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Galectin-1 Is a Novel Functional Receptor for Tissue Plasminogen Activator in Pancreatic Cancer

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Background & Aims: **Tissue plasminogen activator (tPA) exerts many different functions in addition to its role in fibrinolysis. In pancreatic ductal adenocarcinoma (PDA), tPA is overexpressed and plays an important role in proliferation, invasion, and angiogenesis. tPA interaction with cell membrane receptors has been related to increased proteolytic activity and to signal transduction through nonenzymatic mechanisms. The aim was to analyze the role of galectin-1 (Gal-1), an endogenous lectin that also is overexpressed in PDA, as a new functional receptor for tPA.** ***Methods:*** **Gal-1/tPA interaction was analyzed using surface plasmon resonance and pull-down assays. Pancreatic cells and tumors were used to study Gal-1 expression and localization by Western blot and immunostaining. Down-regulation of Gal-1 by small interference RNA was used to analyze the involvement of Gal-1/tPA interaction in extracellular signal-regulated kinase 1/2 activation, cell proliferation, and invasion in pancreatic and fibroblastic cells.** ***Results:*** **Gal-1/tPA interaction is direct, specific, and of high affinity. Gal-1 moderately increases the catalytic activity of tPA. High Gal-1 levels were detected in PDA cells in culture, where it concentrates at the migration front, and in tissues, where it is expressed in epithelial cells and in the stroma. Down-regulation of Gal-1 abolished the effects of tPA on extracellular signal-regulated kinase 1/2 activation, cell proliferation, and invasion, both in pancreatic and in tumor-derived fibroblasts.** ***Conclusions:*** **These findings support a new molecular mechanism by which Gal-1 interaction with tPA contributes to PDA progression involving both transformed epithelial cells and tumor fibroblasts.**

Tissue plasminogen activator (tPA) is a multifunctional protein that regulates a broad range of cellular functions, including fibrinolysis, tissue remodeling, and neuronal plasticity.[1–3]5 Many effects of tPA are mediated by its catalytic activity via plasmin generation. However, tPA also operates as a cytokine that binds to membrane receptors and triggers signaling.[4–9]

Pancreatic ductal adenocarcinoma (PDA) has a dismal prognosis. Its hallmarks include genomic instability, early invasion and metastasis, and extensive desmoplasia.[10] Our previous results showed that tPA is highly expressed in PDA compared with normal pancreas.[11,12] These findings were validated in independent unbiased screens using Serial Analysis of Gene Expression and microarrays.[13,14] In addition to displaying selective tumor distribution, tPA enhances proliferation, invasion, and angiogenesis in tumors.[11,12,15] tPA-induced proliferation in PDA is mediated by a nonenzymatic mechanism leading to extracellular signal-regulated kinase 1/2 (ERK1/2) activation through the epidermal growth factor receptor (EGFR) and annexin A2 (AnxA2).[9] However, inhibition of these two receptors does not abolish the effects of tPA on PDA cells, thereby suggesting the involvement of other proteins. To identify novel receptors, we used affinity capture with tPA-Sepharose (Invitrogen, Carlsbad, CA) and mass spectrometry. In that screen, we found several novel putative tPA receptors, among them galectin-1 (Gal-1).[16]

Gal-1 belongs to the galectin family of proteins with β-galactoside–binding capacity. Galectins are involved in cell growth, apoptosis, adhesion, immune response, and malignant transformation.[17–19] Although at least 15 mammalian galectins have been identified, Gal-1 and galectin-3 (Gal-3) have received the greatest attention. The following information supports the hypothesis that Gal-1 may mediate tPA signaling: (1) Gal-1 is a potent modulator of adhesion and growth regulation via carbohydrate-dependent and carbohydrate-independent mechanisms,[20] and (2) Gal-1 up-regulation in PDA has been

Abbreviations used in this paper: α-SMA1, α-smooth muscle actin; AnxA2, annexin A2; BSA, bovine serum albumin; CK-19, cytokeratin 19; CM, conditioned medium; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; Gal-1, galectin-1; Gal-3, galectin-3; PDA, pancreatic ductal adenocarcinoma; siCtrl, irrelevant small interference RNA; siRNA, small interference RNA; tPA, tissue plasminogen activator; WB, Western blot.

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detected by expression profiling and immunohistochemistry.[21–24]

We report that Gal-1 is a functional tPA receptor that participates in PDA progression. By using surface plasmon resonance and pull-down analysis we show that the tPA–Gal-1 interaction is direct, specific, and of high affinity. Furthermore, Gal-1 is expressed at high levels in cultured PDA cells, where it colocalizes with tPA in the migrating front. By using small interference RNA (siRNA), we show that Gal-1 is involved in tPA-induced ERK1/2 activation, proliferation, and invasion. Finally, in vivo studies of Gal-1 expression and siRNA experiments in fibroblasts support a role for tPA and Gal-1 in the cross-talk between the stroma and cancer cells.

Materials and Methods

For materials and cell culture, see supplementary Materials and Methods (see supplementary material online at www.gastrojournal.org).

Surface Plasmon Resonance Experiments

Bovine serum albumine (BSA), tPA, or urokinase plasminoge activator (uPA) were immobilized at low density for kinetic experiments and at higher densities for specificity experiments as described.[25] Binding was performed at a flow-rate of 20 μL/min; regenerations were achieved with 1 mol/L NaCl pulses. For kinetic analysis, Gal-1 (15 nmol/L to 4 μmol/L), Gal-3 (450 nmol/L to 9 μmol/L), and AnxA2 (30 nmol/L to 4 μmol/L) were used. For competition experiments, glucose, galactose, cellobiose, and lactose (0–20 mmol/L) and Gal-1 at 1 μmol/L were used. Data were processed with the BIAevaluation software (V. 4.0.1 from BIAcore, Uppsala, Sweden); kinetic data were obtained by individual curve fittings and corroborated using the steady-state model.

tPA Catalytic Activity Analysis

tPA (0.5 μg/mL) and plasminogen (0.1 μmol/L) were incubated with plasmin substrate S-2251 (100 μmol/L) alone or in the presence of Gal-1, Gal-3, or AnxA2 (0.5 nmol/L to 5 μmol/L), and absorbance was measured at 405 nmol/L. A detailed description of the data analysis is reported in the supplementary Materials and Methods section (see supplementary material online at www.gastrojournal.org).

Knockdown of Gal-1 Expression by siRNA

Cells were transfected with either siRNA SMARTpool against Gal-1 or with siCONTROL nontargeting siRNA pool (Dharmacon, Lafayette, CO) using the Lipofectamine/PLUS system (Invitrogen, Carlsbad, CA). After transfection, cells were cultured for 72 hours. Inhibition of Gal-1 expression was confirmed by Western blot (WB).

Biochemical and Functional Analyses

Solid-phase assays were performed as described.[26] Detailed descriptions for WB, pull-down, immunoprecipitation, cell surface biotinylation, ERK1/2 activation, cell proliferation, immunofluorescence microscopy, immunohistochemistry, wound healing experiments (migration), and Matrigel (BD Biosciences, Franklin Lakes, NJ) invasion assays are provided in the supplementary Materials and Methods section (see supplementary material online at www.gastrojournal.org).

Statistical Analysis

Results are expressed as mean ± SD of triplicates; the Student *t* test was used to compare control and experimental conditions. Unless indicated, 3 independent experiments were performed.

Results

Interaction of Gal-1 With tPA Is Direct and Displays a High-Affinity Constant

Our previous results identified Gal-1 as a candidate tPA binder in pancreatic cancer cell lysates[16] but these studies did not determine whether Gal-1 binding to tPA was direct or mediated by other proteins. To clarify this point, we performed tPA-Sepharose pull-down experiments with recombinant Gal-1. Recombinant AnxA2 was used as positive control. As shown in Figure 1*A*, Gal-1 was selectively detected in the tPA-Sepharose bound fraction. AnxA2 also was detected in this fraction. Neither of these 2 proteins bound BSA-Sepharose, indicating that Gal-1 interacts directly and specifically with recombinant tPA.

The kinetic constants of the interaction were determined by surface plasmon resonance (Figure 1*B*). Because Gal-3 also is up-regulated in PDA,[16,21] we also examined its association with tPA. As a positive control, AnxA2 binding to tPA was analyzed. The interaction with tPA was referenced to that of BSA. The association response was higher for Gal-3 than for Gal-1. However, Gal-3 dissociated much faster, implying a weaker interaction with tPA. Determination of the thermodynamic dissociation constant (see supplementary Table 1; see supplementary material online at www.gastrojournal.org) shows similar values for Gal-1 and AnxA2. Gal-1 did not bind to immobilized uPA, confirming binding specificity (Figure 1*C*).

