent greater than that seen with either peptide alone. These findings, together with the results reported by Fukuoka et al. (17), raise the possibility that PSTI could be a growth-stimulating factor.

Scott and Seow reported that, despite having investigated a wide range of concentrations of protease inhibitors, such as α1-protease inhibitor and bovine pancreatic trypsin inhibitor, no evidence was found of mitogenic or growth stimulatory activity (36). Although the mitogenic effects of proteases on cells in monolayers have been documented (37–40), few studies have examined the potential role of protease inhibitors as mitogens. However, four lines of evidence, recently provided, indicated that protease inhibitors can be mitogenic: (a) McKeehan et al. (14) have established that PSTI and urinary glycoprotein protease inhibitor, which are both serine protease inhibitors produced in significant quantities by HepG2 cells, do stimulate the growth of human endothelial cells in a serum-free monolayer culture; (b) TGF-β has been shown to induce the production of a metalloproteinase inhibitor that is thought to facilitate anchorage-independent growth by inhibiting extracellular matrix degradation (41); (c) Cook and

Chen (42) reported that three serine protease inhibitors (leupeptin, soybean trypsin inhibitor, and aprotinin) can enhance colony formation in soft agar, but do not have such an effect on monolayer growth; (d) Quan (43) reported that Cystatin C, a thiol protease inhibitor, can stimulate the proliferation of mouse 3T3 fibroblasts. Therefore, it is possible that the mitogenic effect of a PSTI species on fibroblasts or endothelial cells may be a unique property that cannot be mimicked by other protease inhibitors.

We reported that various cancer tissues contained PSTI-immunoreactive cells. We also found that most adenocarcinoma-derived cell lines expressed PSTI and that cultured human cancer cells in protein-free nutrient medium secreted a considerable amount of PSTI into the culture medium. The expression of PSTI by cancer cells was confirmed by the isolation of PSTI cDNA clones from neoplastic tissues (3) and the sequence of PSTI cDNA was completely identical with that of pancreatic PSTI cDNA reported previously (15). The function of PSTI, which is expressed by cancer cells, remains unknown, but the results obtained up to now suggest that PSTI in cancer cells may stimulate the proliferation of fibrous connective tissues or endothelial cells, which is regarded as an inflammatory response of surrounding tissues to cancer cells. Further studies on the isolation of receptor proteins and the gene encoding the PSTI receptor will provide new insight into the expression of PSTI by cancer cells.

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