Plasminogen activator inhibitor 1 is an intracellular inhibitor of furin proprotein convertase

Denis Bernot¹, Jimmy Stalin¹, Pierre Stocker², Bernadette Bonardo¹, Ilse Scroyen¹, Marie-Christine Alessi¹ and Franck Peiretti¹,*

¹Inserm, U626, Université de la Méditerranée, Faculté de Médecine, 27 Boulevard Jean Moulin, 13385 Marseilles Cedex 5, France
²Université Paul Cézanne, Equipe Biosciences iSm2 UMR 6263, FST St Jérome, case 342 13397, Marseilles Cedex 20, France
*Author for correspondence (Franck.Peiretti@univmed.fr)

Accepted 26 November 2010
Journal of Cell Science 124, 1224-1230
© 2011. Published by The Company of Biologists Ltd
doi:10.1242/jcs.079889

Summary
Proprotein convertases (PCs) are a family of serine proteases that are involved in the post-translational processing and activation of a wide range of regulatory proteins. The upstream role of PCs in the control of many physiological and pathological processes generates a growing interest in understanding their regulation. Here, we demonstrate that the serine protease inhibitor plasminogen activator inhibitor 1 (PAI-1) forms an SDS-stable complex with the PC furin, which leads to the inhibition of the intra-Golgi activity of furin. It is known that elevated PAI-1 plasma levels are correlated with the occurrence of the metabolic syndrome and type 2 diabetes, and we show that PAI-1 reduces the furin-dependent maturation and activity of the insulin receptor and ADAM17: two proteins involved in the onset of these metabolic disorders. In addition to demonstrating that PAI-1 is an intracellular inhibitor of furin, this study also provides arguments in favor of an active role for PAI-1 in the development of metabolic disorders.

Key words: ADAM17, Furin, Golgi, Insulin receptor, PAI-1, Convertase

Introduction
The proprotein convertase (PC) family, a class of subtilisin-kexin-like Ca²⁺-dependent serine endoproteases, is responsible for the cleavage of numerous precursor proteins in mammalian cells (Seidah and Chretien, 1999). Processing by PCs is a crucial step in the formation of the biologically active form of proteins synthesized through the secretory pathway. Furin was the first mammalian PC identified (Roebroek et al., 1986; van de Ven et al., 1990) and has been the most extensively studied. Furin is expressed ubiquitously and cleaves proteins C-terminally to a typical basic amino acid sequence [Arg-Xaa-(Lys/Arg)-Arg] (Thomas, 2002). It is a transmembrane protease predominantly localized in the trans Golgi network but it can cycle between the trans Golgi network and the cell surface (Molloy et al., 1999). Furin is regulated at the level of its expression (Guimont et al., 2007; Laprise et al., 2002) and maturation (Anderson et al., 1997; Gawlik et al., 2009). A direct inhibition of furin activity, through association with the cytosolic serine protease inhibitor (serpin) proteinase inhibitor 8, has also been described in human activated platelet releasates (Leblond et al., 2006).

Because of its inhibitory action on plasminogen activators, plasminogen activator inhibitor 1 (PAI-1) is considered to be a major regulator of fibrolysis. However, PAI-1 is potentially able to inhibit several other serine proteases; this inhibition can vary from being marginal to being physiologically relevant, owing to a variable affinity of PAI-1 with its target (Keijer et al., 1991; Olson et al., 2001). PAI-1 is a secreted serpin and, as such, could inhibit serine proteases in the secretory pathway and in the extracellular milieu.

We investigated whether PAI-1 could be a potent inhibitor of furin, and here we provide evidence that it associates with furin and inhibits its activity in the Golgi complex of human cells. Because of the wide variety of furin substrates, this regulation could impact upon many cellular processes. However, given the explanatory potential of a hypothetical diabetogenic mechanism involving the inhibition of PCs by PAI-1 (Griffiths and Grainger, 2006), we analyzed the effect of PAI-1 on the furin-dependent maturation of the insulin receptor (IR) and ‘a disintegrin and metalloproteinase 17’ (ADAM17) (Peiretti et al., 2003; Robertson et al., 1993; Srour et al., 2003; Williams et al., 1990) because both of these proteins are crucially involved in the onset of diabetes. Herein, we show that PAI-1 reduces the maturation of IR and ADAM17, indicating that PAI-1 could be actively involved in the development of insulin resistance.

