DOI 10.1002/pmic.200500376

SHORT COMMUNICATION

A proteomic approach to the identification of new tPA receptors in pancreatic cancer cells

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We have developed a strategy to identify putative tissue-type plasminogen activator (tPA)receptors present in pancreatic cancer cells by affinity capture with tPA-Sepharose followed by 2-DE and MALDI-MS PMF. Proteins pulled down from either total lysates or raft membrane fractions were characterized and compared with those from a total lysate of an endothelial cell line (HUVEC) to identify pancreas-restricted tPA receptors. A total of 31 proteins were found by this approach, including annexin A2, already described as a tPA receptor in pancreas and endothelial cells, other proteins acting as tPA receptors (*i.e.*, enolase, cytokeratins 8 and 18) in other tissues, and additional proteins not previously identified as candidate tPA receptors. Confirmation of the results was performed for some of these proteins using immunoblotting. These studies are the basis for further functional analyses on the role of these proteins in the biological effects of tPA.

Received: May 26, 2005
Revised: September 6, 2005
Accepted: September 19, 2005

Keywords:
Affinity capture / Electrophoresis / Pancreatic cancer / PMF / Raft membrane fraction

Tissue-type plasminogen activator (tPA) is a serine protease whose best documented role, together with urokinase-type plasminogen activator (uPA), is the activation of the zymogen plasminogen to the protease plasmin, which in turn is implicated in the degradation of fibrin clots in blood [1]. The plasminogen system is also involved in the degradation of extracellular matrix and activation of growth factors in processes of tissue remodeling, cell migration and, in the case of cancers, tumor invasiveness [2]. Of the two plasminogen activators, uPA, has been most extensively studied in cancer progression and has been shown to play a role both by activating plasmin and by modulating cell migration through its receptor, uPAR. Its overexpression in tumors is associated with a more invasive behavior and worse prognosis [3]. However, there are some tumor types such as melanomas [4], neuroblastomas [5], acute promyelocytic leukemia [6] and exocrine pancreatic adenocarcinoma [7, 8], where a crucial role for tPA has been demonstrated. In the pancreas, tPA is overexpressed in 95% of ductal tumors, whereas it is undetectable in normal pancreatic ducts, and tPA expression is associated with a more invasive behavior *in vitro* [7, 8]. Furthermore, it has been proposed that tPA may affect tumor progression by increasing angiogenesis and tumor cell proliferation *in vivo* [9, 10]. By analogy with uPA, it has been proposed that tPA activity is linked to its binding to membrane-localized receptors that enhance its proteolytic activity and localize this protein at the cell membrane in the migration front. Annexin A2 (AnxA2) is a well-known receptor for tPA and plasminogen in endothelial cells [11, 12]. AnxA2 is present in several cellular compartments, including membrane rafts [13], and appears to trigger proin-

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Abbreviations: AnxA2, Annexin A2; **tPA**, tissue-type plasminogen activator; **uPA**, urokinase-type plasminogen activator; **WB**, Western blot

vasive activity through tPA activation of plasmin in several tissues, including pancreatic cancer ([14] and Peiró *et al.*, submitted).

Recent work has shown that some effects of tPA–both in pancreatic and other cell types–do not require its proteolytic activity, including the activation of the MAP kinase ERK1/2 signaling pathway ([15] and Peiró *et al.*, submitted). Furthermore, it is likely that other molecules, in addition to AnxA2, participate in tPA binding in pancreatic tumors since AnxA2 can only account for the binding of 50% of tPA to pancreas cancer cells [14]. Therefore, we have set out to identify putative tPA receptors in cultured pancreatic cancer cells, and determine their role in a variety of biological processes.

We have used a proteomic approach relying on an affinity capture (pull-down) initial step using Sepharose-bound tPA, followed by 2-DE and PMF analysis. Several pancreas cancer cell lines were used for the experiments. PANC-1 cells were chosen because they do not produce endogenous tPA [16, 17], and are therefore the focus of this report. To ascertain whether the proteins identified as putative tPA receptors using pull-down were specific for pancreatic cells, the 2-DE profiles of bound proteins isolated from PANC-1 cells were compared with those from human umbilical vein endothelial cells (HUVEC). PANC-1 cells were cultured to confluence in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, and HUVEC cells were cultured as previously described [18]. Total protein lysates were obtained by extraction with Tris-buffered saline (TBS) containing 1% Triton X-100, protease inhibitors (200 mM Pefabloc, 1 mM aprotinin, 20 mM leupeptin) and phosphatase inhibitors (1 mM sodium fluoride, 10 mM sodium pyrophosphate). We also analyzed the raft fractions, prepared as previously described [19] using detergent-free lysis and ultracentrifugation in a discontinuous (5%–35%–45%) sucrose gradient. The raft fraction was collected between 35% and 5% sucrose, and further purified by resuspension in 25 mM MES buffer, 0.15 M NaCl, pH 6.5, and centrifugation at 13 000 rpm for 1 h at 4°C. The pellet was next resuspended in TBS containing 1% Triton X-100, 20 mM octylglucoside and the above-mentioned protease and phosphatase inhibitors.

