Plasminogen activator inhibitor type 2 inhibits cell surface associated tissue plasminogen activator *in vitro*

Potential receptor interactions

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Summary

Regulation of cellular plasminogen activation is necessary for maintenance of tissue homeostasis. Despite increasing evidence for co-expression of tissue type plasminogen activator (tPA) and plasminogen activator inhibitor type-2 (PAI-2; SERPINB2) under patho/physiological conditions, the inhibition of cell-bound tPA-mediated plasminogen activation by PAI-2 has not been addressed. Here we show that PAI-2 can inhibit cell-bound tPA activity *in vitro* and thus prevent plasmin formation. We also examined the potential involvement in this inhibition of the annexin II heterotetramer (AIIt), one of the many well characterized cell-surface co/receptors for tPA and plasminogen that efficiently promotes plasminogen activation. This receptor was of interest because AIIt has also been shown to directly bind PAI-2. Characterization of these potential interactions using purified protein systems revealed that PAI-2 directly bound AIIt via the p11 (S100A10) subunit. However, PAI-2 prevented AIIt/tPA-mediated plasminogen activation by its classic serpin inhibitory activity rather than through competition with tPA/plasminogen for binding. Further analysis showed that PAI-2 inhibited cell bound tPA-induced plasmin activity in both an AIIt-dependent and -independent manner. These data open new possibilities for further investigations regarding the regulation of cellular plasmin generation *in vivo*, especially in tissues where PAI-2 and tPA may be co-expressed.

Keywords

SERPINB2/PAI-2, tPA, plasmin, annexin II heterotetramer, cell surface

Thromb Haemost 2008; 100: 319–329

Introduction

Plasminogen activator inhibitor type 2 (PAI-2; SERPINB2) is an efficient inhibitor of active soluble urokinase (uPA) and tissue type plasminogen activator (tPA) (1, 2). Like most serpins, PAI-2 has a well-conserved tertiary structure, consisting of three β-sheets, nine α-helices, and a reactive centre loop (2–4). The reactive centre loop mediates the formation of covalent uPA:PAI-2 and tPA:PAI-2 inhibitory complexes (4). A unique feature of PAI-2 is an extended 33 amino acid loop between the C and D helices, termed the CD-loop (2). The CD-loop is not involved in the inhibitory activity of PAI-2 but may mediate interactions with other proteins (2, 5, 6).

PAI-2 is a 10-fold more efficient inhibitor of uPA (second order rate constant, 1–2 x 10^6 $M^{-1}s^{-1}$) than tPA (1–2 x 10^5 $mol^{-1}sec^{-1}$) which is suggestive of a preference for uPA inhibition (1, 2). Indeed PAI-2 is not usually associated with fibrinolysis both because PAI-2 is normally undetectable in blood and because it is a poor inhibitor of the free circulating zymogen form of tPA (i.e. single-chain tPA, second order rate constant, ~10^3 $M^{-1}s^{-1}$) (1, 2). In addition, fibrin-bound tPA is protected from inhibition by PAI-2 (7). By comparison, the related uPA and tPA inhibitor PAI-1 (SERPINE1), an established regulator of fibrinolysis, is an efficient inhibitor of single-chain tPA (second order rate constant, 5 x 10^6 $M^{-1}s^{-1}$), though fibrin-bound tPA is also protected from PAI-1 inhibition (1, 2, 8, 9). Nevertheless, in late pregnan-

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Received February 27, 2008
Accepted after major revision June 16, 2008

Prepublished online July 10, 2008
doi:10.1160/TH08-02-0119

cy induction of PAI-2 expression and secretion by placental trophoblast cells leads to measurable plasma levels (up to 250 ng/ml), which has fostered suggestions that PAI-2 is involved in fibrinolysis in late pregnancy (2, 10, 11). However, while one study showed that the amount of PAI-2 expressed by trophoblasts was in excess of the amount required to completely inhibit uPA (12), another study showed that the levels of uPA/tPA as well as PAI-1 and PAI-2 in the circulation also significantly increase as pregnancy progresses, yet the overall fibrinolytic activity remains unchanged (2, 10, 11). One study found that PAI-2 apparently co-localizes with uPA rather then tPA in placental tissues, while PAI-1 is present at sites of uPA and tPA expression (13). Thus, the function of PAI-2 (or indeed uPA/tPA and PAI-1) during pregnancy and any associated potential anti-fibrinolytic role of PAI-2 is still not resolved.

Although PAI-2 is a faster inhibitor of uPA compared to tPA, PAI-2 can still be considered as a very efficient tPA inhibitor and there is evidence to suggest that PAI-2 may play a role in tPA inhibition not associated with fibrinolysis. Indeed, several observations support/suggest that peri/extracellular PAI-2/tPA interactions may occur *in vivo*: (i) PAI-2 is a stress response protein and this can lead to increased local tissue levels of extracellular PAI-2 (14, 15); (ii) tPA plays potentially non-fibrinolytic roles in several patho/physiological processes such as inflammation and neuronal function where PAI-2 levels may also be elevated (16, 17); (iii) tPA/PAI-2 inhibitory complexes have been detected in both saliva (18) and gingival crevicular fluid (19), indicating inhibition of soluble tPA by PAI-2 *in vivo*. As tPA can also interact with cell surfaces, it is possible that PAI-2 may be able to inhibit cell-associated tPA in particular tissue microenvironments where PAI-2 levels may be elevated. Given that there are no studies yet describing the interaction of PAI-2 and tPA at the cell surface, exploration of PAI-2 as a potential inhibitor of cell-surface bound tPA activity using in-vitro cell model systems is warranted.

There are many putative and established tPA and plasminogen receptors, including low-affinity non-specific receptors on the cell surface (20, 21) and moderate-affinity receptors such as annexin II heterotetramer (AIIt). This heterotetramer is composed of two subunits of annexin II (p36) linked by a S100A10 (p11) dimer, which either alone, or within the heterotetramer, serve as cell surface receptors for several extracellular proteins including tPA and plasminogen, and can thus efficiently mediate the conversion of plasminogen to plasmin (22, 23). PAI-2 also interacts with placentally derived cytosolic annexin I and purified annexin I, II and AIIt (6). The interaction between PAI-2 and annexin I was shown to be somewhat dependent on the CD-loop of PAI-2 (6) but the dependence of the CD-loop for binding of PAI-2 to annexin II/AIIt was not addressed. These authors suggested that the abundant intracellular localization of both PAI-2 and annexins is indicative of an intracellular function of the PAI-2/annexin interaction. They also showed that PAI-2 maintains its uPA binding ability in the presence of the cytosolic extracts containing these proteins.

