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Processing of complex between urokinase and its type-2 inhibitor on the cell surface

A possible regulatory mechanism of urokinase activity

Pia Ragno[a], Nunzia Montuori[b], Jean-Dominique Vassalli[c] and Guido Rossi[a,b]

[a]*Centro di Endocrinologia ed Oncologia Sperimentale (CEOS), Consiglio Nazionale delle Ricerche, via Pansini 5, I-80131, Naples, Italy,* [b]*Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli, via Pansini 5, I-80131, Naples Italy* and [c]*Institute of Histology and Embryology, University of Geneva Medical School, CH-1211 Geneva 4, Switzerland*

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Complexes between the urokinase-type plasminogen activator (uPA) and its type-2 inhibitor (PAI-2) are bound by a cell-surface receptor for uPA and rapidly cleaved into two fragments of 70 and 22 kDa. The 70-kDa fragment contains the active site of uPA and PAI-2, while the 22-kDa species was identified as the amino terminal fragment of uPA, that binds specifically to the receptor. When the experiment is performed at 4°C, both fragments remain bound to the cell surface and can be eluted by acid treatment. We therefore postulate that after the binding of the uPA-PAI-2 complex, a new binding site for the 70-kDa species becomes available. This additional binding favours the cleavage of the complex into the 70- and 22-kDa fragments; the 70-kDa species is endocytosed or released, while the 22-kDa fragment remains on the cell surface to prevent the binding of intact uPA.

Urokinase; Urokinase receptor; uPA; uPA-R; PAI-2

1. INTRODUCTION

The proteolytic degradation of the elements of the extracellular matrix is an important step in many physiological processes that involve cellular migration and tissue remodelling. Proteolytic degradation, moreover, seems to play an important role also in such pathological processes as metastasis and inflammation. Plasmin is a broad-spectrum proteolytic enzyme that degrades many components of the extracellular matrix, directly or by activating latent collagenases. It originates from a zymogen present in extracellular fluids, plasminogen, by a single proteolytic cleavage catalyzed by two plasminogen activators (PA): the urokinase-type (uPA) and the tissue-type (tPA). The latter is involved mainly in fibrinolysis, while uPA is considered the key enzyme in the above-mentioned processes [1,2]. The activity of both PAs is regulated by two specific inhibitors, PAI-1 and PAI-2, and their localization is controlled by specific cellular receptors [2,3].

uPA is synthesized in several cells as pre-pro-uPA; the removal of the signal peptide gives the secreted form, pro-uPA, which is almost inactive. Pro-uPA is a single-chain glycosylated polypeptide; a single cleavage after Lys-158 yields the active form consisting of two chains, A and B, linked by a disulfide bridge [1–3].

Many cell types of different species express on their surface a uPA receptor (uPAR) [4–8]: the receptor consists of a single glycosylated polypeptide chain of about 55 kDa, maintained on the cell surface by a GPI anchor [9,10]. uPAR binds with high affinity the active form of the enzyme [4], its zymogen, pro-uPA [11], the enzyme inactivated by diisopropylfluorophosphate (DFP) and, finally, also uPA complexed with one of its inhibitors, albeit with a slightly lower affinity [12,13]. Binding to the receptor involves only the amino terminal portion of the enzyme and does not affect its catalytic activity. Therefore, the A chain and its amino terminal fragment (ATF) binds uPAR [4,5,14], while the B chain and the so-called 'low-molecular-weight uPA' uPA33 (residues 136–158, A chain; and 159–411, B chain) that contain the active site of the enzyme, do not [12,13].

PAI-2 was first identified by Kawano in human placenta [15]; it differs from the other inhibitor, PAI-1, in terms of secretion, localization and stability [2,3], and it inhibits uPA by forming a covalent bond with the active site of the B chain, both in solution and with the enzyme bound to its cellular receptor [12,13].