Results
In vitro inhibition of recombinant furin by PAI-1
The efficacy of PAI-1 to inhibit furin activity in vitro was compared with that of the potent furin inhibitor α1-antitrypsin Portland (α1-PDX), a genetically bioengineered form of serpin A1 (α1-antitrypsin). The concentration of PAI-1 needed for half-maximal furin inhibition was determined to be 15-fold that of α1-PDX (IC₅₀=480 nM compared with IC₅₀=32 nM) (Fig. 1A).

It has been reported that inhibition of furin by α1-PDX and inhibition of plasminogen activators by PAI-1 both involve the formation of covalent SDS-stable complexes (Jean et al., 1998; Levin, 1983; Thorsen et al., 1988). We examined whether such a complex was formed between furin and PAI-1 in vitro with recombinant proteins. Furin and PAI-1 were detected by immunoblotting at their expected molecular masses (~130 kDa) was detected with the anti-furin antibody and, less efficiently, with the anti-PAI-1 antibody (Fig. 1B), suggesting that it results from the association of furin with PAI-1 in a SDS-stable covalent complex. Furthermore, the molecular mass of this protein
complex is in accordance with the sum of the furin and PAI-1 molecular masses. Addition of the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (cmk) prevented the formation of the complex, suggesting that furin activity is necessary for the complex formation.

**Interaction of cell-expressed PAI-1 and furin**

PAI-1 and furin were overexpressed in HeLa cells, and the ability of these two proteins to stably associate was analyzed by immunoblotting. Overexpressed furin was detected as a doublet of ~80–85 kDa, corresponding to the pro- and mature-form of the enzyme (Leduc et al., 1992), and PAI-1 was detected at 45 kDa (Fig. 2A). When both proteins were overexpressed together, a higher molecular mass (120–130 kDa) protein complex was clearly detected with the anti-furin antibody. Detection of the complex with the anti-PAI-1 antibody was possible but relatively ineffective (supplementary material Fig. S1). The formation of this complex appeared as a doublet when total-cell lysates were used for immunoblotting analysis (supplementary material Fig. S2), but the slower migrating band of this doublet was the major form detected in glycoprotein preparations, suggesting that the doublet comprises differently glycosylated forms of the two proteins. However, the differentially glycosylated forms of the complex can still be separated into two distinct forms, possibly representing the complex containing cleaved and uncleaved PAI-1, as has been described for the complex between PAI-1 and tissue plasminogen activator (tPA) (Yamasita et al., 2006).

The complex appeared as a doublet when total-cell lysates were used for immunoblotting analysis (supplementary material Fig. S2), but the slower migrating band of this doublet was the major form detected in glycoprotein preparations, suggesting that the doublet comprises differently glycosylated forms of the two proteins. However, the differentially glycosylated forms of the complex can still be separated into two distinct forms, possibly representing the complex containing cleaved and uncleaved PAI-1, as has been described for the complex between PAI-1 and tissue plasminogen activator (tPA) (Yamasita et al., 2006).

Because hepatocytes are considered a major source of PAI-1 (Konkle et al., 1992), the association between endogenous furin and PAI-1 was studied in the HepG2 hepatocyte cell line. Stimulation of PAI-1 expression by phorbol 12-myristate 13-acetate (PMA) resulted in an increased amount of the PAI-1–furin complex, as judged by immunoblotting and heterodimer immunoassay analysis (Fig. 2B). Knocking down either PAI-1 or furin expression by small interfering RNA (siRNA) led to a decrease in the amount of the detected complex. These results indicate that endogenous PAI-1 and furin can indeed form a complex.