Galactose Is Involved in tPA Binding to Gal-1

Human Gal-1 binds galactose and, with higher affinity, lactose at the central site of its carbohydrate recognition domain.[27] We used surface plasmon resonance to determine the capacity of carbohydrates to interfere with Gal-1/tPA binding. Galactose, but not glucose, inhibited Gal-1/tPA binding in a dose-dependent manner (Figure 2*A*). At 20 mmol/L galactose, a complete inhibition of Gal-1/tPA binding was observed. Because the reducing monosaccharide represents a mixture of isomers (α, β, pyranose, furanose, and the open structure), lactose also was used. This disaccharide, but not

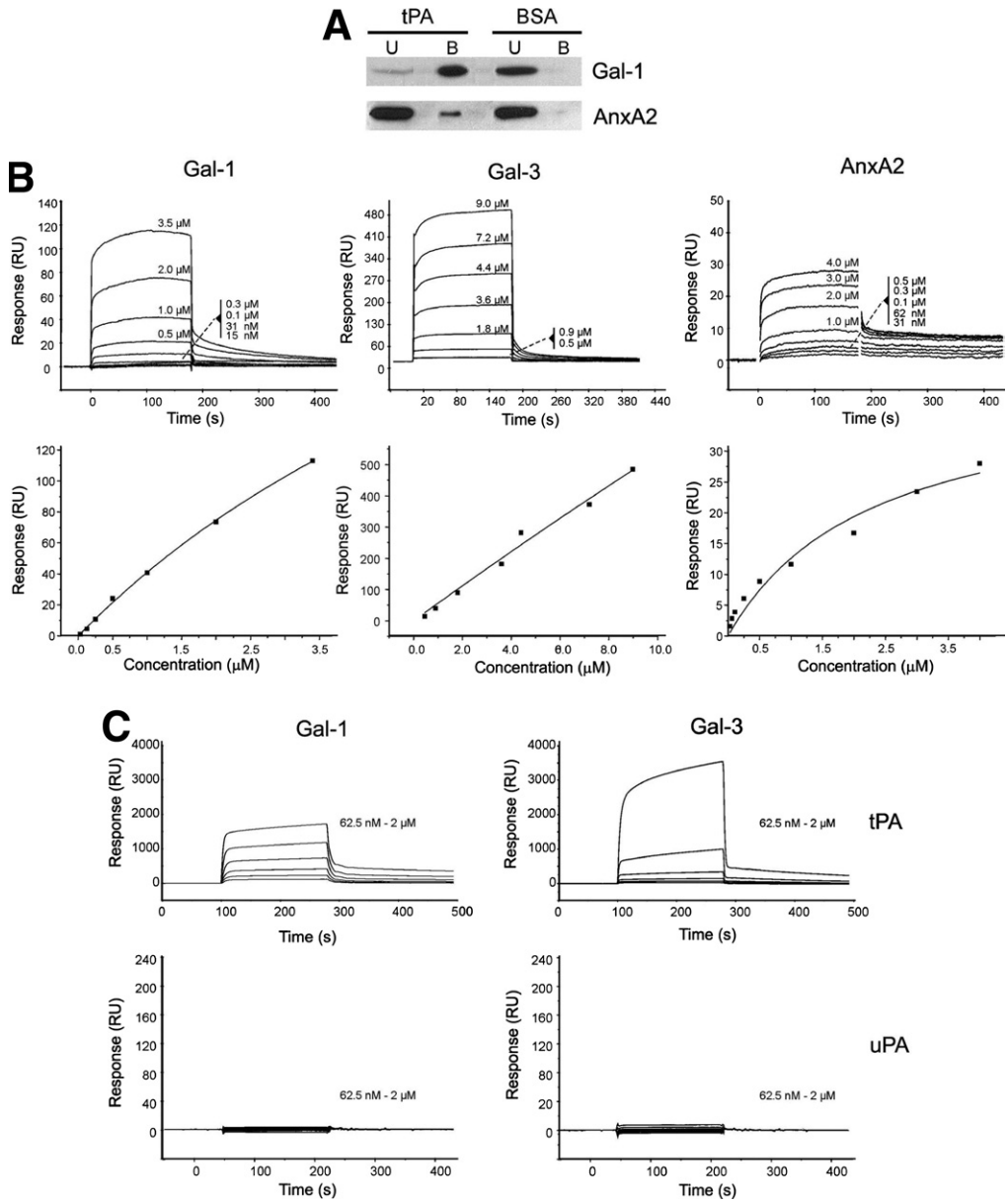

Figure 1. Gal-1 and tPA interact directly in vitro: pull-down and surface plasmon resonance analyses. (*A*) Recombinant proteins were incubated with tPA-Sepharose or BSA-Sepharose. Proteins were detected by WB in unbound (U) and bound (B) fractions. Note that the bound fraction was 10-fold concentrated in comparison with the unbound fraction. (*B*) Gal-1, Gal-3, and AnxA2 sensorgrams and hyperbole fit. Differential surface plasmon resonance was performed with a range of Gal-1, Gal-3, and AnxA2 concentrations over a surface coated with either tPA or BSA. Sensorgrams show a normalized response (RU) of tPA/BSA lanes over time. Bottom graphs show plots of maximum signal vs concentration and the regression to a hyperbole following the steady-state model. AnxA2 was included as a positive control. (*C*) Sensorgrams comparing binding of tPA and uPA with Gal-1 and Gal-3. Note the differences in scale.

cellobiose, completely inhibited the binding at 2 mmol/L. This finding shows the contribution of the carbohydrate recognition domain of Gal-1 to the interaction with tPA and the involvement of galactose in β-anomeric position as the high-affinity epitope.

The carbohydrate-inhibitable nature of the Gal-1/tPA interaction was underscored by independent solid-phase assays (Figure 2*B*). Binding was dependent on the amount of tPA in the matrix, saturable, and almost completely inhibitable by haptenic glycoinhibitors. Sugars without affinity for Gal-1, such as maltose or cellobiose, did not affect Gal-1 binding (not shown).

Gal-1 Binding to tPA Leads to an Increased Proteolytic Activity In Vitro

tPA receptors enhance tPA catalytic activity and plasmin generation. We analyzed the effect of Gal-1 on tPA proteolytic activity by an in vitro enzyme-linked immunosorbent assay using plasminogen as a substrate (Figure 3). tPA increased plasmin generation in the presence of Gal-1 at greater than 500 nmol/L (Figure 3*A*). At these concentrations, Gal-3 had no effect on tPA-mediated proteolysis (Figure 3*B*). In contrast, AnxA2 increased tPA catalytic activity efficiently, at concentrations as low as 50 nmol/L (Figure 3C). Figure 3*D* shows the comparison of the effects of these 3 proteins on tPA proteolytic activity.

Gal-1 Is Expressed at High Levels in Cultured Pancreatic Cells, Interacts With tPA, and Localizes at the Migration Front

To identify suitable in vitro models to examine the functional role of the Gal-1/tPA interaction in PDA, we analyzed Gal-1 expression in a panel of human pan-

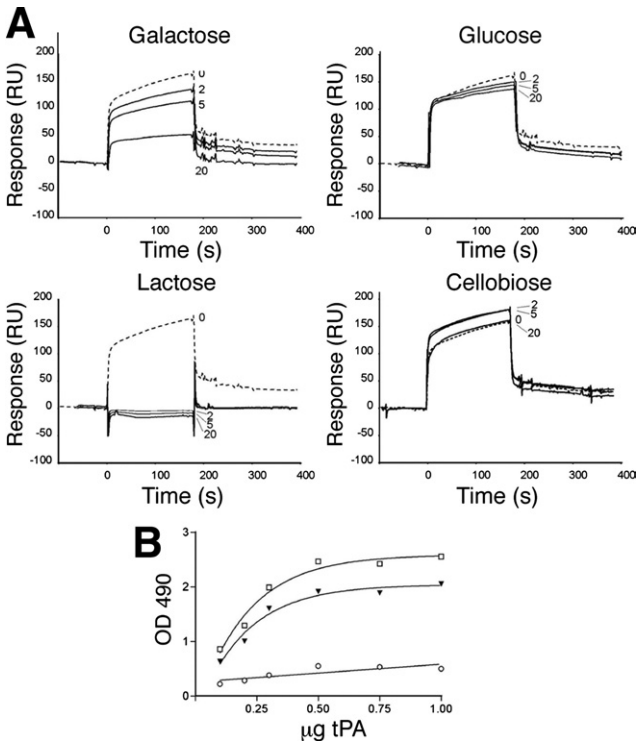

Figure 2. Galactose and lactose inhibit the interaction of tPA with Gal-1 in a concentration-dependent manner. (*A*) Sensorgrams showing tPA binding to Gal-1 on inhibition with galactose, glucose, lactose, and cellobiose. Sensorgrams show normalized response (RU) of tPA/BSA lanes over time. (*B*) Carbohydrate-inhibitable binding of human Gal-1 to surface-immobilized tPA. The extent of total-binding was reduced to background values in the presence of the mixture of haptenic glycoinhibitors (75 mmol/L lactose and 1 mg asialofetuin/mL) to yield the level of carbohydrate-inhibitable binding.[6]

creatic cells with a range of tumorigenic and differentiation properties. Figure 4*A* shows Gal-1 immunodetection in total cell lysates and conditioned medium (CM) using WB. Six of 7 cell lines analyzed showed high Gal-1 expression. tPA levels also were analyzed.

We next analyzed the interaction of tPA and endogenous Gal-1 in lysates from PANC-1 and SK-PC-1 cells using affinity capture with tPA-Sepharose beads. Gal-1 was specifically detected in both lines in the fraction bound to tPA-Sepharose, but not in that bound to BSA-Sepharose (Figure 4*B*). tPA also was detected in Gal-1 cellular immunoprecipitates (Figure 4*C*). To determine whether Gal-1 is present in the plasma membrane of PDA cells, we performed surface biotinylation experiments. Gal-1 was present in the biotinylated fraction of Hs766T cells, indicating its surface distribution (Figure 4*D*). Biotinylated EGFR was used as a positive control.