If the uPA–PAI-2 complex (92 kDa) is bound to the uPAR on the surface of a monocyte-like cell line (THP-1), it originates a degradation product of 70 kDa [13]. At 37°C, both the native complex and the degradation

Correspondence address: P. Ragno, Centro di Endocrinologia ed Oncologia Sperimentale (CEOS), Consiglio Nazionale delle Ricerche, Via Pansini 5, I-80131, Naples, Italy. Fax: (39) (81) 770 1016.

product disappear. These observations suggested that the complex undergoes a partial degradation involving mainly the inhibitor, followed, at 37°C, by endocytosis and total degradation. In this way the uPA–PAI-2 complex would be rapidly removed from the cell surface.

To analyze the steps and the products of the degradation process, we have performed binding experiments on the cell line THP-1, at 4°C. The results show that the degradation process causes the cleavage of the uPA–PAI-2 complex into two parts, both bound to the cell surface: the A chain of uPA and the B chain still complexed with the inhibitor. We propose a model whereby PAI-2 regulates the cell surface-associated proteolytic activity of uPA: a conformational modification of the uPA receptor, followed by the exposure of a new binding site, probably for PAI-2 and the cleavage of the uPA–PAI-2 complex.

2. MATERIALS AND METHODS

2.1. *Cell culture*

THP-1 cells, derived from a human monocytic cell line [16], were a kind gift from Dr J.M. Dayer; they were grown in RPMI 1640 medium (Flow, McLean, VA, USA) supplemented with 10% fetal calf serum (Gibco, Gaithesburg, MD, USA).

2.2. *Reagents*

Human uPA (M_r = 55,000) and uPA33 were obtained from Serono (Denens, Switzerland), human plasminogen from Sigma (St. Louis, MO, USA) and recombinant human PAI-2 from Behring (Marburg, Germany).

2.3. *Iodination of uPA*

uPA was radiolabelled as described [17], by iodogen (Pierce Chemical Co., Rockford, IL, USA) and Na-^{125}I (Amersham International, Amersham, UK).

2.4. *SDS-PAGE and autoradiography*

Samples were electrophoresed in the presence of sodium dodecylsulfate (SDS), under non-reducing conditions, in 12% polyacrylamide gels, in Laemmli buffer [18] and stained with Coomassie brilliant blue R250. Gels were dried and exposed to Kodak XAR films (Eastman Kodak, Rochester, NY, USA).

2.5. *Zymography*

This was performed as described [17], by layering the SDS-12% polyacrylamide gels over a 0.8% agarose resolving gel containing 2% non-fat dry milk (Bio-Rad, Richmond, CA, USA) as a source of casein and 0.4 mg/ml of purified human plasminogen in 0.1 M Tris-HCl buffer, pH 7.5. The plasminogen-dependent caseinolysis appeared as a clear band of lysis on the opaque background.

2.6. *Preparation of preformed complexes*

For binding assays, 5×10^{-9} M [^{125}I]uPA was incubated with 2×10^{-8} M PAI-2 for 1 h, at 4°C in RPMI medium additioned with bovine serum albumin (BSA) (1 mg/ml). The formation of the complexes was ascertained by SDS-PAGE and autoradiography.

2.7. *Binding assay*

Cells were collected, washed three times in phosphate-buffered saline (PBS), BSA (1 mg/ml) was added and stripped of surface-bound endogenous uPA by acid treatment as described elsewhere [12]. Briefly, cells were incubated for 3 min at 4°C in 50 mM glycine, 100 mM NaCl, pH 3.0, half a volume of 50 mM HEPES, 100 mM NaCl, pH 7.4, was then added for neutralization. After 3 washes in RPMI–BSA, cells were incubated, if not otherwise indicated, for 2 h at 4°C, with ^{125}I-labelled uPA alone (5×10^{-9} M) or with preformed [^{125}I]uPA–PAI-2 complexes. After incubation, cells were washed three times with PBS–BSA and the surface-bound radioactivity was measured. Cells were then treated with acid or neutral buffer.

Cell density at each step was 2×10^6/100 μl of medium. To inactivate latent proteases, BSA solutions were acidified by adding HCl to pH 3.0, stirred 2 h at room temperature and then neutralized with NaOH to pH 7.2.