**Intracellular interaction of PAI-1 and furin**

We analyzed the intracellular interaction of PAI-1 and furin in the human LoVo cell line, which lacks furin (Takahashi et al., 1993) and poorly expresses PAI-1. Immunoblotting and heterodimer immunoassay analysis confirmed the above results: concomitant expression of PAI-1 and furin led to their association in a higher molecular mass complex (Fig. 3A). Considering that furin is mainly present in the Golgi network and at the plasma membrane, and that
PAI-1 is a secreted protein, we chose to determine the cellular compartment in which PAI-1 and furin associate. When furin-overexpressing LoVo cells were incubated with recombinant wild-type PAI-1 (Fig. 3B) or stabilized PAI-1 (data not shown), the PAI-1–furin complex was not detected in cell lysates. In addition, when PAI-1-overexpressing LoVo cells were co-cultured with furin-overexpressing cells, we did not find any PAI-1–furin complex (Fig. 3C), indicating that PAI-1 released into the culture medium does not associate with furin. Immunofluorescence microscopy revealed that furin colocalized with the Golgi marker golgin-97 (Fig. 4), which is in accordance with previously reported observations indicating that PAI-1 and furin are localized in the Golgi complex (Ma et al., 2006; Shapiro et al., 1997). Our data suggest that interaction of furin with PAI-1 takes place in the Golgi complex.

Intra-Golgi inhibition of furin by PAI-1

We next investigated whether the association of PAI-1 with furin actually resulted in reduction of its enzymatic activity. To monitor intra-Golgi furin activity, we transfected HeLa cells with the GRAPfurin reporter construct, which encodes an alkaline phosphatase (AP) linked to a Golgi retention signal by the typical furin-cleavage sequence Arg-Gln-Lys-Arg (Coppola et al., 2007). AP activity was detected in conditioned culture medium from GRAPfurin-transfected cells, reflecting endogenous intra-Golgi furin activity (Fig. 5A). As expected, AP activity was increased by furin overexpression (+49%) and almost completely abrogated by the addition of the furin inhibitor cmk. The activity of endogenous furin was significantly, and comparably, inhibited by the wild-type and a stabilized form of PAI-1 (~40%; \( P<0.05 \)). To gauge approximately the efficiency of the intracellular PAI-1 inhibition of furin, we compared its effect with that of \( \alpha_1 \)-PDX; this comparison can be considered reasonable because overexpressed PAI-1 and \( \alpha_1 \)-PDX are necessarily present in large excess over the endogenous furin. The result shows that \( \alpha_1 \)-PDX was approximately twice as effective in inhibiting furin intracellularly compared with PAI-1 (Fig. 5A), suggesting that the efficiency of the PAI-1 inhibition of furin is substantial. Furthermore, overexpression of serpin C1 (antithrombin III) did not inhibit furin (Fig. 5A and inset), indicating that overexpression of a secreted serpin does not inevitably lead to intracellular furin inhibition.

In HepG2 cells, in which we identified an endogenous PAI-1–furin complex (Fig. 2B), the furin activity was significantly increased by knocking down the expression of PAI-1 (Fig. 5B). This result indicates that, in this cell line, endogenous PAI-1 represses furin activity, emphasizing the physiological relevance of the inhibition of furin by PAI-1.

Inhibition of furin-dependent processing by PAI-1

To consider PAI-1 an important regulator of furin, its expression should sufficiently reduce the furin-dependent maturation of physiological substrates such that cellular responses are altered. We, therefore, analyzed the effect of PAI-1 on the furin-dependent maturation of the IR and ADAM17.

Consistent with their furin-deficient phenotype, LoVo cells exhibited poor endogenous IR maturation (Fig. 6A). Furin
overexpression greatly increased the amount of endogenous mature IR, and concomitant expression of PAI-1 reduced this increase (Fig. 6A), confirming that PAI-1 reduces IR maturation by inhibiting furin. As expected, the PAI-1-dependent reduction in IR maturation decreased the amount of phosphorylated IR and Akt measured after insulin stimulation. However, in furin-overexpressing LoVo cells, the insulin-stimulated increase in the phosphorylation of IR was somewhat marginal (+20%). Therefore, to demonstrate clearly the ability of PAI-1 to interfere with IR phosphorylation and insulin signaling, the experiment was repeated in HeLa cells overexpressing human IR. Overexpression of PAI-1 greatly reduced the maturation of coexpressed IR, which strongly reduced the amount of phosphorylated IR (~73%) and the phosphorylation of endogenous Akt, following stimulation by insulin (Fig. 6B). These results show that PAI-1, by inhibiting the furin-dependent maturation of IR, alters insulin signaling.