For the pull-down assay, an initial procedure optimization was required, as commercial recombinant tPA (Actilyse, Roche Molecular Biochemicals) contains a 300-fold molar excess of arginine as stabilizer which effectively competes with tPA for CNBr-Sepharose binding sites, and results in very low immobilization yields. Attempts to remove arginine by either dialysis or ultrafiltration on Centricon (Millipore) membranes resulted in the precipitation of tPA. However, overnight dialysis of the commercial tPA solution against 0.9 M guanidine hydrochloride avoided precipitation and the resulting arginine-free tPA solution could be used to obtain satisfactory tPA substitution levels (ca. 25 mg/mL dried CNBr-Sepharose). This resin was then used for affinity capture of tPA-binding proteins. tPA-Sepharose (50 μL, dry volume) was incubated with ca. 5 mg total protein from the cell lysates. After 2 h at 4°C, the supernatant was discarded, the resin washed three times with lysis buffer, and bound proteins were eluted with the IEF buffer [7 M urea, 2 M thiourea, 4% CHAPS (Amersham Biosciences), 0.5% IPG buffer 3–10 non-linear (NL) (Amersham Biosciences) and 1% DTT]. BSA-coupled Sepharose was used as control to assay for nonspecific binding.

IEF was carried out on 24-cm, pH 3–10 NL IPG strips, and followed by 2-DE on 12.5% Bis/acrylamide precast gels (Amersham Biosciences). Protein spots were visualized first by CBB R-350 and, in some experiments, destained and restained with silver, and then excised, and digested with trypsin (Promega). Control 2-DE runs included BSA-Sepharose (see above) and, due to the spontaneous leaking of recombinant tPA from the matrix, tPA-coupled Sepharose resin incubated with buffer alone (Fig. 1D). Figure 1 shows the 2-D gels of PANC-1 cell lysates (Fig. 1A, C) compared with those of HUVEC (Fig. 1B). Spots selected for PMF analysis are indicated by arrows and the spots corresponding to proteins selected for further characterization as putative tPA receptors are specified.

Digest solution (10 μL) was passed through an Empore column (Proxeon) and the tryptic peptides were eluted with 1 μL 70% ACN in 0.1% TFA containing 20 mg/mL CHCA. A Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) operating in the reflectron mode was used to generate PMFs, which were searched against the NCBI database using MASCOT search engine (http://www.matrixscience.com) with a mass tolerance of 50 ppm. PANC-1 total cell lysates were analyzed in quadruplicate; HUVEC total cell lysates and PANC-1 raft fraction experiments were performed in duplicate.

The protein identification process yielded 31 tPA receptor candidates identified in gels of PANC-1 cell pull-downs, either from total lysates or from raft fractions (Table 1). Since several proteins previously shown to act as tPA receptors cannot strictly be classified as *bona fide* membrane proteins, we have chosen to report all reliably identified (*i.e.*, reproducibly observed by PMF with a significant sequence coverage) candidates, assuming they may include either authentic tPA receptors, or proteins associated with authentic tPA receptors, or even ligands with no apparent physiological relevance.

Some of the listed proteins have already been described as tPA receptors, such as AnxA2, enolase, cytokeratins 8 and 18, and tubulin [20–22], thereby validating the analytical methodology used. Among them, AnxA2 is the only protein previously identified as a tPA receptor in pancreas cancer cells ([15] and Peiró *et al.*, submitted).

Thioredoxin peroxidase has been recently identified as capable of binding AnxA2 [23], and thus might have been indirectly bound by tPA-Sepharose. Along similar lines, vimentin has been described as a mediator of PA inhibitor and its receptor in platelets [24].