We then examined Gal-1 expression in confluent and wounded cell monolayers (Figure 4*E*). Gal-1 was undetectable in confluent monolayers but it clearly was detected at the migration front after wounding. The specificity of the staining was determined (supplementary Figure 1; see supplementary material online at www.gastrojournal.org). These data support the notion that Gal-1 redistributes and concentrates at the plasma membrane upon a migration signal. Following this evidence, wound healing experiments were performed using SK-PC-1 cells expressing endogenous tPA. Colocalization of tPA and Gal-1 after wound healing was studied by double immunofluorescence confocal microscopy. tPA was expressed at high levels and colocalization with Gal-1 was detected at the migration front (Figure 4*F*, *merged panels*), suggesting that the interaction between these 2 proteins is involved in cell migration.

Gal-1 Mediates tPA-Induced ERK1/2 Activation, Cell Proliferation, and Invasion in Pancreatic Cells

We previously showed that tPA exerts mitogenic effects on pancreatic cells through ERK1/2 activation.[9] To establish whether Gal-1 is involved therein, we knocked-down Gal-1 using siRNA. As a control, we used irrelevant siRNA (siCtrl). WB analysis using anti–Gal-1 antibody confirmed Gal-1 knockdown (Figure 5*A*). Treatment of HPDE cells with tPA led to ERK1/2 activation in untransfected cells (-) and in cells transfected with siCtrl (Figure 5*B*). In contrast, Gal-1 knockdown was associated with reduced phospho-ERK1/2. ERK1/2 activation by growth factors was unaffected by Gal-1 knockdown, indicating that these effects are specific for tPA. We then examined whether Gal-1 was required for tPA-induced cell proliferation. HPDE and PANC-1 cells were treated with tPA in the presence of Gal-1 siRNA or siCtrl and cell proliferation was measured. tPA increased proliferation in both cell lines (Figure 5*C*). siGal-1 transfection decreased [^{3}H]-thymidine uptake to baseline values whereas siCtrl showed no effect. In contrast, down-regulation of Gal-1 did not affect serum/growth factor–induced cell proliferation, indicating that Gal-1 is involved mainly in the response to tPA. Altogether, these results show that Gal-1 mediates tPA-induced ERK1/2 activation and proliferation in pancreatic cells.

tPA also increases PDA cell invasion in vitro.[11] To determine whether Gal-1 participates in this process, in vitro Matrigel invasion assays were performed. We selected 3 pancreatic cell lines with distinct phenotypes: SK-PC-1 and HPDE cells, which express high levels of tPA, and PANC-1 cells lacking detectable tPA. SK-PC-1 cells showed high in vitro invasiveness, as reported,[11] whereas HPDE and PANC-1 cells showed moderate invasiveness (Figure 5*D*). In PANC-1 cells, the addition of exogenous tPA led to increased invasion. In all cells, Gal-1 knockdown (Figure 5*D*, *right*) led to reduced invasion, thereby indicating the involvement of Gal-1. Transfection with an irrelevant siRNA did not affect invasion. Although we cannot rule out that Gal-1 could be involved in modulating a general invasion mechanism, the results

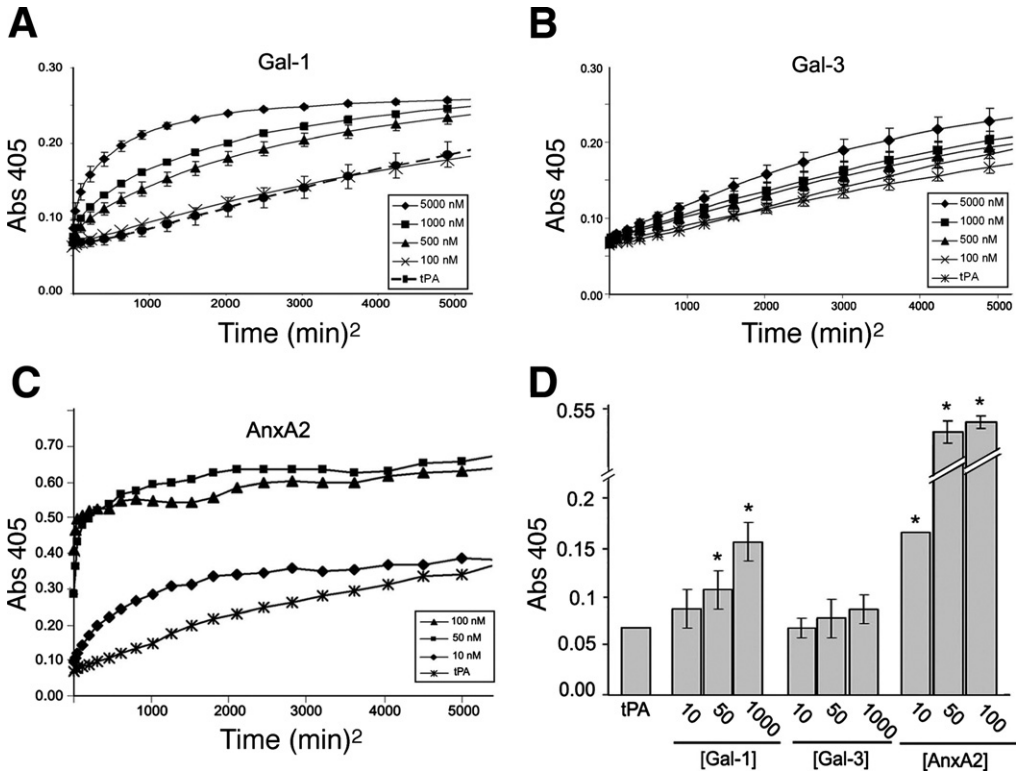

Figure 3. Gal-1 increases the catalytic activity of tPA in vitro. tPA and plasminogen were incubated with plasmin chromogenic substrate in the absence or presence of (*A*) Gal-1, (*B*) Gal-3, and (*C*) AnxA2 at a range of concentrations. Time-dependent changes in absorbance (405 nm) were measured. (*D*) Representative quantification and statistical analysis (*$P < .05$) of tPA catalytic activity in the presence of Gal-1, Gal-3, and AnxA2 at 1 hour.

obtained with PANC-1 cells indicate thgeneral invasion mechanism, the resultsat Gal-1/tPA interaction is relevant for PDA invasion.

Gal-1 Is Strongly Expressed in Ela-myc Pancreatic Tumors and Localizes With tPA Focally in Ductal Cells and at the Epithelial–Stromal Interface

To extend this analysis, we examined Gal-1 and tPA expression in normal pancreas and in tumors from Ela-myc mice. We previously showed that tPA is overexpressed in Ela-myc ductal tumors and contributes to tumor progression.[12] Gal-1 was undetectable in all cell types in normal pancreas (Figure 6*A*, *a*). In contrast, pancreatic tumors expressed high levels of Gal-1 both in ductal tumoral cells (Figure 6*A*, *b*, *arrows*) and in the stroma (Figure 6*A*, *c*, *arrows*). To determine whether tPA and Gal-1 colocalize, we used double immunofluorescence. tPA and Gal-1 displayed largely complementary distributions: Gal-1 was expressed strongly in tumor stroma whereas tPA was detected mainly in tumor cells (Figure 6*B*, *a* and *b*). However, the merge analysis indicates colocalization of both proteins focally at the interface of epithelial cells and stromal fibroblasts (Figure 6*B*, *c*, *arrowheads*). To conclusively determine whether Gal-1 is present in epithelial cells or fibroblasts, we performed double immunofluorescence staining of Gal-1 with cytokeratin 19 (CK-19) or α-smooth muscle actin (α-SMA1). Gal-1 was found in both tumor cells expressing CK-19 (Figure 6*B*, *d–f*) and in activated fibroblasts expressing α-SMA1 (Figure 6*B*, *g–i*). In contrast, double immunostaining for tPA and CK-19 or α-SMA1 showed that tPA expression is restricted to ductal tumoral cells (Figure 6*C*). These data suggest that tPA and Gal-1 participate in the cross-talk between epithelial tumoral cells and activated fibroblasts.