Given that AIIt is involved in plasminogen activation at the cell surface and that PAI-2 binds directly to AIIt and potentially maintains its serpin activity, it is possible that the PAI-2/AIIt interaction may impact on tPA-mediated plasminogen activation. Herein, we describe biochemical and cellular analyses of the interaction between PAI-2 and AIIt (and its individual components p36 and p11) in the absence and presence of tPA and plasminogen and assess the potential role of these interactions on the regulation of AIIt and tPA-dependent plasminogen activation. For these cellular analyses we utilized two cell systems that differ in their endogenous expression levels of the two subunits of AIIt. These include HeLa cells as a model cell system expressing both subunit components at the cell surface (22) and MCF-7 cells as a model cell system predominately expressing p36 (24). As both cell lines express low to no endogenous cell surface localized uPA, tPA, PAI-1 or PAI-2 this allowed analysis of PAI-2 binding affects in the absence or presence of exogenous tPA without the confounding effects of endogenous plasminogen activator or serpins that could compete for PAI-2 interactions with AIIt. We thus show that while PAI-2 can directly bind AIIt via p11, it does not act as co-receptor for PAI-2 and tPA/plasminogen. Rather, PAI-2 inhibits AIIt- and cell-bound tPA via its serpin activity to prevent plasmin formation.

Materials and methods

Proteins, antibodies and reagents

Nitrocellulose membrane (Trans-Blot, 0.45 μm) and HRP-conjugated secondary antibodies were from Bio-Rad (Hercules, CA, USA). Mouse anti-PAI-2 (#3750, recognizes both free PAI-2 and uPA:PAI-2 or tPA:PAI-2), anti-uPA (#394) and anti-plasminogen (#3641) monoclonal antibodies, goat anti-tPA (#387), rabbit anti-tPA (#385) polyclonal antibodies, two-chain recombinant tPA (#170), human high molecular weight uPA (#128) and Spectrozyme PL were from American Diagnostica Inc. (Stamford, CT, USA). Goat anti-human S100A10 IgG was from R&D Systems Inc (Minneapolis, MN, USA). Goat anti-annexin II (C-16) polyclonal antibody (sc-1924) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). BSA, Tris, D-Phe-Pro-Arg chloromethylketone (PPACK, Dihydrochloride), Hank's balanced salts, fibrinogen, IgG rabbit serum, IgG goat serum, FITC-conjugated anti-goat and anti-rabbit antibodies and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). SuperSignal West Pico Chemiluminescent substrate was from Pierce (Rockford, IL, USA). The ProteoExtract Native Membrane Protein Extraction kit was purchased from Calbiochem (San Diego, CA, USA). Protein A sepharose was obtained from Amersham Biosciences (Piscataway, NJ, USA). Plasminogen was purified from human plasma as described (25) and tested for absence of plasmin activity (data not shown).

Hexa-His-tagged human wild-type PAI-2 and PAI-2 ΔCD-loop (CD-loop mutant, residues 66–98 deleted) (approx. 49 kDa and 46 kDa, respectively) were expressed and purified from *E. coli* as previously described (5, 26). Un-tagged human recombinant PAI-2 (47 kDa) (27) was used where indicated. Purified human AIIt, annexin II (p36) and S100A10 (p11) proteins were a kind gift from Professor D. Waisman (University of Calgary, Canada). Bovine AIIt was purchased from Meridian Life Science (Saco, ME, USA). The integrity of both the human and bovine AIIt was confirmed by SDS-PAGE and western blotting. Human and bovine AIIt have very similar properties and have been used interchangeably in previous studies (22).

Ligand dot blot analysis of PAI-2, tPA and plasminogen binding to immobilized AIIt, p36 and p11

This was performed as previously described (27) with the following modifications. Purified AIIt, p36 and p11 (1 μg each) as well as BSA (1 μg, as a negative control) were dotted onto nitrocellulose membrane strips and dried. The efficient attachment of AIIt, p36 and p11 to the membranes was confirmed using specific primary antibodies against these proteins and detection by enhanced chemiluminescence (data not shown). In addition, membrane strips dotted with 0.5 μg uPA (for PAI-2), 1 μg each of either PAI-1 (for tPA) or fibrinogen (for plasminogen) were utilized as positive ligand binding controls. All membranes were blocked by incubation with Tris-buffered saline containing Tween 20 (0.05%), 1 mM Ca^{2+}, pH 7.4 (TBST-Ca) and 10% fat-free milk for 30 minutes (min) at room temperature. After three washes with TBST-Ca, the membranes were incubated with various ligands and antibodies as follows. For direct binding interactions between the ligands (PAI-2, tPA or plasminogen) and the immobilized proteins, 100 nM PAI-2, tPA or plasminogen were incubated with the membranes for 1 hour (h) at room temperature in TBST-Ca/1% gelatin. After another three washes with TBST-Ca, the membranes were incubated with anti-PAI-2, anti-tPA or anti-plasminogen antibodies (1:5,000 dilution) in TBST-CA/10% fat-free milk. To account for potential non-specific interactions of the ligand detecting antibodies controls were conducted in which the ligands were omitted and the membranes incubated with the detecting antibodies alone. The detecting antibodies were incubated with the membranes for 1 h at room temperature, washed with TBST-Ca, then incubated with corresponding HRP-conjugated secondary antibody (1:5,000) in TBST-Ca/ 10% fat-free milk for 1 h at room temperature. After further extensive washing, membrane associated HRP activity was detected using enhanced chemiluminescence.

For co-binding of PAI-2 and tPA or PAI-2 and plasminogen to the immobilized AIIt, p36 and p11 proteins, 100 nM tPA or plasminogen were first incubated with the membranes in TBST-Ca/1% gelatin as above, washed, then incubated with 100 nM PAI-2 in TBST-Ca/1% gelatin for 1 h at room temperature. After washing, the membranes were incubated with primary antibodies for either plasminogen, tPA or PAI-2 in TBST-CA/10% fat-free milk, washed, incubated with appropriate HRP-conjugated secondary antibodies and developed using enhanced chemiluminescence. In some experiments immobilized tPA was inactivated by incubation with the specific proteinase inhibitor PPACK Dihydrochloride (1 mM) for 5 min at room temperature.

The lysine dependency of the interaction of tPA and plasminogen (and potentially PAI-2) with AIIt and its subunits was determined by performing the tPA, plasminogen or PAI-2 incubation steps in the absence or presence of 1 mM tranexamic acid, then directly assaying for the presence of the bound ligands with specific antibodies.

Surface plasmon resonance (SPR) analysis

Wild-type PAI-2, PAI-2 ΔCD-loop or tPA were immobilized to CM5 BIAcore chips (Melbourne, Australia) according to manufacturer's instructions. Briefly, the chip was activated using a 1:1 mixture of 0.2 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccimide. Ligands were coated onto the chips at 40 μg/ml in 10 mM sodium acetate (pH 3.0) to a level of ~10,000 response units. Unoccupied binding sites were blocked using 1 M ethanolamine, pH 8.5. The analyte, AIIt was diluted into running buffer [HEPES (10 mM) pH 8.0, NaCl (140 mM), $CaCl_2$ (1 mM) and 0.05% Tween-20] before applying to the BIAcore chip at 40 μl/min over 3 min. The affinity surface was regenerated between subsequent sample injections of AIIt using 100 mM H_3PO_4. All buffers were filtered and degassed before use and blank reference cells (activated and processed as above in the absence of ligand as controls for the contribution of the change of bulk refractive index) were run in parallel and subtracted from ligand binding sensograms. Attempts to immobilize AIIt gave high non-specific binding of ligand to the chips, as seen by the inability to properly elute non-covalently bound ligand before and after the deactivation step. Thus the SPR analyses could not be conducted with AIIt as the ligand.