Galectin-1, one of the proteins identified for which a membrane localization has been reported in a variety of cell types, has been proposed to play an important role in tumor

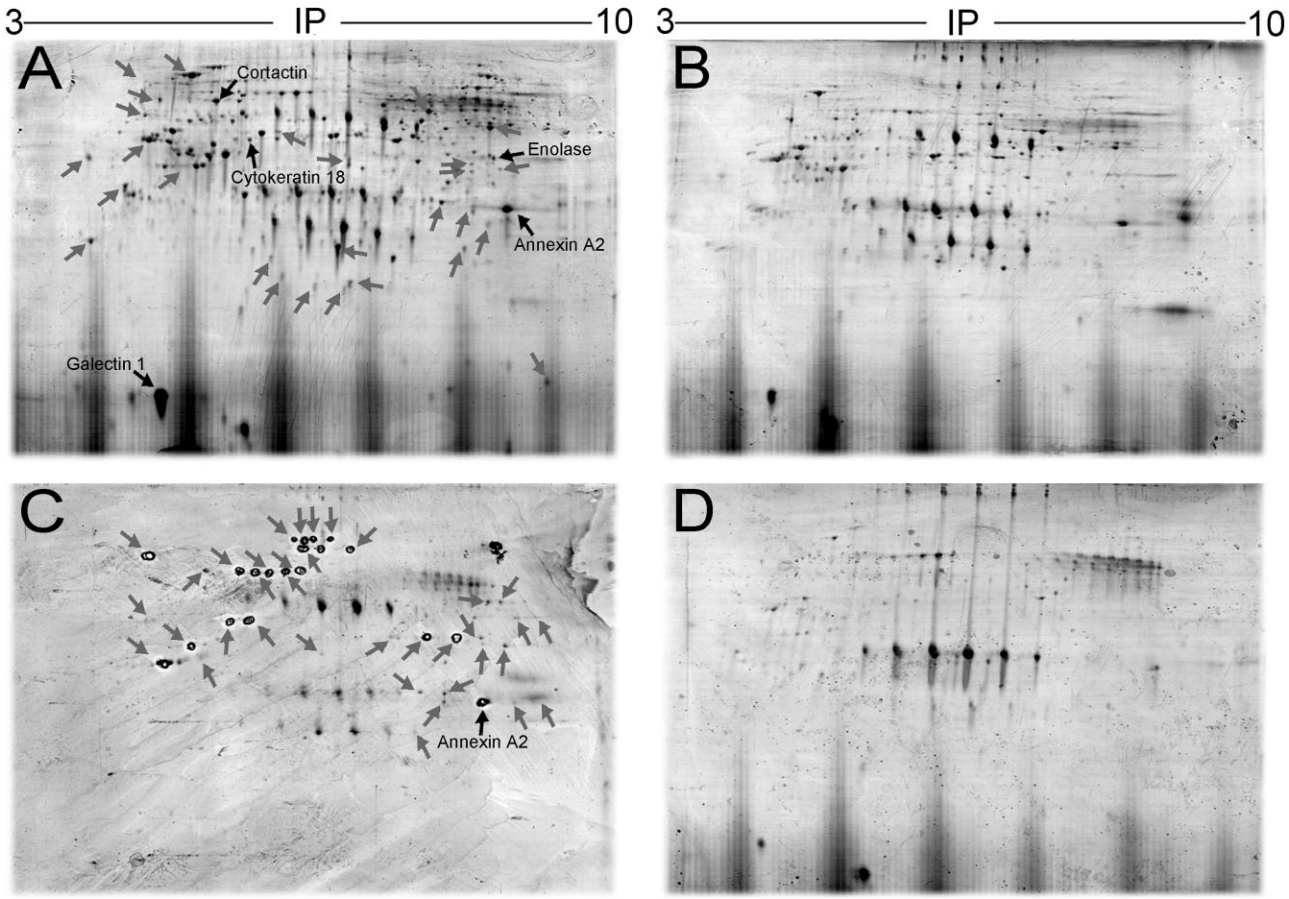

Figure 1. 2-DE of proteins bound to tPA-Sepharose in the pull-down assays. Pull-down experiments were done as described in the text using total cell lysates of PANC-1 (A) or HUVEC (B) and the raft fraction of PANC-1 cells (C). A control experiment with tPA-Sepharose incubated with lysis buffer was also carried out (D); all spots in this gel correspond to tPA. Protein spots are CBB stained and, in the case of raft fraction of PANC-1, destained and re-stained with silver for improved visualization. Spots excised from both PANC-1 gels (total lysate and raft fraction) are marked with arrows. Proteins most extensively studied (see text), *i.e.*, enolase, AnxA2, cortactin, cytokeratin 18 and galectin-1 (black arrows), are explicitly labeled.

progression, partly by modulation of immune cells [25], and also through its interaction with the product of the H-Ras proto-oncogene [26].