Gal-1 Is Expressed in Tumor Fibroblasts and Mediates the Functional Effects of tPA

Abundant desmoplasia is a hallmark of PDA and emerging data suggest that the interaction between cancer cells and stromal fibroblasts plays an essential role in tumor progression.[28] We therefore examined whether Gal-1 acts as a functional tPA receptor in tumor-derived fibroblasts (F88.2 cells). High Gal-1 levels were detected both in F88.2 cell lysates and in CM (Figure 7*A*), whereas tPA was undetectable. tPA-Sepharose pull-down experiments showed that Gal-1 was specifically detected in the tPA-bound fraction, indicating that fibroblast-derived Gal-1 interacts in vitro with tPA. To determine the distribution of Gal-1, immunofluorescence was used. As described for pancreatic cells, Gal-1 was barely detectable in confluent F88.2 cultures, whereas it localized at the membrane at the migration front after wounding (Figure 7*B*). Treatment of F88.2 cells with tPA induced a strong activation of ERK1/2, which was dramatically reduced on Gal-1 knockdown (Figure 7*C*), indicating that Gal-1 also is involved in tPA-induced ERK1/2 activation in fibroblasts. We also analyzed the involvement of Gal-1/tPA in

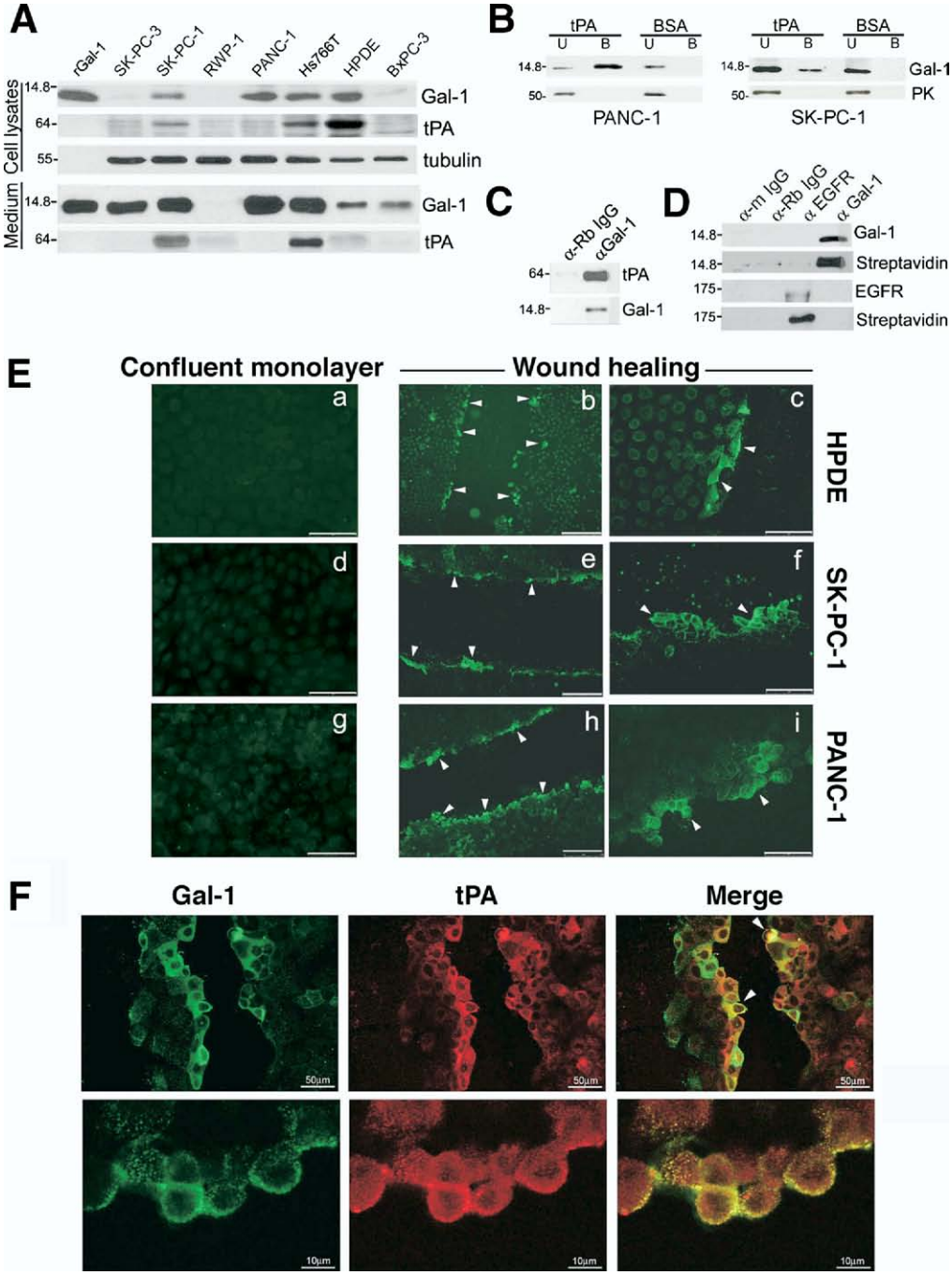

Figure 4. Gal-1 is expressed in cultured pancreatic cells, interacts with tPA, and both proteins colocalize at the migration front. (*A*) Gal-1, tPA, and tubulin expression in cultured pancreatic cells analyzed by WB. Expression in both lysates and CM is shown. (*B*) Pancreatic cancer cell lysates were incubated with tPA (or BSA)-Sepharose. Gal-1 was immunodetected by WB in unbound (U) and bound (B) fractions. Note that the bound fraction was 10-fold concentrated in comparison with the unbound fraction. Detection of pyruvate kinase (PK) was used as an internal control to determine binding specificity. (*C*) Hs766T lysates were used to immunoprecipitate with anti–Gal-1 polyclonal antibody or with irrelevant immunoglobulin (Ig)G using protein A–Sepharose. tPA was detected by WB. (*D*) Hs766T cells were incubated with biotin and lysates were immunoprecipitated with anti–Gal-1, anti-EGFR (positive control), or with irrelevant IgG. Biotinylated proteins were detected by WB using streptavidin–horseradish-peroxidase. (*E*) Confluent cells were scratched with a micropipette tip, allowed to migrate, and Gal-1 was detected in living cells by immunofluorescence. Gal-1 was barely detectable in a confluent monolayer (*a*, *d*, and *g*), whereas it redistributed at the migration front of HPDE (*b* and *c*), SK-PC-1 (*e* and *f*), and PANC-1 cells (*h* and *i*). Bars: 75 μm (*a*, *c*, *d*, *f*, *g*, *i*) and 250 μm (*b*, *e*, *h*). (*F*) Localization of Gal-1 (*green*), tPA (*red*), and overlay (*merge*) at the migration front of SK-PC-1 cells.

F88.2 proliferation: the tPA-induced increase in [^{3}H]-thymidine uptake was abolished on Gal-1 siRNA transfection (Figure 7*D*); siCtrl had no effect. We examined the involvement of Gal-1 in fibroblast invasion using Matrigel-coated filters. F88.2 cells were moderately invasive in basal conditions. tPA induced a marked increase in invasiveness (Figure 7*E*), which was reverted to baseline on Gal-1 siRNA transfection, thereby showing the participation of the tPA–Gal-1 interaction. To further analyze Gal-1 involvement in tumor/fibroblast cross-talk we examined the effects of pancreatic CM on fibroblast invasion in the absence or presence of Gal-1 siRNA. CM from SK-PC-1 cells, secreting high amounts of tPA, significantly increased F88.2 fibroblast invasion (Figure 7*F*). In contrast, PANC-1 CM, lacking tPA, had no effect on F88.2 cells. The addition of recombinant tPA to PANC-1 CM resulted in increased invasion. The enhanced invasiveness of F88.2 cells resulting from the addition of SK-PC-1 CM or exogenous tPA was reverted with Gal-1 siRNA, indicating its requirement for tPA-induced invasion. Because invasion involves extracellular matrix degradation, we analyzed the involvement of tPA catalytic activity in Matrigel invasion. The addition of plasminogen activator inhibitor 1 resulted in decreased invasion to