Flow cytometry analysis of tPA-dependent binding of PAI-2 to the cell surface

The ability of PAI-2 to bind the cell surface in tPA-dependent manner was assayed as following. Adherent cells were detached by incubation with PBS/ 5 mM EDTA, pH 7.4 at 37°C for 5 min. After washing with ice-cold binding buffer (Phenol red free Hanks buffered salt solution, pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% BSA) the cells were resuspended in binding buffer at the concentration 10^6 cells/ml and aliquoted into round bottom 96-well plates, 200 μl/well, in triplicate. All the following manipulations were done on ice to maintain the integrity of interactions at the cell surface. The cells were incubated with 50 nM of active or PPACK-inactivated tPA for 30 min, washed and incubated with 100 nM PAI-2:Cy5 for 30 min. After washing with PBS cell surface associated Cy5 fluorescence was then analysed by dual colour flow cytometry with propidium iodide (PI) to distinguish between viable and non-viable cells as previously described (24, 25). This technique allows non-viable (PI positive) cells to be gated out so that potential intracellular p36 and/or p11 interactions are excluded from the analysis.

Cell-surface analysis of AIIt expression and co-localization with exogenous PAI-2

MCF-7 human breast and HeLa human cervical carcinoma cell lines were routinely cultured as previously described (24, 28). Cell-surface p36 and p11 expression was analyzed by indirect immunofluorescence staining using dual colour flow cytometry with propidium iodide (PI) to distinguish between viable and non-viable cells as described above. Co-localization studies were performed by confocal fluorescence microscopy essentially as previously described (24, 29), with the following modifications. Adherent MCF7 cells were washed with ice-cold binding buffer and incubated with 100 nM PAI-2:Cy5 for 1 h on ice. Following three washes with ice-cold binding buffer the cells were fixed with 3.75% paraformaldehyde for 15 min on ice. Cell surface p36 was detected by incubating the cells with 10 μg/ml anti-p36 polyclonal antibody followed by incubation with a 1:50 dilution of an anti-goat IgG-FITC secondary antibody. All samples were examined using a Leica confocal microscope and the images analyzed using TCS NT software, version 1.6.587 (Leica Microsystems GmbH, Wetzlar, Germany).

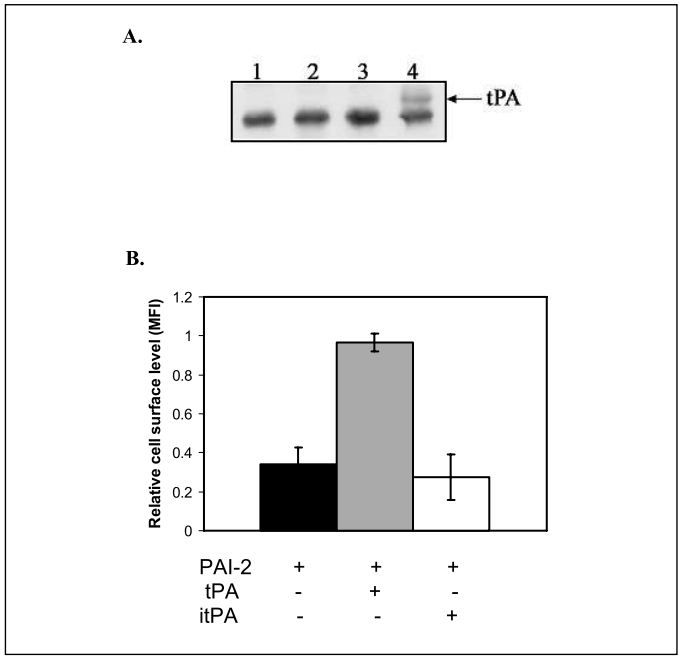

Figure 1: PAI-2 binds to cells in a tPA-dependent manner. A) tPA binds to the cell surface. HeLa cells were pre-incubated in the absence (Lanes 1 and 2) or presence of 50 nM tPA (Lanes 3 and 4) on ice, washed and then membrane associated proteins extracted. Following tPA specific immunoprecipitation (Lanes 2 and 4), protein extracts were analysed by non-reducing SDS-PAGE and Western blotting. As controls for Protein A sepharose non-specific binding, the protein extracts were incubated without antibodies (Lanes 1 and 3). B) tPA-dependent PAI-2 binding to the cell surface. HeLa cells were pre-incubated in the absence (black bar) or presence of 50 nM tPA active (grey bar) or PPACK-inactivated (itPA) (open bar) on ice, washed and then incubated with 100 nM PAI-2:Cy5. Cell surface-associated Cy5 fluorescence was then analysed by dual colour flow cytometry. Autofluorescence was subtracted from the data sets. Values shown are the mean fluorescence intensity (MFI) ± SEM of triplicate determinations from a representative experiment. Similar results were obtained for MCF-7 cells (data not shown).

Co-immunoprecipitation of cell AIIt components with surface associated tPA

Adherent HeLa cells were washed twice with ice cold binding buffer and incubated in the absence or presence of 50 nM tPA for 30 min on ice. The cells were washed three times with ice-cold binding buffer and membrane associated proteins extracted using the ProteoExtract Native Membrane Protein Extraction kit according to the protocol supplied by the manufacturer. Following extraction, samples were incubated with or without 1 μg/ml of polyclonal anti-tPA antibodies for 2 h on ice, after which Protein A sepharose beads were added and the mixtures incubated overnight at 4ºC. After washing the beads extensively with ice-cold PBS, the beads were boiled in non-reducing SDS-PAGE sample buffer and extracted proteins fractionated by electrophoresis. Following protein blotting, the membranes were incubated with primary antibodies for either tPA, p36 or p11 in TBST/5% fat-free milk, washed, incubated with appropriate HRP-conjugated secondary antibodies and developed using enhanced chemiluminescence.

Plasmin activity assay

The ability of PAI-2 to inhibit AIIt-bound tPA-dependent plasminogen activation in solution was performed essentially as described by Kwon et al. (22). Briefly, AIIt (1 μM) in 20 mM Tris/ 150 mM NaCl/ 1 mM Ca^{2+}, pH 7.4 (TBS-Ca) was preincubated with 10 nM tPA for 5 min at 25ºC. Then an equal volume of buffer containing 200 nM plasminogen/ 0.5 mM of the plasmin amidolytic substrate Spectrozyme PL/ TBS-Ca with or without 200 nM PAI-2 was added and colour development immediately recorded at 405 nm at 25ºC in a temperature-controlled plate-reader spectrophotometer (SpectraMax Plus, Molecular Devices, VIC, Australia). All assays were performed in triplicate. As controls, samples lacking plasminogen or tPA were used which were subtracted from all values.

A fibrin-based activity assay was performed as above except that human fibrin at final concentrations of 0.25, 0.5 and 0.75 mg/ml was used instead of AIIt. To confirm the ability of PPACK to inhibit tPA, the experiment was repeated with fibrin at 0.25 mg/ml in presence of 1 mM PPACK, Dihydrochloride.