2.8. *Cross-linking*

After binding with uPA or uPA–PAI-2 complexes, cells were washed and incubated with or without 1 mM disuccinimidyl suberate (DSS) for 15 min at room temperature. The reaction was stopped by adding 1/10 (v/v) of 100 mM ammonium acetate. After 10 min, cells were washed, lysed and analyzed by SDS-PAGE and autoradiography.

2.9. *Immunoprecipitation*

After binding with uPA–PAI-2 complexes, 50 μl of acid eluates were incubated for 2 h at 4°C with 1 μg of an anti-ATF monoclonal antibody (Biopool, Umea, Sweden) or with 1 μl of normal mouse serum. Then protein A-Sepharose (Pharmacia, Uppsala, Sweden) was added, v/v, for 30 min at room temperature. The resin had been previously washed with 0.5% Nonidet P-40 in 0.5 M NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.1 (NET buffer) and diluted to 10% in NET buffer containing 0.05% Nonidet P-40. The immunoprecipitate was sequentially washed in NET buffer supplemented with 1% Triton X-100 and 0.2% SDS, NET buffer containing 1% SDS supplemented with 60 mg/ml urea and in 0.5 M NaCl, 50 mM Tris-HCl, pH 8.1, 0.1% SDS. Finally the immunocomplexes were eluted by boiling the samples for 10 min in Laemmli buffer, and analyzed by SDS-PAGE and autoradiography.

3. RESULTS

3.1. *Binding of uPA–PAI-2 complexes on THP-1 cells*

All the following experiments were performed with THP-1 cells that express a uPA receptor that has the same characteristics as the uPA receptor of another monocyte-like cell line, U937. In contrast to the latter, however, THP-1 cells do not produce high levels of PAIs [13].

To investigate the interaction between uPA, PAI-2 and their cellular binding sites, THP-1 cells, stripped of endogenous uPA by acid treatment, were incubated for 2 h at 4°C with preformed uPA–PAI-2 complexes or with uPA alone. Binding of complexes could also be achieved by sequential incubation of acid-treated cells with uPA, for 2 h at 4°C, and then with PAI-2 1 h at 4°C, without changing the final result. After the incubation with the ligands, cells were treated with acid, to elute all bound material from the surface, and the eluates were analyzed by SDS-PAGE under non-reducing conditions.

The electrophoretic analysis of the commercial uPA used throughout the experiments, showed that it was a mixture of 55- and 33-kDa molecules (Fig. 1, lane 2). Accordingly, preformed uPA–PAI-2 complexes presented two bands of 92 and 73 kDa apparent molecular mass (Fig. 1, lane 1). In order to saturate the enzyme with the inhibitor [12,13] we used a fourfold excess of

PAI-2 with respect to uPA. Under these conditions, however, the electrophoretic analysis showed that a small fraction of the enzyme was still free.

The SDS-PAGE analysis of the acid eluates showed two bands corresponding to the molecular weights of uPA55–PAI-2 complexes and free uPA (92 and 55 kDa, respectively), and two additional major bands at 70 and 22 kDa, which were apparently, degradation products of the 92-kDa complex (Fig. 1, lane 3). The same result was obtained with lysates of cells not treated with acid (not shown). As expected, there were no bands of 33 and 73 kDa since uPA33 does not bind to the cell surface. In the absence of PAI-2, the only major band observed was of 55 kDa, corresponding to free uPA (Fig. 1, lane 5).

3.2. *Analysis of the degradation products of uPA–PAI-2 complexes bound to THP-1 cells*

To identify the components of the bands observed in the presence of PAI-2 (92, 70, 55 and 22 kDa), the same samples previously analyzed by SDS-PAGE were analyzed by zymography and immunoprecipitation with specific antibody.