The mature form of ADAM17 was modestly represented in LoVo cells (Fig. 7A) when compared with that of its proform. Furin overexpression increased the maturation of ADAM17, and PAI-1 expression diminished this effect. To assess ADAM17 activity, we measured the accumulation of TNF-R2 (tumor necrosis factor receptor 2; also known as TNFRSF1B) in the culture medium, as this results from an ADAM17-dependent shedding process (Reddy et al., 2000; Solomon et al., 1999). Soluble TNF-R2 was detected in the culture medium of LoVo cells upon overexpression of TNF-R2 (Fig. 7B) and this release was not altered by coexpression of PAI-1. Furin overexpression drastically increased TNF-R2 release, and concomitant expression of furin and PAI-1 significantly reduced TNF-R2 shedding when compared with that caused by furin alone (~27%; P<0.05). To ensure that the observed effect was due to an alteration of a shedding process, we overexpressed a spontaneously secreted form of TNF-R2 (TNF-R2mut) and observed that neither furin nor PAI-1 modified the ADAM17-unrelated continuous release of TNF-R2mut (Fig. 7C).

Discussion

Our results demonstrate that PAI-1 inhibits furin in the secretory pathway. Both proteins colocalize in the Golgi, where furin is predominantly, if not exclusively, localized (Gawlik et al., 2010) and where PAI-1 is detected when its expression increases (Ma et al., 2006). Association of PAI-1 with furin leads to the formation of a higher molecular mass SDS-stable complex and a decrease in intra-Golgi furin-dependent maturation processes. This is the first demonstration of an intracellular regulation of furin activity by a physiological serpin in mammalian cells. However, serpin-mediated inhibition of furin has been suggested previously; indeed, the best-characterized furin inhibitor is a genetically engineered variant of α1-antitrypsin (α1-PDX). α1-PDX forms a stable complex with furin and inhibits processing of precursors within the constitutive secretory pathway (Benjannet et al., 1997). We evaluated that the
none of these serpins possess the minimal furin cleavage site represented by arginine residues at positions P1 and P4 [notation of Schechter and Berger (Schechter and Berger, 1967)] (supplementary material Table S1). However, PAI-1 and some serpins possess an arginine residue at position P1, which is mandatory, but not sufficient, for an efficient cleavage by furin. Indeed, α1-antitrypsin (serpin A1) does not inhibit furin, whereas its variant form with an arginine at position P1 (α1-antitrypsin Pittsburg) inhibits furin (Oda et al., 1992; Wasley et al., 1993). However, introduction of an arginine residue into position P4 of α1-antitrypsin Pittsburg (which gives α1-PDX) tremendously increases the inhibition efficiency (Anderson et al., 1993). Among the serpins with an arginine residue at position P1, which are most similar to PAI-1, are serpin E2 (protease nexin I) and serpin C1 (antithrombin III). As PAI-1 and serpin E2 both inhibit plasminogen activators it is probable that serpin E2 also inhibits furin. However, results obtained with the GRAPfurin assay indicate that serpin C1 does not inhibit intracellular furin, which suggests that other elements, in addition to the arginine residue at position P1, have to be considered when explaining why a serpin can inhibit furin. In line with this suggestion, it was shown that serpin C1 only becomes a furin inhibitor upon activation by heparin (Brennan and Nakayama, 1994), which involves modification of the conformation of its reactive-site-loop (Langdown et al., 2004).