The ability of PAI-2 to inhibit cell-bound tPA-dependent plasminogen activation was measured as follows. Cells were seeded at 15 x 10^3 cells/well in triplicate into 96-well plate 48 h prior the experiment. The cells were washed twice with ice-cold binding buffer (see above) then incubated in the absence or presence of 50 nM tPA in binding buffer for 30 min on ice. After two washes, 100 μl of binding buffer containing 200 nM plasminogen / 0.5 mM Spectrozyme PL +/- wild-type PAI-2 (50–500 nM) was added to the cells and colour development immediately recorded at 405 nm at 37ºC over time. Controls included no plasminogen (+/- 200 nM PAI-2) or no tPA pre-incubation. These gave identical background level values which were subtracted from all values.

Statistical analysis

Results are means ± standard error of the mean (SEM) of experiments performed in triplicate. The Student's t-test was used. P-values are $p<0.05$ unless otherwise indicated.

Results

PAI-2 regulates tPA-dependent plasmin activity on the cell surface – potential contribution of AIIt

The ability of PAI-2 to inhibit cell bound tPA was examined using HeLa and MCF-7 cells, as they have low to non-detectable levels of endogenous receptor-bound uPA or cell-associated tPA as assessed by flow cytometry (24) (and data not shown). This was confirmed by (i) the lack of measurable endogenous tPA in membrane fractions as determined by immunoprecipitation (Fig. 1A, lane 2), (ii) the very low levels of PAI-2 binding to the cell surface observed in the absence of exogenous activators (Fig. 1B), and (iii) by the absence of measurable intrinsic cellular plasmin activity (Fig. 2A, B). Pre-incubation with exogenous tPA led to measurable cell membrane associated tPA (Fig 1A, lane 4), caused a significant increase in cellular plasmin activity (Fig. 2A, B) in a dose-dependent manner (Fig. 2A, inset) and also increased subsequent cell-surface binding of exogenous PAI-2 (Fig. 1B). Preincubation with inactivated tPA did not cause an increase in cell-surface PAI-2 binding (Fig. 1B). This suggested

that PAI-2 formed inhibitory complexes with cell surface-bound tPA and indeed PAI-2 significantly inhibited cell-bound tPA-dependent plasmin formation in a dose-dependent manner (Fig. 2). Incubation with 500 nM PAI-2 completely or substantially inhibited tPA-induced plasmin activity on HeLa and MCF-7 cells, respectively (Fig. 2A, B). Significant inhibition was also observed on both cell lines using PAI-2 at equimolar or lower concentrations to plasminogen (i.e. 200 nM).

Having established the ability of PAI-2 to inhibit cell surface tPA, we then attempted to ascertain the potential tPA/plasminogen receptor(s) responsible. Previous studies showed that HeLa cells express components of AIIt (i.e. p36 and p11), a well characterized tPA/plasminogen co-receptor (22), and we confirmed the cell surface expression of p36 and p11 on HeLa cells (Fig. 3A, B). MCF-7 cells have very low/no cell surface p11 but very high levels of cell surface p36 (24) (Fig. 3C, D). Given that exogenous tPA bound to MCF-7 cells led to a substantial increase in plasmin activity (Fig. 2B), this implied that tPA may have been binding to the considerable amounts of p36 on these cells. However, attempts to block this interaction with a reported blocking p36 polyclonal antibody (30) on these or on HeLa cells were not successful (data not shown). Blocking experiments with anti-p11 polyclonal antibodies and HeLa cells resulted in only small (approximately 10%) reductions in tPA-dependent plasminogen activation. Attempts to co-immunoprecipitate AIIt components with cell surface bound tPA in experiments where tPA was successfully detected (see Fig. 1A), failed to detect either p11 or p36 (data not shown). Taken together these results suggest either a) inefficiency of the blocking antibodies and/or, b) AIIt and/or its components were not the major tPA/plasminogen receptors on these cells.

PAI-2 has been shown to interact with intracellular annexins as well as purified annexin II (p36) and AIIt (6). MCF-7 cells were thus also useful for determining whether PAI-2 interacted with cell surface p36 without the potential confounding effects of endogenous p11, or endogenous uPA/tPA, with which PAI-2 could form inhibitory complexes. As previously reported (24), using confocal microscopy we observed high density p36 distribution at the cell surface (Fig. 3E) but no p11 (data not shown) as expected from the flow cytometry results. When the cells were incubated with saturating amounts of PAI-2 (28) and then washed, only low-density PAI-2 binding was observed (Fig. 3E). Where PAI-2 binding was detected, it appeared to co-localize with p36. However, if PAI-2 interacted with cell surface p36 then the distribution of PAI-2 should have resembled that of p36 on these cells, which it clearly does not. The apparent areas of co-localisation may be due to secondary interactions that either stabilized PAI-2/p36 (e.g. within possible rare AIIt complexes that may exist on these cells) or were not related to p36 at all. The biochemical nature of the interaction between PAI-2/AIIt components is further addressed below.

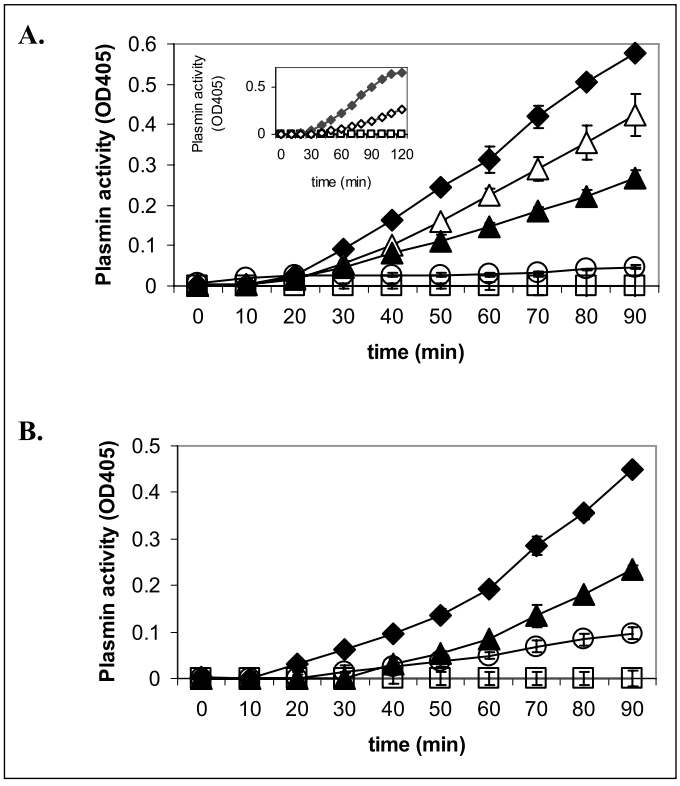

Figure 2: PAI-2 regulates cell surface tPA-dependent plasmin activity. HeLa (A) or MCF-7 (B) cells were pre-incubated with 50 nM tPA. After washing 200 nM plasminogen/0.5 mM Spectrozyme PL was added to the cells in the absence (filled diamonds) or presence of 50 nM (open triangles), 200 nM (filled triangle) or 500 nM (open circles) PAI-2 and the change in OD405 measured over time at 37°C. As a measure of intrinsic plasminogen activation activity, the cells were assayed in the presence of plasminogen without the tPA preincubation step (open squares). Background values were subtracted from all data sets. Values shown are the means ± SEM of triplicate determinations from a representative experiment. A, inset: Functional assay of dose-dependent tPA binding to the cell surface. HeLa cells were preincubated in the absence (open squares) or presence of 5 nM (open diamonds) or 50 nM (filled diamonds) tPA and tPA-dependent plasmin activation was monitored as above.