The zymographic analysis (Fig. 2) showed that a caseinolytic activity, indicating the presence of the B chain, was associated only with the bands of 92, 70 and

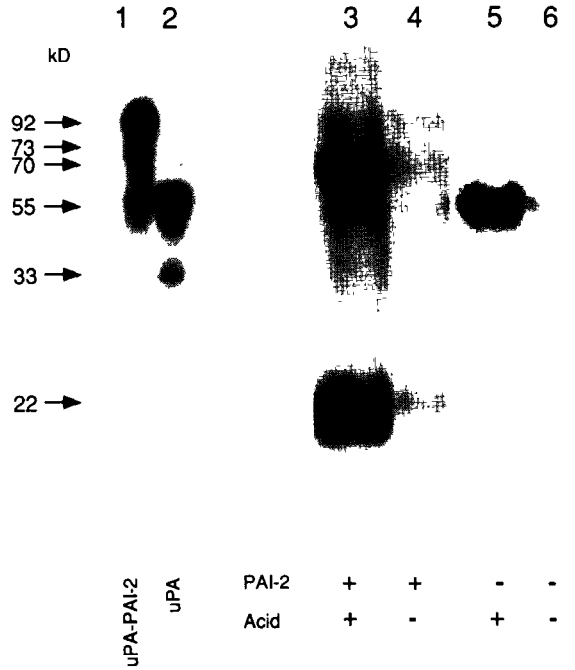


Fig. 1. Binding of [^{125}I]uPA–PAI-2 complexes to THP-1 cells. THP-1 cells were incubated with [^{125}I]uPA–PAI-2 complexes (lanes 3 and 4) or with uPA alone (lanes 5 and 6), then treated with acid (lanes 3 and 5) or with neutral buffer (lanes 4 and 6). Preformed uPA–PAI-2 complexes (lane 1), uPA (lane 2) and eluates (lanes 3–6) were analyzed by SDS-12% PAGE and autoradiography. In this figure and in the following ones, the molecular weights were estimated from the migration of standards.

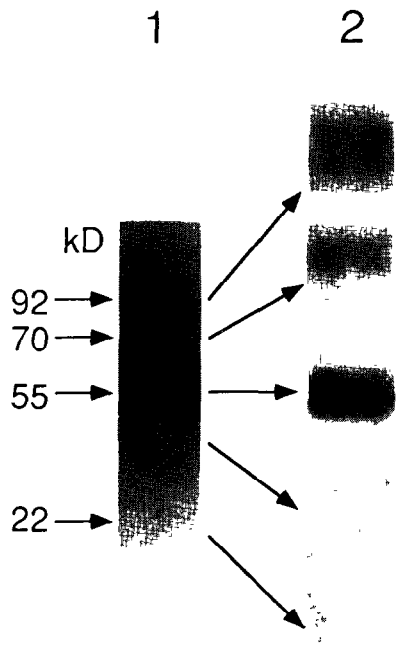


Fig. 2. Zymographic analysis of uPA–PAI-2 complexes and their degradation products bound on THP-1 cells After incubation with uPA–PAI-2 complexes THP-1 cells were treated with acid. The eluate was analyzed in duplicate by SDS-12% PAGE followed by zymography of intact gel (panel 1) and strips corresponding to different molecular weights (panel 2)

55 kDa; there was no enzymatic activity in association with the 22-kDa band, which probably represented the A chain or a part of it (panel 1). The two bands of 92 and 70 kDa were very weak, probably due to the presence of the inhibitor [19] and merged into a smear, when the incubation was prolonged; therefore we cut the gel in strips corresponding to different molecular weights and incubated them separately for the time required to give distinct bands (panel 2).

Immunoprecipitation with a monoclonal antibody against the ATF yielded only the three bands of 92, 55 and 22 kDa (Fig. 3, lane 3). Therefore the 70-kDa degradation product did not contain the ATF region of uPA. Finally, analysis by 10–20% SDS-PAGE under reducing conditions showed that the 22 kDa fragment co-migrated with the A chain of uPA (not shown).

3.3. *Effects of the degradation process on the uPA receptor*

THP-1 cells, after binding with uPA or uPA–PAI-2 complexes, were acid-stripped, incubated again with labelled uPA for 2 h at 4°C, lysed and analyzed by SDS-PAGE. The results showed that preincubation with complexes or uPA alone did not affect the capacity of THP-1 cells to bind newly added uPA (Fig. 4, lanes 4 and 6) as compared to control THP-1 cells that had not been preincubated (lane 2). Moreover, if the cells were treated with DSS before the SDS-PAGE analysis, there