As an acute-phase protein, PAI-1 is rapidly produced and released by cells in a number of clinical situations. Consequently, a potential furin-dependent role for PAI-1 should be studied in pathologic contexts in which PAI-1 synthesis is increased. The circulating levels of PAI-1 are strongly correlated with the metabolic syndrome (Alessi and Juhan-Vague, 2008; Alessi et al., 2007), a cluster of defined metabolic disorders predisposing individuals to cardiovascular disease and type 2 diabetes. Recently, a hypothetical diabetogenic mechanism involving the inhibition of PCs by PAI-1 was suggested (Griffiths and Grainger, 2006), and our present results, showing that PAI-1 is a furin inhibitor, led us to study the effect of PAI-1 on the maturation of IR and ADAM17, two furin substrates crucially implicated in the onset of the metabolic syndrome. We demonstrated that PAI-1 inhibits furin-dependent processing of IR, which reduces the amount of phosphorylated IR and, hence, subsequent Akt phosphorylation. Consequently, through furin inhibition, PAI-1 impairs the cellular insulin response, which could contribute to development of insulin resistance. However, a further characterization of the metabolic phenotype of the cell will be necessary in order to make definitive conclusions about the physiological consequences of such effects. Several studies provide arguments for suspecting the involvement of PAI-1 in metabolic disorders, such as obesity and insulin resistance. In mice, PAI-1 deficiency leads to reduced weight and better insulin sensitivity (Ma et al., 2004). However, although PAI-1 expression can impair glucose uptake, especially in adipocytes (Liang et al., 2006), its direct participation in the development of insulin resistance has been questioned (Schafer et al., 2001) and might be linked to an effect on the inflammation state. Interestingly, the furin-dependent processing of the key inflammatory enzyme ADAM17 is also reduced by PAI-1, resulting in a decrease in its activity. ADAM17 is a metalloproteinase primarily described as the enzyme responsible for TNF-α shedding from the cell surface (Black et al., 1997; Moss et al., 1997). The involvement of ADAM17 in the TNF-mediated inflammatory response is reinforced by its participation in the cleavage of the TNF receptors TNF-R1 and TNF-R2 (Reddy et al., 2000; Solomon et al., 1999). ADAM17 is
also implicated in the development of obesity and insulin resistance (Serino et al., 2007; Xu et al., 2002a; Xu et al., 2002b). The emerging concept is that the synthesis of TFN-α increases in hypertrophied adipose tissue, whereas its processing rate by ADAM17 decreases, resulting in elevated transmembrane TFN-α, which strongly inhibits insulin action at this site. Our results suggest that the increased synthesis of PAI-1 by adipose tissue in obese individuals (Alessi et al., 1997) could be responsible for the local decrease in TFN-α processing.

Considering the two furin substrates analyzed in this work, it appears that the inhibition of furin by PAI-1 could have a direct role in the onset of metabolic disorders in which high levels of PAI-1 are observed. Taken as a whole, our results show that highly expressed PAI-1 can directly impair intracellular processing by furin, and they provide a molecular basis for the emerging concept of PAI-1 being more than just a marker of metabolic disorders such as obesity and the metabolic syndrome (Alessi et al., 2007; Griffiths and Grainger, 2006). However, considering the wide variety of furin substrates, it is evident that its regulation by PAI-1 could impact upon a broad spectrum of biological processes. Furthermore, the high similarity between the catalytic domains of the PCs suggests that PAI-1 could inhibit several other PCs and not just furin.

Materials and Methods

Reagents

Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (cmk), a furin inhibitor, and the internally quenched fluorescent furin substrate butoxycarbonyl-Arg-Val-Arg-7-aminooxy-4-methylcoumarine (Boc-RVRR-AMC) were purchased from Bachem Bioscience. p-Nitrophenyl phosphate was from Sigma–Aldrich. Recombinant furin was obtained from R&D Systems. Recombinant human bioengineered t(1)-antitrypsin (Portland t(1)-PDX) was from Calbiochem. Recombinant PAI-1 (Vleugels et al., 2000) and anti-PAI-1 monoclonal antibodies for immunofluorescence microscopy (33H1F7) and enzyme-linked immunosorbent assay (ELISA) coating (15H12) were kindly provided by Paul Declerck (University of Leuven, Leuven, Belgium). Anti-ADAM17 polyclonal and biotinylated polyclonal antibodies were from Alexis Biochemicals and R&D Systems, respectively. Anti-PAI-1 monoclonal and anti-(insulin receptor) polyclonal antibodies were from Alexis Biochemicals and R&D Systems, respectively. Anti-PAI-1 monoclonal and anti-(insulin receptor) polyclonal antibodies were from Alexis Biochemicals and R&D Systems, respectively. Anti-PAI-1 monoclonal and anti-(insulin receptor) polyclonal antibodies were from Alexis Biochemicals and R&D Systems, respectively. Anti-PAI-1 monoclonal and anti-(insulin receptor) polyclonal antibodies were from Alexis Biochemicals and R&D Systems, respectively. Anti-PAI-1 monoclonal and anti-(insulin receptor) polyclonal antibodies were from Alexis Biochemicals and R&D Systems, respectively.