Characterization of PAI-2 binding to AIIt, p36 and p11 in the absence of tPA and plasminogen

In certain physiological settings, AIIt may represent the major tPA/plasminogen receptor on cells and PAI-2 may be able to inhibit cell surface AIIt-bound tPA activity by forming a classic inhibitory complex with bound tPA. However, given the known ability of PAI-2 to directly bind AIIt, it may interfere with the known AIIt/p11/plasminogen binding interaction and thus prevent tPA-dependent plasminogen activation via a non-serpin mechanism. To address this we characterized the interactions between AIIt subunits and PAI-2 in the absence and presence of tPA and plasminogen under the more controlled conditions of purified protein components. To this end we used non protein-denaturing systems, as detergents have been shown to disrupt the interaction of PAI-2 with placental cytosolic annexins (6). We first confirmed the ability of PAI-2 to interact with AIIt in the absence of tPA or plasminogen using ligand blotting in which native AIIt and its subunit proteins were immobilized onto a nitrocellulose membrane. PAI-2 bound to AIIt and to p11 but no binding was detectable under these conditions with p36 (Fig. 4A). Similar data was obtained using bovine AIIt and PAI-2 (untagged – data not shown). As expected, PAI-2 also bound to immobilized uPA but not BSA (Fig. 4A), suggesting that any binding interactions

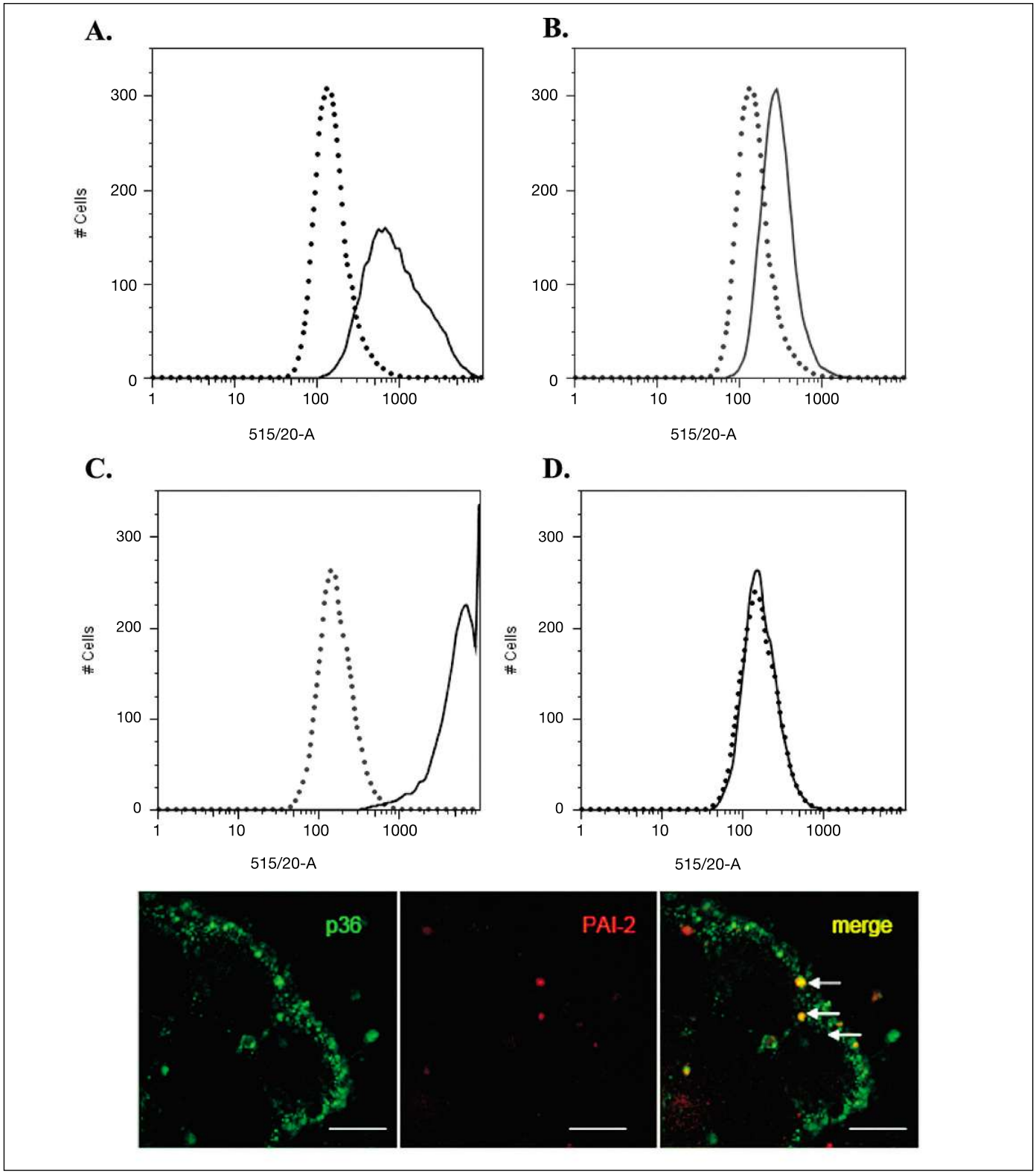

Figure 3: Cell-surface expression of AIIt subunits and potential co-localization with PAI-2. A-D) Cell-surface detection of p36 and p11. HeLa (A and B) and MCF-7 (C and D) cells were detached and incubated for 30 min on ice with 10–20 μg/ml of anti-p36 (C-16 pAb) (A and C), or anti-p11 (B and D) goat polyclonal antibodies (solid lines), or matched isotype control antibodies (dotted lines). Cells were then washed and incubated for 45 min on ice in the dark with a 1:200 dilution of FITC-conjugated rabbit anti-goat IgG secondary antibodies. All cells were then washed and cell surface associated FITC fluorescence analysed by dual colour flow cytometry. E) Adherent MCF-7 cells were incubated with anti-p36-cy3 antibodies (green) and PAI-2-cy5 (red) then analysed by confocal microscopy. White arrows highlight the minimal areas of PAI-2 and p36 colocalization (yellow) at the cell surface. Scale bar 10 μm.