Nine of the proteins in Table 1 are cytoskeletal, suggesting their implication in the cell motility function of tPA. Immunocytochemical studies to determine their co-localization with tPA in the migration front of pancreatic cells are ongoing.

We have also identified the cytosolic protein ERK 1, in agreement with its role in tPA mitogenic signaling ([14] and Peiró *et al.*, submitted), also suggesting that the methodology used may allow the identification of proteins not directly bound to tPA but implicated in complexes with other tPA-interacting proteins.

For other proteins listed in Table 1, a discernible role as tPA receptors cannot be identified, and the biological relevance of their *in vitro* interaction with tPA has to be established. This is particularly the case for those proteins localized in cellular organelles were the presence of tPA has so far not been established (*i.e.*, the nucleus or mitochondria). Intriguingly, some of them have been described as overexpressed in pancreatic cancer [27, 28], and others might have a yet unknown function in this system. For example, valosin-containing protein, although not strictly described as membrane bound, has been implicated in membrane fusion events [29] and has been consistently observed in our experiments, therefore suggesting that the interaction is real and that it is a plausible tPA receptor candidate. Even though RNA-binding proteins and chaperones in Table 1 may at first sight be labeled as likely false positives, the recent finding of AnxA2 as an RNA binding protein [30] would recommend a more cautious course.

Twelve of these proteins (labeled ψ in Table 1) were overrepresented in PANC-1 cells, a property that made them particularly attractive for further validation given the selectivity of their expression. Five of these proteins (AnxA2,

Table 1. Proteins identified by tPA-Sepharose pull-down assay and 2-DE of pancreatic cell fractions[a)]

Protein name	Lysate	Raft	Reference	Best coverage	p*I*	Mass (kDa)	Localization	Function
Annexin A2	+	+	gi|16306978	48%	7.6	39	Membrane, cytoplasm, nucleus	Signal transduction; cell communication
Enolase$^{\psi}$	+	+	gi|4503571	22%	7.0	47	Membrane, cytoplasm,	Metabolism
Galectin-1$^{\psi}$	+		gi|42542978	51%	5.3	15	Membrane, cytoplasm, nucleus	Receptor binding; immune response
Cortactin$^{\psi}$	+		gi|182087	35%	5.2	61	Cytoskeleton	Structural component
Cytokeratin 8$^{\psi}$	+	+	gi|181573	33%	5.5	53	Cytoskeleton	Structural component
Cytokeratin 18	+	+	gi|30311	48%	5.3	47	Cytoskeleton	Structural component
Tubulin$^{\psi}$	+	+	gi|2119276	29%	5.0	50	Cytoskeleton	Structural component
Vimentin	+	+	gi|2119204	54%	5.1	54	Cytoskeleton	Structural component
Actin	+	+	gi|3157976	38%	5.3	42	Cytoskeleton	Structural component
ARP3$^{\psi}$	+	+	gi|5031573	32%	5.6	47	Cytoskeleton	Structural component
Cytokeratin 19	+		gi|24234699	49%	5.0	44	Cytoskeleton	Structural component
Enigma proteins with LIM and PDZ domains$^{\psi}$	+		gi|13994151	39%	6.6	36	Cytoskeleton	Receptor signaling complex scaffold
Cystathionine-beta-synthase	+		gi|4557415	43%	6.2	61	Cytoplasm	Metabolism
Pyruvate kinase 3		+	gi|31416989	18%	7.9	58	Cytoplasm	Metabolism
Placental thrombin inhibitor	+		gi|20141722	53%	5.1	43	Cytoplasm	Protease inhibitor
Translin associated protein X	+		gi|6136057	60%	6.1	33	Cytoplasm	Transporter
Eukaryotic translation initiation factor 3	+		gi|4503513	37%	5.4	37	Cytoplasm	Translation regulation
CTP synthetase	+		gi|20981706	24%	6.0	67	Cytoplasm	Ligase; nucleotide and nucleic acid metabolism
Chaperonin (acute related morphine dependence protein)$^{\psi}$	+		gi|4502643	28%	6.2	58	Cytoplasm	Chaperone activity; metabolism
Thioredoxin peroxidase$^{\psi}$	+		gi|9955007	45%	5.4	22	Cytoplasm, nucleus	Peroxidase activity; metabolism
ERK 1$^{\psi}$	+		gi|20986531	19%	6.5	42	Cytoplasm, nucleus	Kinase activity; signal transduction; cell communication
Valosin containing protein$^{\psi}$	+		gi|6005942	47%	5.1	90	Cytoplasm, ER, nucleus	ATPase activity
Heterogeneous nuclear ribonucleoprotein H1	+		gi|5031753	37%	5.9	49	Nucleus	Ribonucleoprotein
ER-associated DnaJ protein 3	+		gi|18203497	33%	5.8	41	ER	Chaperone
DnaJ (Hsp40) homolog	+		gi|5453980	29%	5.8	58	ER	Chaperone
Eukaryotic translation elongation factor	+		gi|39644794	27%	6.3	50	ER	Translation regulator activity
Eukaryotic initiation factor 4B	+		gi|18146614	79%	5.4	69	Ribosome	Translation regulator activity; metabolism
PWP1-interacting protein 4	+		gi|14579002	33%	5.8	41	Mitochondria	DNA binding protein
Elongation factor Tu, mitochondrial precursor$^{\psi}$	+		gi|34147630	51%	7.3	46	Mitochondria	Translation regulator activity
H+-transporting two-sector ATPase		+	gi|16359160	17%	9.2	59	Mitochondria	Ion channel activity; transport
Ubiquinol-cytochrome-c reductase		+	gi|731047	20%	6.9	53	Mitochondria	Catalytic activity; metabolism