Expression vectors

The PAI-1 coding sequence was inserted into vector pcDNA3 (Invitrogen). The human furin and TFN-R2 expression vectors (pcDNA3) were as previously described (Peiretti et al., 2003). To generate the soluble N-terminal form of TFN-R2 (TFN-R2mut; amino acids 1–248 of the extracellular domain), the TFN-R2 expression vector was digested with ApaI and then re-ligated. The plasmid encoding the GRAPfurin vector (Coppola et al., 2007) expresses human insulin receptor, human antithrombin III and t(1)-PDX were kindly provided by Tarik Isaad (Institut Cochin, Paris, France), Delphine Borgel (Université Paris Sud, Paris France) and Gary Thomas (Oregon Health & Science University, Portland, OR), respectively.

Cell culture and transfection

The LoVo cell line was cultured as previously described (Peiretti et al., 2003). The HeLa cell line was cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Transient transfections of LoVo and HeLa cells were performed with ICAFection 441 (Eurogentec), as specified by the manufacturer. For all co-transfections, empty plasmid was used to keep the amount of DNA constant. The HepG2 cell line was cultured in MEM and Ham’s F-12 mixed medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. HepG2 cells were transfected with siRNA (Eurogentec) using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

In vitro furin activity and association assays

Recombinant furin (0.021 μg/ml) was incubated in 96-well microplates, with various concentrations of inhibitors, at room temperature for 45 minutes in buffer (25 mM Tris-HCl pH 9, containing 0.5% Triton X-100 and 1 mM CaCl2). Then Boc-RVRR-AMC was added (10 μM final concentration) and fluorescence was measured (excitation at 380 nm and emission at 460 nm) every 30 seconds with a microplate spectrophotometer reader (TECAN Infinite 200; TECAN Austria). Data were derived from the initial linear rates of substrate hydrolysis. For the association assay, recombinant furin and PAI-1 were coincubated at a molar ratio of 1:10. The reaction was stopped by the addition of SDS-PAGE sample buffer, and the samples were immediately analyzed by immunoblotting with antibodies against furin or PAI-1.

Immunofluorescence microscopy

Cells cultured on microscope chambers (Labtek) were treated to enable immunofluorescence detection of furin, golgin-97 and PAI-1. Briefly, cells were washed, fixed on ice with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 in PBS and saturated for unspecific binding with 2% BSA for 30 minutes. After 1 hour of incubation with primary antibody, cells were incubated with the appropriate secondary antibody coupled to Alexa-Fluor-488 or Alexa-Fluor-546. Finally, cells were mounted in medium containing DAPI and visualized using a microscope (Leica DMIRb with a Plan ×60 objective). Images were captured using a camera (ProgRes CFCool; Jenoptik) and ProgRes Capture Pro software (version 2.6). Images were prepared for presentation using ImageJ software with minimal processing.

Immunoblotting

Total cell lysates or glycoproteins purified from cell lysates, isolated as previously described (Peiretti et al., 2003), were boiled for denaturation. Then the proteins were separated by SDS-PAGE under reducing conditions and transferred onto polyvinylidene fluoride membranes (Millipore). Membranes were blocked for 1 hour in 5% (wt/vol) skimmed-milk powder and proteins were immunodetected using appropriate antibodies.

ELISAs

For the PAI-1–furin heterodimer immunobassay, the wells of a microtiter plate were coated overnight at 4°C with 2 μg/ml anti-PAI-1 capture antibody (15H12). After two washes, the remaining protein-binding sites were blocked for 2 hours with a solution of 5% BSA in PBS. The cell lysates, diluted five times, were added to each well and incubated overnight at 4°C. After two washes, biotinylated anti-furin antibody (0.25 μg/ml) was added for 2 hours. Detection was performed by incubating the lysates with streptavidin-conjugated horseradish peroxidase (HRP) for 1 hour followed by addition of TMB solution. The reaction was stopped by adding H2SO4 (1 M final concentration), and the optical density was read at 450 nm. The levels of human TFN-R2 in the conditioned medium and cell lysate were assayed using an ELISA kit from R&D Systems, according to the manufacturer’s protocol. Phosphorylated insulin receptor was quantified with the human phosphorylated insulin receptor ELISA kit (R&D Systems), according to the manufacturer’s protocol.