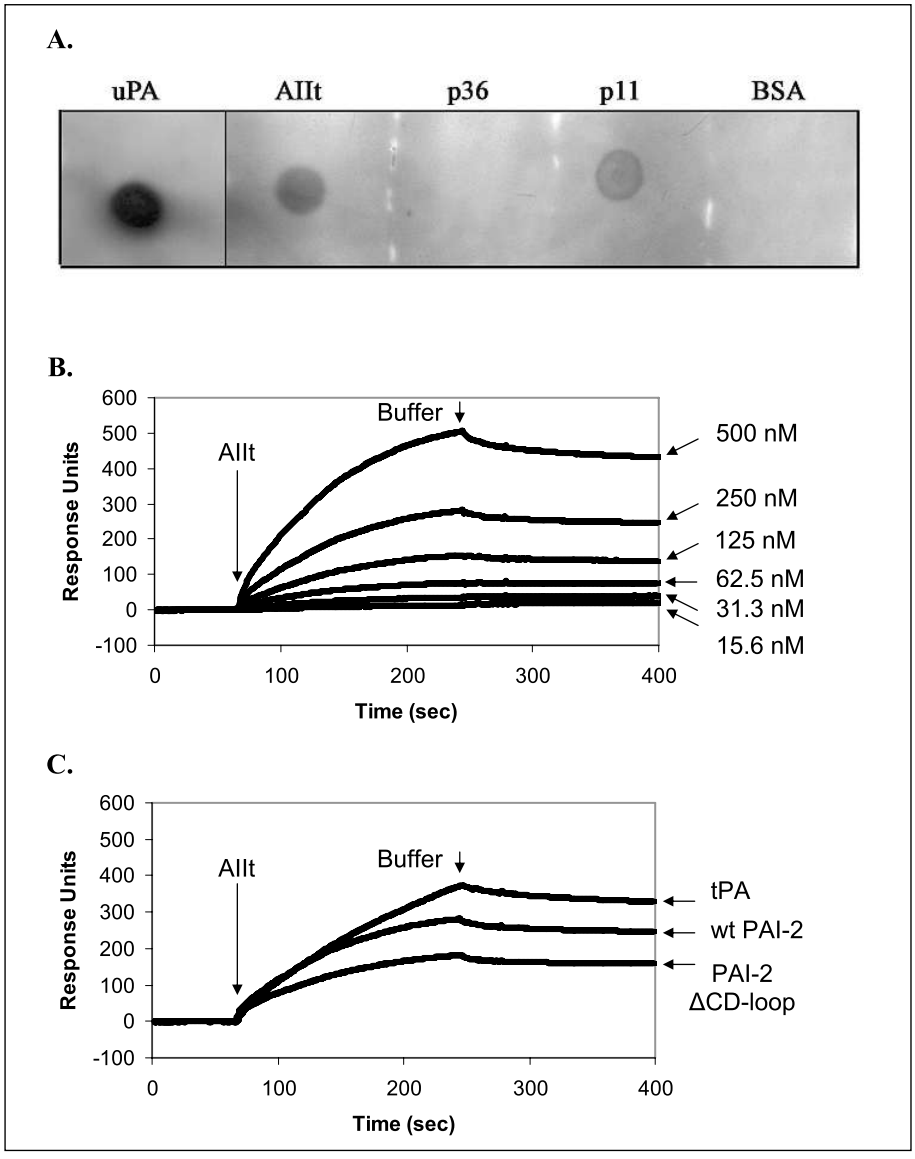

Figure 4: Characterization of the interaction between purified PAI-2 and AIIt.
A) Ligand dot blot analysis of the interaction between PAI-2 and AIIt, p36 and p11. Immobilized uPA (0.5 µg), AIIt, p36, p11 and BSA (1 µg each) were incubated with 100 nM PAI-2. Bound PAI-2 was immunodetected and visualized by enhanced chemiluminescence. B) Surface plasmon resonance (SPR) analysis of the interaction between AIIt and PAI-2. Sensorgrams show the interaction of a range of concentrations (500 – 15.625 nM) of the analyte AIIt with immobilized wild-type PAI-2 as the ligand. Arrows indicate the start and end of the sample injection (start of association and dissociation phases, respectively).
C) SPR analysis of the interaction between AIIt and PAI-2, PAI-2 ΔCD-loop or tPA. Sensorgrams show the interaction of the analyte AIIt (250 nM for the PAI-2 forms or 200 nM for tPA) with immobilized wild-type PAI-2, PAI-2 ΔCD-loop or tPA as the ligands. Arrows indicate the start and end of the sample injection.

observed were specific. Negligible non-specific binding of the detecting PAI-2 antibody to the immobilized proteins was observed when PAI-2 was omitted (see Fig. 5D) further confirming the specificity of the PAI-2/AIIt (and uPA) interaction. SPR analysis showed that the binding of AIIt to immobilized PAI-2 was dose-dependent (Fig. 4B). The association of AIIt with PAI-2 was moderately slow and equilibrium binding was not observed under these experimental conditions (as visualized by the contours of the apparent steady-state phase – between arrows, Fig. 4B). Furthermore, dissociation appeared to be mediated by an initial brief but fast and second very slow dissociation event, suggestive of a heterogenous or multi-site interaction between the two proteins. Indeed, PAI-2 could potentially interact with both p11 subunits within AIIt. Specificity analysis using overlay plots of sensograms showing interaction between immobilized wild-type and ΔCD-loop mutant forms of PAI-2 indicated that AIIt bound somewhat more strongly to the wild-type than the ΔCD-loop form of PAI-2 (Fig. 4C). Stronger binding of AIIt to tPA than to PAI-2 was also revealed (Fig. 4C). The CD-loop therefore contributes to the interaction of PAI-2 with AIIt, as previously suggested (6) but another component within PAI-2 must also be involved. Thus, given the potential complexity of the interaction of PAI-2 with AIIt as implied from both these ligand blot and the SPR sensogram results, further kinetic analyses were not pursued.

Characterization of PAI-2 binding to AIIt, p36 and p11 in the presence of tPA and plasminogen

We analysed the ability of PAI-2 to interact with AIIt in the presence of tPA or plasminogen. Both plasminogen and tPA bind to the C-terminal lysines of p11 within AIIt via lysine-binding sites within their kringle domains (22). Specific binding of radiolabeled tPA to annexin II (p36) has also been reported (23). We confirmed that tPA and plasminogen bound to AIIt/p11 (Fig. 5A, B) and that this was lysine-dependent, as these interactions were inhibited in the presence of the lysine analogue tranexamic acid

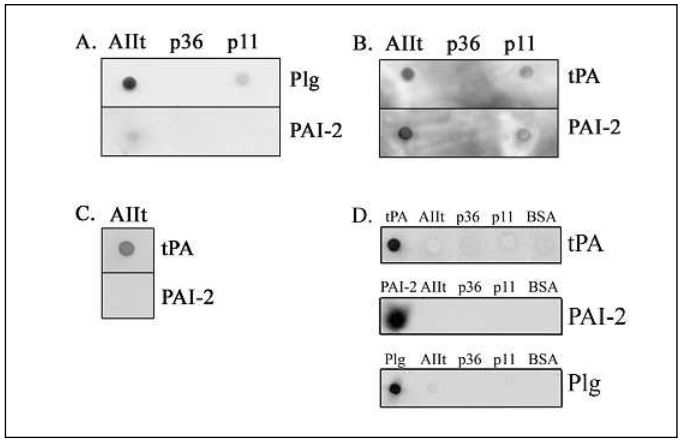

Figure 5: Ligand dot blot analysis and functional outcome of the ability of AIIt and its subunits to act as co-receptor of PAI-2 and tPA or plasminogen. Immobilized AIIt, p36 and p11 (1 μg each) were first incubated with 100 nM plasminogen (A), 100 nM active tPA (B) or 100 nM PPACK-inactivated tPA (C) followed by incubation with 100 nM PAI-2. After that individual membrane strips were tested for the presence of plasminogen (A, upper panel), tPA (B and C, upper panels) or PAI-2 (A, B and C; lower panels) using specific antibodies for the proteins as shown to the right of each panel as visualized by enhanced chemiluminescence. D) Additional control blots confirming the specificity of the ligand/AIIt interactions. Membrane strips also dotted with 1 μg each tPA, PAI-2 or plasminogen (as positive antibody detection controls) were incubated without the ligand-binding step but with anti-tPA, anti-PAI-2, or anti-plasminogen antibodies, respectively.