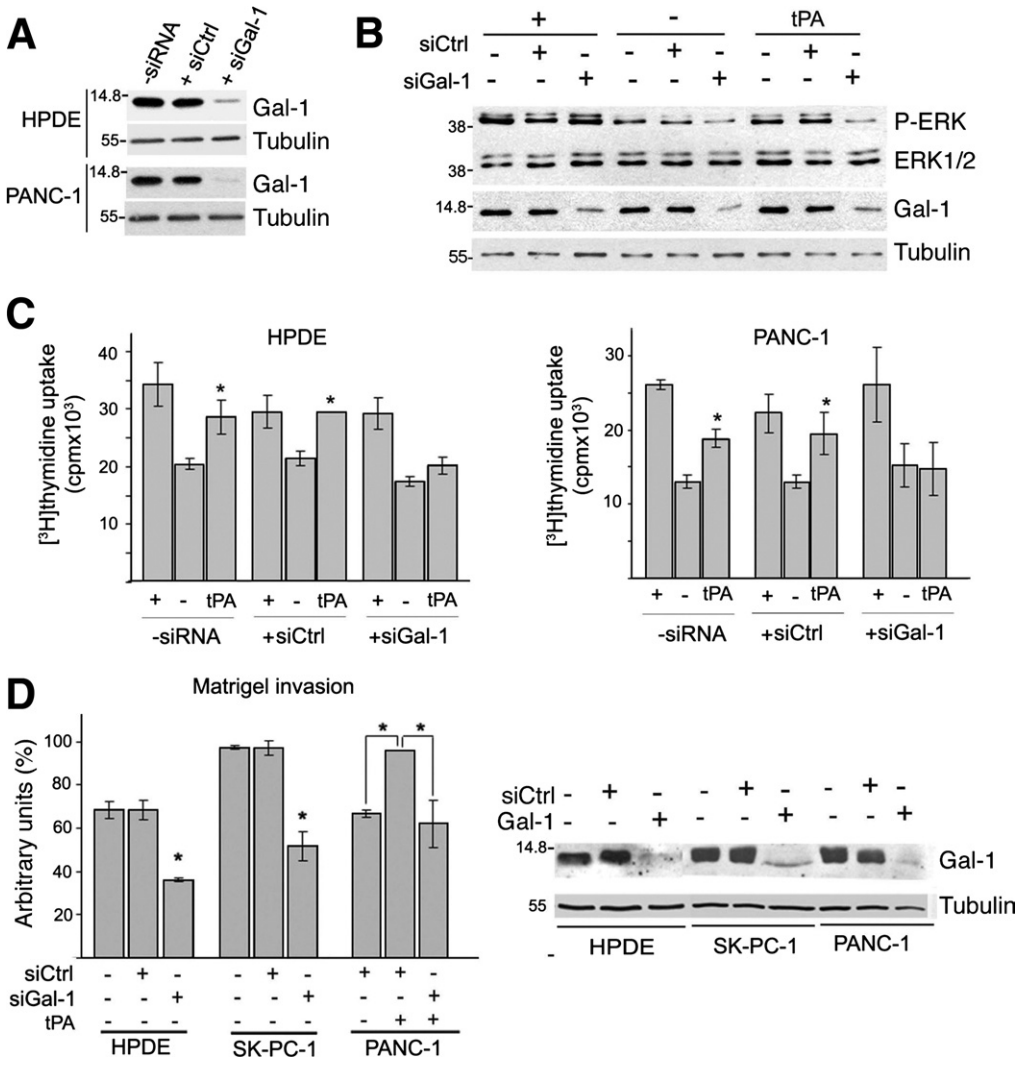

Figure 5. Gal-1 mediates tPA-induced ERK activation, proliferation, and invasion in cultured pancreatic cells. (*A*) Gal-1 knockdown using siRNA. Cells were transfected transiently with siRNA against Gal-1 (siGAL-1) or an irrelevant target (siCtrl) and Gal-1 was analyzed by WB 5 days later. (*B*) HPDE transfected with siGal-1 or siCtrl RNA were untreated (-), or treated with tPA or growth factors (+); ERK1/2 activation was assessed using anti–P-ERK; total ERK1/2, Gal-1, and tubulin levels also were assessed. (*C*) Cells were transfected with either siGal-1 or siCtrl RNA, treated with tPA or growth factors (+), and 24-hour [^{3}H]-thymidine uptake was measured. Results shown are representative of 4 independent experiments (*$P < .05$). (*D*) HPDE, SK-PC-1, and PANC-1 cells transfected with siGal-1 or siCtrl were cultured on Matrigel-coated filters for 72 hours. Matrigel invasion was determined using crystal violet (*$P < .05$). The decrease in the expression of Gal-1 is shown.

basal levels, indicating that tPA catalytic activity is required for this process (Figure 7*F*). These data suggest that, during invasion, Gal-1/tPA interaction contributes to increased tPA catalytic activity. Altogether, these results show the involvement of Gal-1 in tPA-induced ERK1/2 activation, proliferation, and invasion of tumor-derived fibroblasts, supporting a role of Gal-1 and tPA in epithelial-stroma cross-talk in PDA.

Discussion

In this study, we identify the endogenous lectin Gal-1 as a new functional receptor for tPA. Gal-1 has been reported to play a fundamental role in many functions related to cancer, such as angiogenesis, cell adhesion, proliferation, and migration.[29,30] We show that Gal-1 is expressed strongly in pancreatic cancer cells and tumoral fibroblasts both in vitro and in vivo. In cell culture, Gal-1 expression was detected at the front of migrating cells and colocalized with tPA. In pancreatic tumors, Gal-1 and tPA were present at the interface between tumor cells and stromal fibroblasts. Importantly, the inhibition of Gal-1 expression by siRNA in pancreatic cancer cells and in tumor-derived fibroblasts resulted in the abolishment of tPA-mediated effects on cell signaling, proliferation, and Matrigel invasion. We conclude that the Gal-1/tPA interaction reported here provides further insight into the mechanisms by which tPA participates in pancreatic cancer progression and associated desmoplasia.

tPA is a secreted protease best characterized for its role in fibrin degradation through the conversion of plasminogen to plasmin. Recent research has emphasized the role of tPA in other cellular functions both in physiologic and pathologic situations. Although several cell membrane receptors mediate the catalytic and noncatalytic activities of tPA, the molecular mechanisms involved are not well characterized. In the pancreas, AnxA2 and EGFR have been shown to be required for the effects of tPA during pancreatic tumor progression.[8,9] Interestingly, the inhibition of AnxA2 or EGFR does not completely abolish the effects of tPA, which suggests the involvement of other receptors. By using a proteomic approach involving tPA-

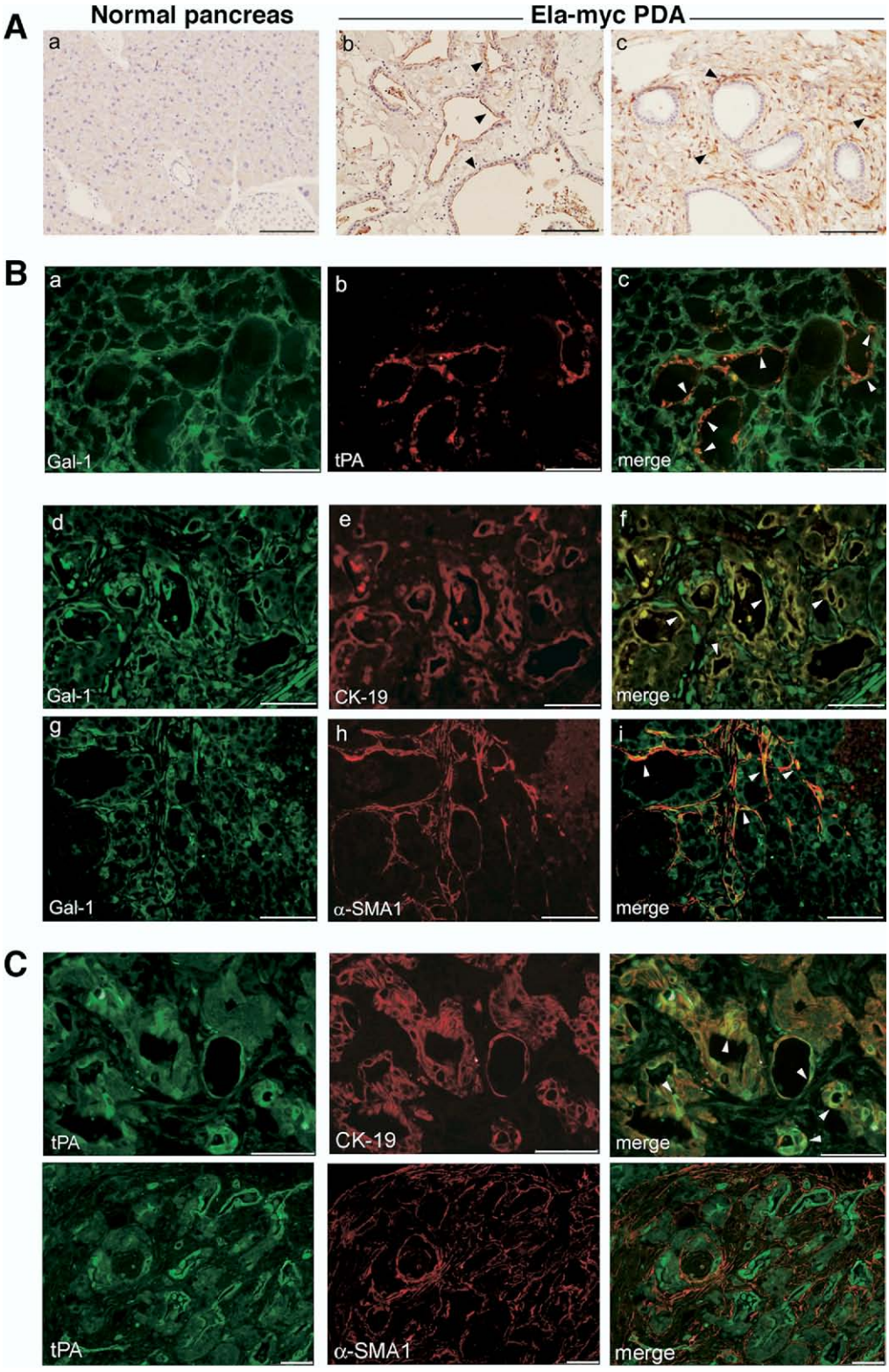

Figure 6. Gal-1 and tPA expression in pancreatic cancer tissues. (*A*) Sections of formalin-fixed, paraffin-embedded normal and neoplastic (PDA) tissues from Ela-myc mice were analyzed for Gal-1 expression. (*B*) Frozen sections of Ela-myc pancreatic cancer tissues were used to show colocalization of Gal-1 with tPA (*a–c*), CK-19 (*d–f*), or α-SMA1 (*g* and *h*). Signal overlay is shown in the *merge panels*. *Arrowheads* show focal areas of colocalization. (*C*) Double immunostaining for tPA and CK-19 (*a–c*) or α-SMA1 (*d–f*). Signal overlay (*merge*) is detected only for CK-19 (*arrowheads*). Bar, 75 μm.