a) Proteins present in PANC-1, but not in HUVEC, total cell lysates are marked with a superscript ψ. Cell localization and function of each protein were obtained from Human Protein Reference Database (http://www.hprd.org)

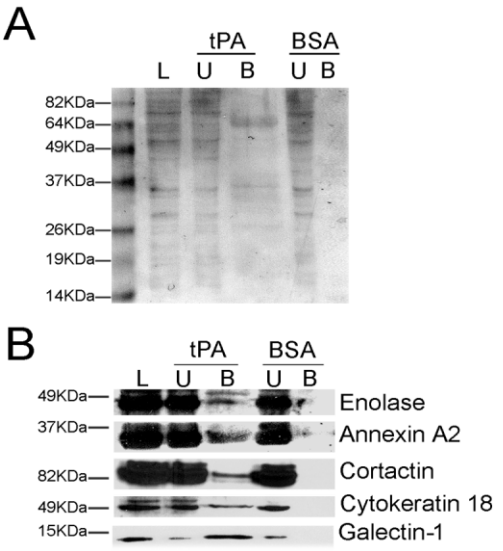

Figure 2. WB analysis to examine the presence of selected proteins identified by pull-down in cell lysates and in the tPA-Sepharose pull-down fractions. PANC-1 cell lysates were incubated with either tPA-Sepharose or BSA-Sepharose, as described in the text. One half of the bound fraction (B) and 5% of the unbound lysate (U) of both tPA and BSA pull-downs were loaded, along with the corresponding amount of unfractionated cell lysate (L). Proteins were separated by 1-DE (8% and 12% Bis/acrylamide gels) and transferred to NC membranes. (A) Ponceau staining. For WB analysis (B), filters were blocked with 5% skim milk, incubated with primary antibodies against enolase, AnxA2, cortactin, cytokeratin 18 and galectin-1, washed and incubated with peroxidase-conjugated secondary antibodies. Proteins were detected by the enhanced chemiluminescence system (Amersham Biosciences) according to manufacturer's instructions. The following antibodies were used: rat mAb against enolase (kind gift of Dr. G. Adamus, Oregon Health Science University, Beaverton, OR); rabbit polyclonal anti-AnxA2 produced in our laboratory [10]; rabbit polyclonal antibody against cortactin (kind gift of Dr. J. Cheng, Mayo Clinic, Rochester, MN, USA) [34]; mouse mAb LE61 recognizing cytokeratin 18 (kind gift of Dr. E. B. Lane, University of Dundee, UK) [35]; and rabbit polyclonal antibody detecting galectin-1 [36].

cytokeratin 18, enolase, cortactin, and galectin-1) were selected for confirmation of the 2-DE and PMF-based protein identifications by further analysis using tPA-Sepharose pull-down (as described above) followed by 1-DE and Western blot (WB). Anx2 and cytokeratin 18 were included as positive controls because their interaction with tPA has been previously reported. An additional reason to include cytokeratin 18 was that it is a highly abundant cytoskeletal protein that has been reported to be accessible at the extracellular side of the membrane in cancer, but not normal, epithelial cells [31]. Figure 2 shows the detection of these proteins in total cell lysates, in the tPA-Sepharose pull-down fraction, and in the unbound material, using WB as a semi-quantitative approach.