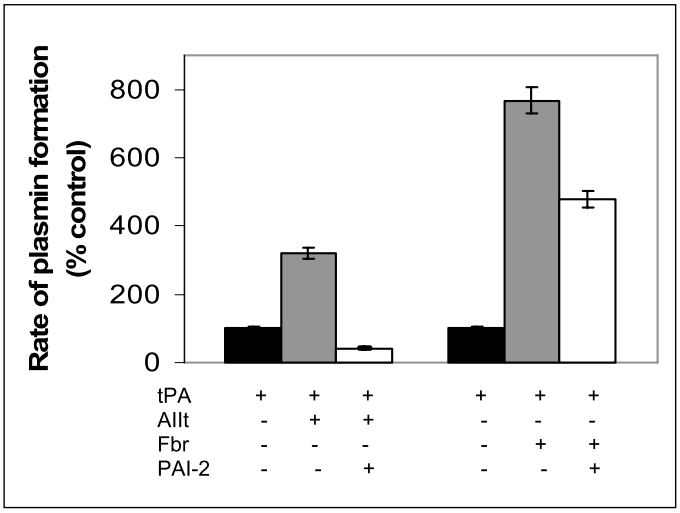

Figure 6: Regulation of tPA-dependent plasmin activity by PAI-2 in the presence of AIIt or fibrinogen (Fbr). Bovine AIIt (1 μM) and human fibrinogen (0.25 mg/ml) were prebound with tPA (10 nM) after which an equal volume of 200 nM plasminogen/0.5 mM Spectrozyme PL was added in the absence (gray bar) or presence of 200 nM PAI-2 (unfilled bar) and the rate (i.e. change in OD405 over the linear part of the plasmin activation curve) of plasmin formation was determined. tPA-mediated plasminogen activation in the absence of AIIt and fibrinogen is also shown (black bar) and represent 100% activity (control). Values shown are means of triplicate determinations ± SEM with blanks subtracted.

(data not shown). However, no binding with either ligand was observed with p36 under the conditions used in our experiments (Fig. 5A, B), which may not be of sufficient sensitivity to detect these particular interactions and/or may require limited proteolysis of p36 for enhanced binding.

As PAI-2 did not bind to AIIt or isolated p11 in a lysine-dependent manner (data not shown), we expected that AIIt could act as a co-receptor for PAI-2 and plasminogen and/or form complexes with bound tPA. To this end we employed the ligand blotting technique with immobilized native AIIt, p36 and p11 and sequential incubation steps with either a) plasminogen then PAI-2, or b) tPA then PAI-2, after which the various bound ligands were detected with antibodies (Fig. 5A-C). Fibrinogen and PAI-1 were also blotted onto the same membranes as positive ligand-binding controls for plasminogen and tPA, respectively (data not shown). Negligible non-specific binding of the detecting antibodies was observed when the ligands were omitted (Fig. 5D), confirming the specificity of the binding interactions observed. Firstly, we tested whether PAI-2 could either co-bind or displace plasminogen pre-bound to immobilized AIIt and p11 (Fig. 5A). When these blots were assayed for the presence of plasminogen (Fig. 5A, upper panel) or PAI-2 (Fig. 5A, lower panel), plasminogen remained bound to AIIt and p11, while PAI-2 at best very weakly co-bound with plasminogen to AIIt but not at all to p11. Neither PAI-2 nor plasminogen bound to p36 (Fig. 5A). This suggested that AIIt or p11 alone cannot act as a co-receptor for plasminogen and PAI-2.

We next tested the ability of tPA and PAI-2 to concurrently bind immobilized AIIt and p11 (Fig. 5B). When tPA was pre-bound to the blots and then incubated with PAI-2, tPA was detected (Fig. 5B, upper panel) indicating that it remained bound to AIIt and p11. PAI-2 was also detected (Fig. 5B, lower panel). As PAI-2 was unable to bind to AIIt-bound inactivated tPA (Fig. 5C), it most likely formed complexes with AIIt/p11-bound tPA, rather than co-binding with tPA.

When we repeated these experiments except reversing the plasminogen or tPA and PAI-2 incubation steps, again none of the ligands were detected bound to p36, while tPA or plasminogen appeared to displace the pre-bound PAI-2 as only tPA and plasminogen were detected bound to AIIt or p11 (data not shown). The fact that in this case PAI-2 was not co-detected with tPA suggests that free tPA (which is in large excess compared to the bound ligands) displaced PAI-2 and/or possible PAI-2/tPA complexes formed on the blot. These results further confirmed that AIIt/p11 cannot act as a co-receptor for tPA/plasminogen and PAI-2.

Given the ability of PAI-2 to form a complex with AIIt-bound tPA, we next determined the capacity of PAI-2 to inhibit tPA-mediated plasminogen activation in the absence and presence of AIIt. Studies performed with p36 or p11 showed negligible or low plasmin activities, respectively (data not shown), as would be expected from the apparently negligible/lower binding interactions between tPA/plasminogen and p36 or p11 compared to AIIt. When tPA was pre-incubated with AIIt, however, there was a significant enhancement in the rate of plasmin formation, which was significantly inhibited by 100 nM PAI-2 to levels well below the activity in the absence of AIIt (Fig. 6). This confirmed that PAI-2 can regulate tPA-mediated plasmin formation, most

likely via formation of a stable inhibitory complex with AIIt-bound tPA. In comparison, in the presence of fibrin and 100 nM PAI-2 there was an attenuation of inhibition of tPA activity, but this was still significantly above the activity seen in the absence of fibrin (Fig. 6). This confirmed that PAI-2 was not an efficient inhibitor of fibrin-bound tPA activity, which is in line with previous observations showing that fibrin has known tPA-protective functions (7).

Discussion

The accumulated knowledge to date regarding PAI-2 supports the notion that PAI-2 is a multi-functional, bi-topological protein with distinct intracellular and extracellular roles (14). In this study we show PAI-2 is also an effective inhibitor of tPA activity when associated with the cell surface. Further, as AIIt is a known cell surface receptor for tPA/plasminogen and a binding partner for PAI-2 we also characterized these interactions. While PAI-2 can directly bind AIIt via p11, it does not act as co-receptor for PAI-2 and tPA/plasminogen but rather inhibits AIIt- and cell-bound tPA via its serpin activity to prevent plasmin formation. PAI-2 bound directly to AIIt via the p11 subunit, although the presence of p36 might stabilize this interaction within the hetero-tetramer as we observed an apparently stronger interaction between PAI-2 and AIIt compared with p11 alone (see Fig. 4A). This phenomenon was also observed for the binding interaction between tPA and plasminogen and is a notion not without precedent. For example, the affinity and plasminogen activation potential of tPA and plasminogen for the AIIt complex is stronger than for the p11 subunit alone (31). Furthermore, PAI-2 binding to AIIt appears to be mediated by its CD-loop and at least another site. All of these factors point to potential multi-site interactions between AIIt and PAI-2, as reflected in the complicated binding kinetics suggested by SPR analysis.