Sepharose affinity capture of cellular lysates, we identified Gal-1 as a new protein that interacts with tPA in pancreatic cells[16] and here we found—using pull-down and surface plasmon resonance—that this interaction is direct, specific, and of high affinity. In addition, we show that Gal-1 is involved in tPA-mediated effects on pancreatic cancer cells and tumor-associated fibroblasts. The evidence for a direct Gal-1/tPA interaction in vitro does not rule out the involvement of a multiprotein complex in cells. Indeed, for uPA a multiprotein complex includ-

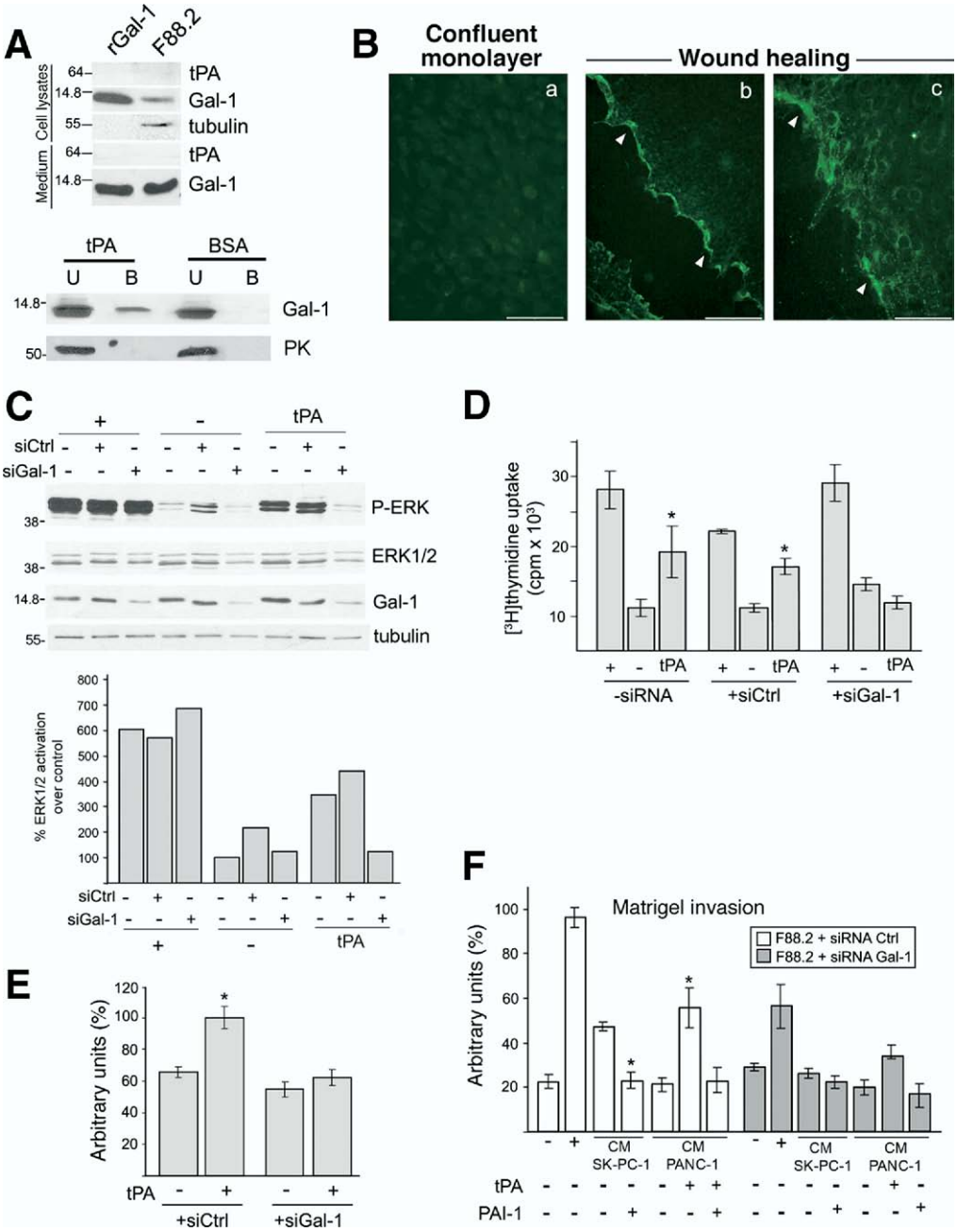

Figure 7. Functional interaction of Gal-1 and tPA in human F88.2 tumor-derived fibroblasts. (*A*) Gal-1 and tPA levels in F88.2 fibroblasts (cell lysates and CM) analyzed by WB. *Lower part of the panel* shows Gal-1/tPA interaction in F88.2 cells using a pull-down assay. Gal-1 was immunodetected by WB in unbound (U) and bound (B) fractions. Detection of pyruvate kinase was used as an internal control to determine binding specificity. (*B*) Immunofluorescence localization of Gal-1 in F88.2 fibroblasts. Gal-1 expression is barely detectable in a confluent monolayer (*a*), whereas it is localized at the migration front (*b* and *c*, *arrowheads*). Bars, 75 μm (*a* and *c*) and 250 μm (*b*). (*C*) ERK1/2 activation determined in F88.2 cells transfected with siGal-1 or siCtrl and treated with tPA or fetal bovine serum (+). Densitometric analysis of P-ERK1/2 vs total ERK1/2 is shown in the *lower panel*. Gal-1 and tubulin levels also were assessed. (*D*) Cells transfected with either siGal-1 or siCtrl were treated with tPA or fetal bovine serum (+) and 24-hour [^{3}H]-thymidine uptake was measured. (*E*) F88.2 cells transfected with siGal-1 or siCtrl were cultured with or without tPA on Matrigel-coated Transwells for 72 hours. Invasion was determined using crystal violet. (*F*) Effect of SK-PC-1 or PANC-1 CM was tested in F88.2 Matrigel-invasion assay as described in the supplementary data (see supplementary material online at www.gastrojournal.org). tPA or plasminogen activator inhibitor 1 (PAI-1) was added when indicated. Results shown are representative of 3 independent experiments (*$P < .05$).

ing EGFR and integrins has been described.[31] We hypothesize that tPA could interact similarly with a complex comprising Gal-1, AnxA2, and EGFR. The molecular interaction between these proteins requires further experiments. One interesting possibility is that Gal-1, through its carbohydrate recognition domain, clusters these proteins by recognition of glycosylated chains from EGFR or other membrane glycoproteins. Indeed, our preliminary

data using 1-deoxymannojirimycin to inhibit complete glycan processing showed that tPA-mediated signaling is abolished by the treatment, suggesting that mature glycosylation of membrane proteins is required (supplementary Figure 2; see supplementary material online at www.gastrojournal.org).

Special attention should be given to the role of cellular glycosylation as a modulator of the Gal-1/tPA interaction because tumorigenesis almost universally is associated with alterations in glycosylation.[32] Our data indicate that galactose in the β-anomeric position could interfere with Gal-1/tPA binding, thereby suggesting that tPA glycosylation is required for the interaction. Binding affinity is higher for Gal-1 than for Gal-3, thus showing selectivity among the galectin family. The modulation of glycosylation, with functional implications via galectin binding, has been reported in the case of $p16^{INK4a}$ tumor suppressor by increasing susceptibility to carbohydrate-dependent induction of anoikis in pancreatic cancer cells.[33] Interestingly, $p16^{INK4a}$ inactivation occurs almost universally in PDA through a variety of mechanisms[34] and thus may contribute, in part, to the malignant properties of PDA through altered glycosylation of proteins. The identification of Gal-1 as a new tPA functional receptor, and the ability to block the carbohydrate-binding activity of Gal-1 by custom-made inhibitors,[35] provides new insights for the design of novel targeted therapies.

In functional terms, our study shows that inhibition of Gal-1 expression by siRNA in pancreatic cells markedly decreases tPA-induced ERK1/2 activation and proliferation, thus providing strong evidence that Gal-1 is a crucial component of tPA mitogenic signaling. In this context, it is of interest to note that Gal-1 interacts with oncogenic H-Ras and is essential for its membrane anchorage and transforming activity.[36] These observations suggest that H-Ras might be an upstream element in the activation of ERK1/2 cascade by tPA and Gal-1 interaction. Whether K-Ras, whose constitutive activation occurs in the majority of PDA, also may interact with Gal-1 in pancreas cancer cells and contribute to ERK1/2 activation remains an interesting possibility. We also report that the tPA proliferative effects on fibroblasts are related to ERK1/2 activation. However, the involvement of additional signaling pathways cannot be excluded. Interestingly, it has been reported that platelet-derived growth factor-C, one of the main inducers of desmoplasia, increases fibroblast proliferation by a mechanism that requires tPA proteolytic activity.[37] Our data indicate that tPA interaction with Gal-1 leads to a modest increase of tPA proteolytic activity in vitro. Whether this could result in the activation of platelet-derived growth factor in our system remains an open question.