The interaction of tPA with these proteins was also analyzed in three additional pancreatic cell lines, selected on the basis of their different tumorigenic and differentiation properties: SK-PC-1 cancer cells display a well-differentiated phenotype [27, 32]; Hs766T are less differentiated; and HPDE are an immortalized non-tumorigenic cell line obtained by infection of human pancreatic ductal cells with a retrovirus containing the E6 and E7 genes of the human papilloma virus [33]. Hs766T and SK-PC-1 were grown using the same conditions as described above for PANC-1. HPDE cells were cultured in KSFM medium (Gibco) supplemented with epithelial growth factor (0.1–0.2 ng/mL) and bovine pituitary extract (25 μg/mL) [33]. Figure 3 shows that binding of AnxA2, cytokeratin 18, cortactin and galectin-1 to tPA could be verified by WB analysis in all pancreatic cells. In contrast, enolase was barely detectable despite its abundant expression. Cytokeratin 7, an abundant cytoskeletal protein expressed by pancreatic cancers that did not appear among the proteins identified in the pull-downs, was included as a negative control. Surprisingly, although cytokeratin 7 binding to tPA was not detected in the three cancer cell lines examined, its binding to the non-tumorigenic HPDE cells suggests differential binding properties of tPA to either normal or tumor cells.

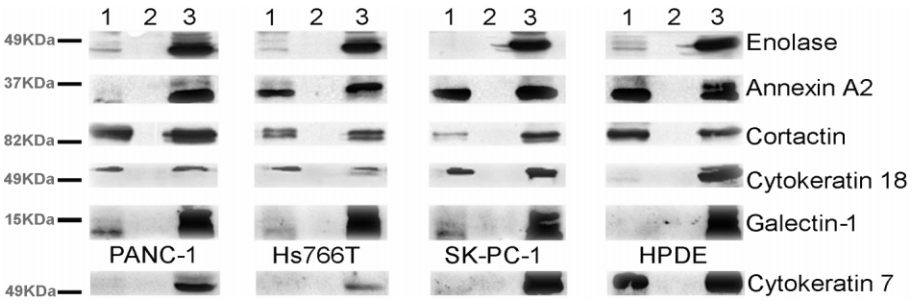

Figure 3. WB detection of six putative tPA receptors by pull-down assay using four pancreatic cell lines. Lysates from four different pancreatic cell lines (PANC-1, Hs766T, SK-PC-1, and HPDE) were incubated with tPA-Sepharose (1) or BSA-Sepharose (2) as described, followed by separation by 1-DE and WB as described in Fig. 2 with antibodies against enolase, AnxA2, cortactin, cytokeratin 18, galectin-1, and cytokeratin 7. Total cell lysates (3) were included in the analyses for reference. Cytokeratin 7 was detected using mouse mAb RCK105 (kind gift of Dr. F. Ramaekers, University of Maastricht, The Netherlands).

The work reported here represents the first step of a systematic attempt of the structural and functional characterization of putative tPA receptors. Biochemical validation of the interactions, demonstration of the colocalization of the proteins in cultured cells and tissues, and functional analysis using siRNA or antisense strategies should provide stronger evidence about the role of these proteins in the biological effects of tPA, both in pancreatic cancer and in other diseases. In addition, these studies should provide clues for the design of selective pharmacological strategies.

This work was supported by grants from Instituto de Salud Carlos III, Spain (00/0462 to P.N., C03/010 to F.X.R., PI040885 to D.A.), the Spanish Ministry of Science and Technology (SAF2002-00718 to P.N., BIO2002-04091-C03-01 to D.A.) and the European Community Biomed Programme (QLG1-CT-2002-01196 to F.X.R.). O.R. is supported by a predoctoral fellowship from the Spanish Ministry of Education and Science.

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