GRAFPurin alkaline phosphatase assay

After transient transfection of the GRAFPurin vector (Coppola et al., 2007), cell medium was replaced with uncoloured DMEM for 5 hours. The medium was then centrifuged at 20,000 g, mixed with the alkaline phosphatase substrate p-nitrophenyl phosphate in a 96-well plate and incubated for 1 hour, to allow color development, and absorbance was read at 405 nm. For each sample, the absorbance value was normalized to the total protein content of cell lysate (absorbance of supernatant over the protein content of transfected cell)–(absorbance of supernatant over the protein content of untransfected cell)×1000. Results are expressed in arbitrary units.

Statistics

All experiments were performed at least in triplicate and at least three times (unless specified in figure legends). Results are expressed as means±s.d., and statistical analysis was performed using Student’s t-tests with GraphPad Prism software. Significance was taken as at least P<0.05.

We thank Christin Hamilton and Alnawaz Rehentulla for providing the GRAFPurin expression vector, Paul Declerck for providing recombinant PAI-1 and anti-PAI-1 monoclonal antibodies, and Tarik Isaad, Delphine Borgel and Gary Thomas for sharing expression vectors for human insulin receptor, human antithrombin III and t(1)-PDX, respectively. This work was supported by grants from Agence Nationale de la Recherche (ANR-07-PHYSIO-019-01) and from the Fondation pour la Recherche Médicale (DEQ20071210508).

Supplementary material available online at http://jcs.biologist.org/cgi/content/full/124/8/1224/DC1

References


Table S1. Amino acid sequence alignment of the reactive-center-loop of the secreted human serpins.

<table>
<thead>
<tr>
<th>Serpins:</th>
<th>P15</th>
<th>P4</th>
<th>P1</th>
<th>f</th>
<th>P'1</th>
<th>P'10</th>
<th>% id.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1</td>
<td>GTVASSSTAVIVSA</td>
<td>R369</td>
<td>MAPPEEIIMDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinA5</td>
<td>GTRAAAATGTF</td>
<td>R372</td>
<td>SARLNSQRVL</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinB2</td>
<td>GTETAAAAGTGVMTG</td>
<td>R380</td>
<td>TGHGPGFVVA</td>
<td>33.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinC1</td>
<td>GSTAATVIAAG</td>
<td>R425</td>
<td>SLNPNRVTFK</td>
<td>46.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinE2</td>
<td>GTKASAAATTILIA</td>
<td>R365</td>
<td>SSSPWFIVDR</td>
<td>53.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinG1</td>
<td>TGVEAAASISA V</td>
<td>R403</td>
<td>MSLSSFSVNR</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinI1</td>
<td>GSEAAASISVA</td>
<td>R408</td>
<td>TLLVF EVQQP</td>
<td>26.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinA2</td>
<td>GSEAAAV SGMIAIS</td>
<td>R362</td>
<td>MAVLYPQ VIV</td>
<td>26.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinA3</td>
<td>GTETAGPHLEEKKA</td>
<td>W464</td>
<td>SKYQTVMF NR</td>
<td>26.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinA4</td>
<td>GTEAASATAVKILT</td>
<td>L383</td>
<td>SALVETTRTIV</td>
<td>46.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinA10</td>
<td>GTEAVAGILSEITA</td>
<td>Y408</td>
<td>SMPVPVKVD R</td>
<td>26.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinD1</td>
<td>GTQATTVTVTGFMPL</td>
<td>L463</td>
<td>STQVRFTVD R</td>
<td>33.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serpinA1</td>
<td>GTEAGAMFLEAP</td>
<td>M362</td>
<td>SIPEVKFNK</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-PDX</td>
<td>GTEAGAMFLEIRIP</td>
<td>R382</td>
<td>SIPEVKFNK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

f indicates the protease cleavage sites. % id. indicates the percentage identity with PAI-1.
The α1-PDX sequence is shown for comparison with serpin A1 (α1-antitrypsin).