Both tPA and Gal-1 are overexpressed in PDA, as shown by gene expression microarrays and proteomic analyses.[11–14,21–24] Here we provide a link between the overexpression of these 2 proteins through the identification of a direct interaction that contributes to tumor progression. tPA is overexpressed by the tumor cells in PDA and plays a relevant role by inducing cell proliferation, tumor invasion, and angiogenesis. Previous immunohistochemical studies had suggested that Gal-1 expression was restricted to the tumor stroma in human pancreatic cancers.[21,23] We now show that Gal-1 also is expressed in cultured human pancreatic cancer cells, localizing at the cell membrane during cell migration. In tumors from Ela-myc mice, using double staining with cell type–specific markers, we observed strong Gal-1 staining in epithelial and stromal cells and colocalization of tPA and Gal-1 focally at the interface of tumor cells and stromal fibroblasts. We propose that, on contact with the surrounding stroma, pancreatic epithelial cells redistribute Gal-1 to the membrane. In addition, strong Gal-1 expression in the stroma could result, at least in part, from its secretion by pancreatic cancer cells, thus acting in a paracrine manner on the desmoplastic reaction. Figure 8 shows a working model of this molecular mechanism: tPA secreted by pancreatic cancer cells would act in a dual autocrine and paracrine fashion on both epithelial tumor cells and fibroblasts by stimulating proliferation and cell migration/invasion via an interaction with Gal-1. At the same time, the regulation of Gal-1

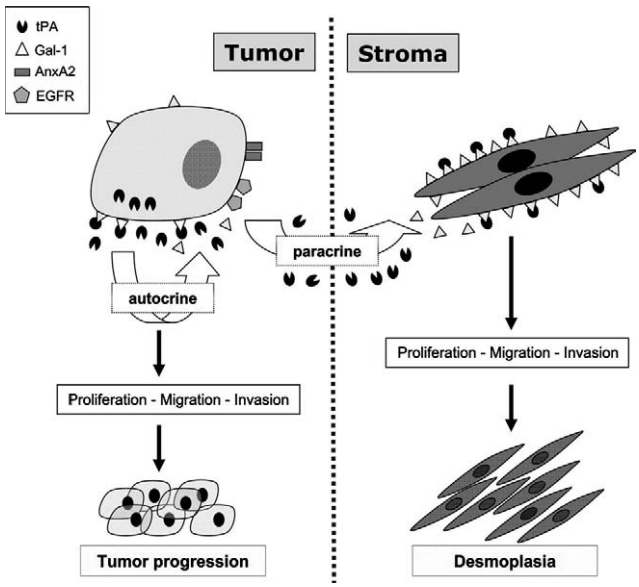

Figure 8. Epithelial-stromal cross-talk in pancreatic cancer involves tPA and Gal-1. A model is proposed whereby tPA and Gal-1 participate in the generation of a signaling microenvironment in the transition zone between epithelial and stromal cells. Gal-1 is synthesized by both fibroblasts and epithelial tumor cells, although levels are higher in the former. Gal-1 concentrates at the migration front in cells and it also can be secreted to the extracellular medium. tPA binding to Gal-1 induces cell proliferation, migration, and invasion. Effects can occur on the 2 cell types, thus generating a dual autocrine–paracrine signaling loop.

expression and subcellular distribution in epithelial cells and fibroblasts could modulate the effects of tPA during pancreatic tumor progression. It is remarkable that desmoplasia is one of the main hallmarks of PDA and recent work has shown that stromal fibroblasts enhance invasiveness, tumor growth, and metastasis.[38] Stroma also is believed to contribute to aberrant epithelial-mesenchymal interactions, which are partially accountable for acquired drug resistance during cancer treatment.[39] The identification of the Gal-1/tPA interaction as a new mechanism involved in fibroblast proliferation, migration, and invasion sheds some light on the molecular basis for the cross-talk between malignant epithelial cells and the surrounding stroma, a critical step to deciphering the pathogenesis of tumor progression.

Despite substantial advances in the understanding of PDA on a molecular basis, the prognosis of this tumor still remains very poor and finding new molecular targets is urgently needed to improve the clinical outcome of pancreatic tumors. Identification of Gal-1 as a new tPA functional receptor therefore provides a promising therapeutic strategy for PDA.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.12.039.

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O.R. and E.O.–Z. made equivalent contributions to this article and share first authorship.

Conflict of interest

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Materials and Methods

Materials

All reagents were obtained from Sigma (St. Louis, MO) unless stated otherwise. Polyclonal antibodies against galectins, including specificity controls against a panel of human galectins, were produced in our laboratories and characterized as reported elsewhere.[1,2] Mouse α-tPA monoclonal antibodies 374-B, 373, and 387 were from American Diagnostica (Stamford, CT). Rabbit α-phospho-ERK1/2 and α-total-ERK1/2 antibodies were purchased from Cell Signalling (Boston, MA) and Upstate Laboratories (Charlottesville, VA), respectively. Alexa 488– and Alexa 555–labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA). For immunofluorescence experiments, biotinylated donkey anti-goat immunoglobulin (Ig) (Jackson ImmunoResearch Laboratory) followed by rhodamine-streptavidin were used for the detection of tPA. Mouse anti–α-tubulin and α-SMA1 antibodies were from Sigma. CK-19 TROMA-III antibody was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Plasminogen activator inhibitor 1 reagent was a gift from P. Muñoz (Center for Genomic Regulation, Barcelona, Spain). Chips, reagents, and buffers for the BIAcore experiments were obtained from BIAcore. SMARTpool and siCONTROL nontargeting siRNA pool were from Dharmacon. Recombinant AnxA2 was prepared from BL21 *Escherichia coli* transformed with the pET21b(+) vector containing the human AnxA2 complementary DNA, which was kindly provided by Dr K. A. Hajjar (Cornell University Medical College, New York, NY). Recombinant Gal-1 and Gal-3 were produced as described.[3] Recombinant tPA (Actilyse) was from Boehringer (Ingelheim, Germany). The chromogenic substrate for plasmin S-2251 was from Chromogenix (Milan, Italy). [^{3}H]-thymidine was purchased from Amersham Biosciences (Uppsala, Sweden).

Cell Culture

PANC-1,[4] SK-PC-1, SK-PC-3,[5] and BxPC-3[6] were obtained from primary pancreatic tumors. Hs766T[7] and RWP-1 cells[8] were obtained from metastases. F88.2 are spontaneously immortalized fibroblasts obtained from a breast tumor. Cells were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum (Invitrogen-Gibco, Long Island, NY). Human papilloma virus E6/E7 immortalized HPDE cells[9] were cultured in keratinocyte serum-free medium supplemented with EGF and bovine pituitary extract (Invitrogen–Gibco).

WB

Cells were lysed in buffer A (0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, 1% Triton X-100, pH 7.2) containing protease and phosphatase inhibitors. After centrifugation, proteins in the supernatant were quantified and boiled with Laemmli buffer. Proteins (20 μg/lane) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. WB was performed as described elsewhere.[10] For CM, cells were grown until confluence, washed, and grown in cell culture medium without fetal bovine serum for 72 hours. CM was collected and concentrated using Centricon filters (Millipore, Bedford, MA). After the addition of Laemmli buffer and boiling, 10 μL of sample (corresponding to 5% of the total CM) was loaded for each cell line and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and WB. In those experiments in which total cell lysates and CM are shown, the relative amount of CM is 2.5-fold enriched in comparison with total cell lysates, although the relative lysate/medium ratios are the same for all cell lines shown.

Data Analysis of tPA Catalytic Activity Assay

Absorbance at 405 nmol/L was measured every 5 minutes for up to 1 hour in an Elx808 Ultra Multiplate reader (Bio-tek Instruments Inc, Winooski, VT). Samples were analyzed in quadruplicate in 3 independent experiments. The rate of plasmin generation was calculated using linear regression analysis of plots of absorbance at 405 nmol/L vs time squared, as described.[11,12]

Pull-Down Experiments

tPA or BSA were coupled to Sepharose as reported.[13] Recombinant proteins (5 μg) or 1 mg of cell lysates (prepared as mentioned earlier) were incubated with tPA-Sepharose beads in phosphate-buffered saline (PBS) (2 h, 4°C). After 3 washes with cold PBS, bound proteins were eluted with Laemmli buffer. Thirty percent of the bound fraction (B) and 3% of the unbound lysate (U) of both tPA and BSA pull-downs were loaded for WB analysis.

Immunoprecipitation

Cell lysates in buffer A were precleared and immunoprecipitated with anti–Gal-1 antibodies or with rabbit IgG for 3 hours at 4°C. Immunoprecipitates were washed in buffer A, boiled in sample buffer, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane.

Cell Surface Biotinylation

Hs766T pancreatic cancer cells were rinsed twice in ice-cold PBS, and incubated for 15 minutes at 4°C with 0.4 mg/mL of sulfo-NHS-biotin (Pierce, Rockford, IL) in PBS, with slight shaking. After washing with Dulbecco's modified Eagle medium and PBS, cells were lysed and immunoprecipitated with anti–Gal-1, anti-EGFR, or irrelevant IgGs as described earlier. Biotinylated proteins were detected by WB using streptavidin–horseradish-peroxidase.

ERK1/2 Activation Analysis

HPDE and F88.2 cells were processed for Gal-1 knock-down as described earlier and starved in growth

factor–depleted medium. They then were treated with tPA (20 μg/mL) for 10 minutes. EGF/bovine pituitary extract (HPDE) or 5% fetal bovine serum in Dulbecco's modified Eagle medium (F88.2) were added as positive controls; untreated cells were used as negative control. Detection of activated or total ERK1/2 was detected by WB as previously described.[10] Gal-1 knock-down was confirmed for each experiment by WB analysis.

Inhibition of Glycosylation With 1-Deoxymannojirimycin

HPDE or F88.2 cells were pretreated for 48 hours with the glycosylation inhibitor 1-deoxymannojirimycin (0.3 mmol/L), which inhibits conversion of high-mannose-type to complex-type N-linked glycans.[14] ERK1/2 activation then was determined as described earlier.