The physiological role of this direct AIIt-PAI-2 interaction still remains unclear. Jensen et al. (6) showed that PAI-2 binds to placentally-derived cytosolic annexins and to purified AIIt and bovine p36. Due to its inefficient secretion signal a large proportion of expressed PAI-2 remains intracellular and non-uPA/tPA inhibitory functions have been attributed to this pool of PAI-2 (14). Given that AIIt/p36 has been implicated as an intracellular protein in endocytosis and exocytosis (32), it may be possible that in the cytosol where tPA and plasminogen are absent that PAI-2 directly binds AIIt and affects these AIIt related functions. A potential reason for our conflicting data regarding lack of direct binding of PAI-2 to p36 may be that Jensen et al. (6) used radiolabeled PAI-2, which may increase the sensitivity of detection compared to the methods used in our study. These authors did not show data comparing the PAI-2 binding affinity of p36 versus AIIt. Regardless, a direct AIIt/PAI-2 interaction does occur and requires further physiological characterization.

Our data suggests that AIIt was just one of the many potential cell-surface tPA receptors as the inhibition of tPA-mediated plasmin generation by PAI-2 was also AIIt/p11-independent if p11 was absent from the cell surface. There are a number of potential cell-surface receptors other than AIIt including actin (33), amphoterin (34, 35) human type-II transmembrane protein p63 (CKAP4) (36); α-enolase (37, 38) and cytokeratin 8/18 (39, 40). Furthermore, a number of low-affinity but high-density non-specific binding sites on the cell surface have been reported (20, 21, 41). The presence of many possible tPA receptors and non-specific binding sites that may exist on the surface of the cells used in our study made it extremely difficult to attribute cell surface tPA inhibition entirely/specifically to AIIt-mediated activity. Hence, attempts to assess the contribution of cell surface AIIt to tPA binding/activation and its inhibition by PAI-2 were not possible in these models. There are several cell lines that have reported high AIIt on the cell surface including HT1080 and HUVECs (22). However, not only is there clonal variation in expression of AIIt by these cells but they also express PAI-1, PAI-2, uPA or tPA (42, 43). This could interfere with analyses of interactions between exogenous PAI-2 and tPA, and were therefore considered unsuitable for our studies. Attempts to co-immunoprecipitate AIIt with tPA pre-bound to the cell surface were unsuccessful (unpublished observations), also suggesting that AIIt is at best one of the potentially many tPA/plasminogen receptors – at least under our conditions. Importantly, published studies reporting immunoprecipitation experiments using whole cell lysates may not quantitatively reflect the cell surface situation, especially given that AIIt is also found intracellularly. Our studies were always performed under conditions that maximized cell viability and/or excluded non-viable cells so as to ensure cell surface measurements only. Other methods to confirm the interaction between cellular AIIt/p11– and PAI-2-mediated inhibition of tPA could utilize modulation of p11 cell surface expression via siRNA or transfection but there are significant complicating factors. For example, silencing of either p11 and p36 in HeLa cells in order to test the function of AIIt in the structural organization and dynamics of endosomal membranes affected endosome trafficking (44) which may impact on other potential tPA/plasminogen receptors on the cell surface.

Regardless of the nature of the cell surface tPA receptor, PAI-2 clearly inhibits surface-bound tPA *in vitro*. For PAI-2 to be a physiological regulator of cell-bound tPA activity *in vivo* there must be elevated PAI-2 levels in peri/extracellular spaces at sufficient concentrations. As discussed previously, PAI-2 is a stress-response protein, and this can lead to increased blood (during late pregnancy) and local tissue levels of extracellular PAI-2. For example, there is a strong correlation between tPA and PAI-2 elevated levels in gingival crevicular fluid during inflammation (16, 45), where tPA and PAI-2 exist as high-molecular-weight SDS-stable complexes – a good indication of inhibitory complex formation (19). The concentrations of PAI-2 in gingival pockets in fact reach up to 5 μg/ml (16, 19) (~ 100 nM), providing an example of how high local concentrations of PAI-2 might exist in tissue microenvironments. Whether PAI-2 inhibits cell-associated tPA in these and other tissue microenvironments has not yet been addressed, but since cell-bound tPA-mediated plasmin activation was significantly inhibited by 50 and 200 nM PAI-2 in our study, this suggests that PAI-2 could possibly inhibit cell-bound tPA in such specialized micro-environments. While the concentrations of PAI-2 reached in plasma during late pregnancy (250 ng/ml, ~ 5 nM) may not be sufficient to efficiently inhibit tPA, a potential role of elevated blood PAI-2 in both fibrinolytic and non-fibrinolytic tPA-mediated events cannot not be excluded yet. In terms of potential cell-surface interactions, components

of AIIt, tPA and PAI-2 might coexist in the brain. For example, S100A10 (p11), annexin II (p36) and tPA are all present in the brain and believed to play an important role for brain function (17, 46–48). Importantly, PAI-2 is over-expressed immunohistochemically after brain damage (49) and found in the brains of Alzheimer's disease patients (50). While neuroserpin (SERPINI1) and tPA co-localize in the brain, which has led to suggestions that neuroserpin is the selective inhibitor of tPA in this organ (48), it is possible that PAI-2 may also participate in regulation of tPA activity during brain damage.

In conclusion, as PAI-2 is able to regulate tPA-induced plasmin activity *in vitro* in both an AIIt -dependent and -independent manner, PAI-2 should be further considered as a member of the cohort of proteins controlling tPA-induced plasmin activity at the cell surface. Recent observations of co-localization of PAI-2/tPA complexes in the fluids of inflamed tissues, and our results reported here, open new possibilities for investigations to further explore the complex mechanism of regulation for plasmin in-site generation *in vivo*.

Acknowledgements

The authors wish to thank Professor T. Ny (Umea University, Sweden) for the generous gift of wt PAI-2 and PAI-2 ΔCD-loop expression vectors, Professor D. Waisman (University of Calgary, Canada) for the generous gift of purified AIIt and its components, and Dr P. Navarro (Unitat de Biologia Cellular i Molecular, Barcelona, Spain) for the generous gift of the annexin II antiserum. M. Ranson and D.N. Saunders are Cancer Institute NSW Research Fellows, and D.R. Croucher was a Cancer Institute NSW Research Scholar. We also thank Dr B. Kinnby (School of Dentistry, Malmö, Sweden) for fruitful discussions.

Abbreviations

PAI-2, plasminogen activator inhibitor type 2; serpin, serine protease inhibitor; tPA, tissue type plasminogen activator; uPA, urokinase plasminogen activator; AIIt, annexin II heterotetramer; CD-loop, loop connecting helices C and D in PAI-2; SPR, surface plasmon resonance; BSA, bovine serum albumin; PPACK, D-Phe-Pro-Arg chloromethylketone.

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