The efficiency of the deglycosylation was determined by enzyme-linked immunosorbent assay using biotinylated Gal-1 as a probe. Confluent HPDE or F88.2 cells in 96-well Nunclon plates (Nunc, Naperville, IL), treated or not with 1-deoxymannojirimycin (0.3 mmol/L, 72 h), were equilibrated for 1 hour at 37°C with incubation buffer (11 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, 3 mmol/L $MgCl_2$, 1 mmol/L glucose, and 1% BSA) and 5–20 nmol/L biotinylated Gal-1 then was added for 15 minutes at 37°C. After 3 washes with incubation buffer, cells were fixed with methanol (5 min at –20°C), washed 3 times with 0.02% Tween 20 in TBS, and incubated with alkaline phosphatase–coupled streptavidin (1 h, 37°C). Enzymatic activity was measured using 4-methylumbelliferyl phosphate (1 mg/mL in triethanolamine buffer, pH 9.5) for 30 minutes at room temperature. Quantification was performed by measurement of absorbance at 460 nm using a Cytofluor 235 instrument (Millipore).

Cell Proliferation Assays

HPDE, PANC-1, and F88.2 cells were processed for Gal-1 knock-down as described earlier and starved in growth factor–depleted medium. [^{3}H]-thymidine incorporation assays were performed as described.[10] Inhibition of Gal-1 expression was confirmed for each experiment by WB.

Immunofluorescence Microscopy

Cells cultured on cover slips were incubated with anti–Gal-1 antibody for 15 minutes at 37°C, washed, and fixed. For colocalization, cells were incubated with anti-tPA antibody 373 for 1 hour at 37°C. After washing, Alexa-488–anti-rabbit (for Gal-1) or Alexa-555–anti-mouse (for tPA) secondary antibodies were added for 1 hour at 37°C. Cover slips were mounted and analyzed by fluorescence microscopy using a Leica DMRB microscope adapted to a DC300F camera (Leica Lasertechnik GmbH, Mannheim, Germany). Images were obtained with the 40× objective. Confocal images were acquired with a TCS S2 microscope adapted to a DMIRBE inverted microscope (Leica) for sequential image acquisition. Images were obtained with 63× and 40× Leica Plan-Apocromatco lenses and analyzed using Studio Lite 1.0 software.

Immunohistochemistry

Tissue samples obtained from Ela-myc mice[15] were fixed with formalin (paraffin-embedded sections) or with cold acetone (fresh-frozen sections). Primary antibodies were added overnight at 4°C. The following secondary antibodies were used: Envision+ anti-rabbit Ig reagent (Dako, Glostrop, Denmark), Alexa-488–anti-rabbit Ig (for Gal-1); biotinylated anti-mouse Ig (for tPA) and streptavidin-rhodamine (for CK-19); and Alexa-555–anti-mouse Ig (for α-SMA1). Images were acquired with 20× or 40× magnification as described earlier. Nonimmune (Dako) or pre-immune rabbit serum were used as negative controls.

Wound Healing Experiments

Cells were grown to confluence on cover slips and the monolayer was wounded with a micropipette tip. After gentle washing, they were allowed to migrate for variable time periods, fixed, and processed by immunofluorescence. For the competition control experiment, anti–Gal-1 polyclonal antibody was co-incubated with the recombinant protein (1:5, respectively) in unpermeabilized living cells. For the time zero control experiment, anti–Gal-1 was added 15 minutes before the wound and cells were fixed immediately after the scratching.

Cell Invasion Assays

For invasion assays on Matrigel-coated Transwells (Costar, Cambridge, MA), cells were washed and seeded in Dulbecco's modified Eagle medium plus 1% BSA. Recombinant tPA (20 μg/mL) was added 3 hours later. Cells were allowed to invade for 72 hours; cells that passed to the lower compartment of the filter were fixed, and quantified after crystal violet staining for 5 minutes at room temperature and 10% acetic acid extraction. To test the effect of pancreatic cell CM on fibroblast invasion, SK-PC-1 or PANC-1 cells were grown to confluence in the lower chamber of the plates and F88.2 cells were transfected with siGal-1 or siCtrl were seeded on Matrigel-coated Transwells as described earlier. Cell invasion was measured after 72 hours by crystal violet staining. tPA (20 μg/mL) or plasminogen activator inhibitor 1 (5 μmol/L) was added in the lower wells when indicated.

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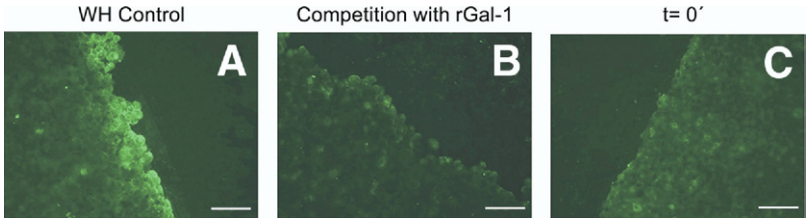
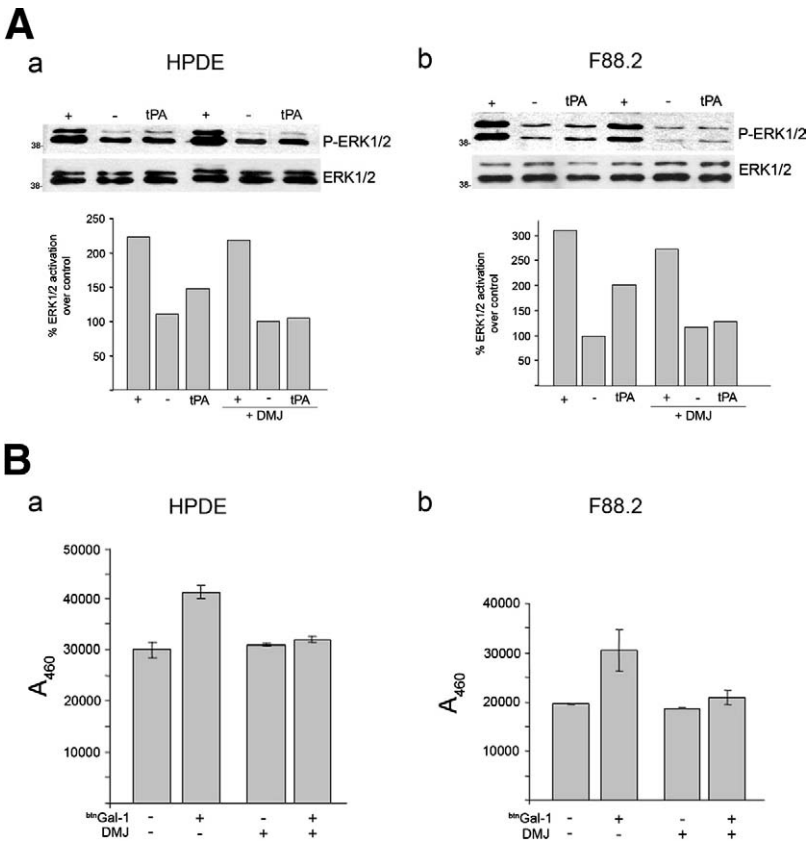
Supplementary Table 1. Hyperbolic Fit of Surface Plasmon Resonance Data for the Interaction of Gal-1, Gal-3, and AnxA2 With tPA

	Gal-1	AnxA2	Gal-3
K_d	9.1×10^{-6}	2.3×10^{-6}	1.7×10^{-4}
Chi-square	1.8	3.5	486.8
r^2	0.993	0.933	0.998

NOTE. Maximum signal vs concentration graph was fitted to a hyperbolic regression with the equation $y = ax/(1 + bx)$ following the steady-state model. Quality of the fit was determined by chi-square, assessing a good correlation of the model in the case of Gal-1 and AnxA2. For Gal-3 chi-square value indicates a poor correlation. K_d corresponds to the dissociation constant (molar) of the ligand/tPA interaction. r^2 indicates the goodness of the K_d value in the regression. K_d values for both Gal-1 and AnxA2 were of similar magnitude. The high chi-square value for Gal-3 indicated a poor fit of the model, in agreement with a higher K_d and a weaker interaction with tPA.



Supplementary Figure 1. Wound healing controls. (*A*) Gal-1 is expressed at the migrating front of PANC-1 cells. To verify that this staining is not an artifact of the wound healing technique, competition with recombinant protein and zero time controls were performed as described in the supplementary Materials and Methods section. Gal-1 is undetectable when the (B) anti–Gal-1 polyclonal antibody is co-incubated with recombinant Gal-1 (5:1 ratio, respectively) or when (*C*) cells are fixed immediately after wounding the confluent monolayer (zero time). *Bars*, 100 μm.



Supplementary Figure 2. tPA signaling is not affected by inhibition of glycosylation. (*A*) HPDE (a) or F88.2 cells (b) were treated or not with 0.3 mmol/L 1-deoxymannojirimycin (DMJ), which inhibits conversion of high-mannose type to complex-type N-linked glycans. After 48 hours, cells were untreated (−), treated with growth factors (HPDE) or fetal bovine serum (F88.2) (+), or with tPA (tPA), and levels of phospho-ERK1/2 and total ERK1/2 were analyzed. Densitometric analysis of P-ERK1/2 vs total ERK1/2 is shown in the *lower panel*. (*B*) HPDE (a) or F88.2 (b) cells were untreated or treated with 0.3 nmol/L DMJ for 72 hours. After blocking, biotinylated Gal-1 10 nmol/L (^{btn}Gal-1) was added and binding was detected using alkaline phosphatase–labeled streptavidin and spectrophotometer quantification of absorbance at 460 nm.