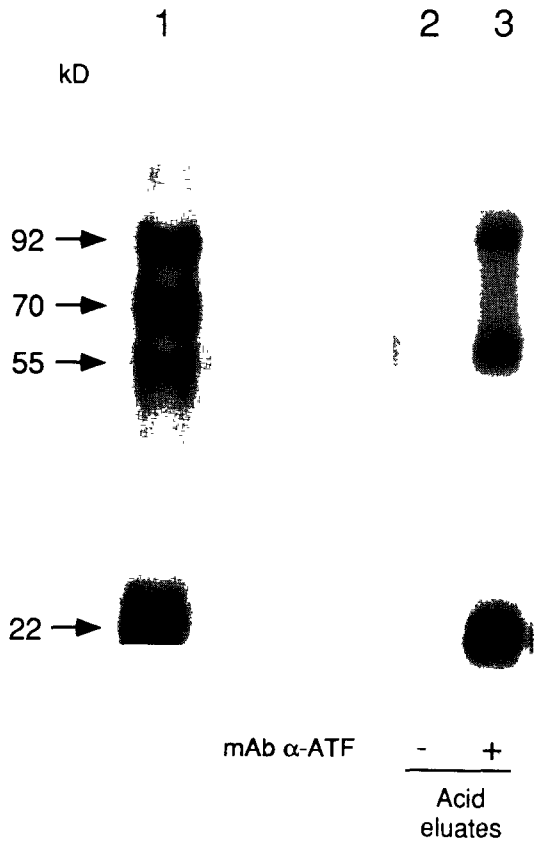


Fig. 3. Immunoprecipitation of [^{125}I]uPA–PAI-2 complexes and their degradation products bound to THP-1 cells. After the binding of [^{125}I]uPA–PAI-2 to THP-1 cells, acid eluates were incubated with an anti-ATF mouse monoclonal antibodies (lane 3) or a nonimmune mouse serum (lane 2). The immunocomplexes were precipitated by protein A-Sepharose, eluted in Laemmli buffer and analyzed by SDS-12% PAGE and autoradiography. Lane 1: acid eluate from THP-1 cells incubated with [^{125}I]uPA–PAI-2 complexes before the immunoprecipitation.

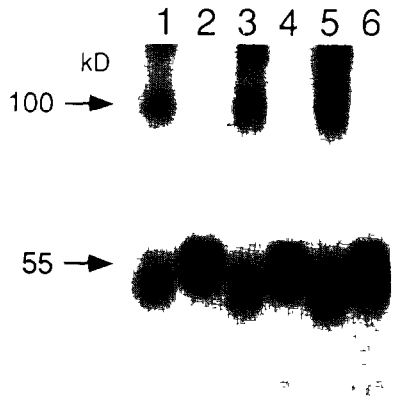
was no shift of the 100 kDa band representing uPA bound to uPAR (lanes 3 and 5) as compared to control cells (lane 1). These two observations suggest that the degradation process does not affect the functioning of the receptor and that the products of this process (70 and 22 kDa) do not contain significant amounts of uPAR or fragments of it.

THP-1 cells preincubated with uPA–PAI-2 complexes, not acid-stripped and incubated again with labelled uPA, did not bind the newly added enzyme (not shown), thus indicating that all uPA receptors were occupied even after degradation of the complex. Moreover, the 22-kDa fragment could still be eluted by acid treatment, even after 150 min at 37°C (Fig. 5, lane 2). It is interesting to note that after 150 min at 37°C, all bound 92-kDa uPA–PAI-2 complexes had been processed and that also the 70-kDa species had disappeared, probably following endocytosis or release into the medium. In the absence of PAI-2 (Fig. 5, lanes 3 and 4), uPA remained intact, even after 150 min.

4. DISCUSSION

In this study of the interaction between the uPA–PAI-2 complex and the uPA receptor we provide evidence of a new cellular binding site for the degraded complex.

We first performed the binding between THP-1 cells, which express a uPA receptor, and the uPA–PAI-2 complex at 4°C, to block endocytosis, and then we analyzed by SDS-PAGE the radioactive products bound to the cells. Unexpectedly, in addition to the bands corresponding to uPA (55 kDa), the native uPA55–PAI-2 complex (92 kDa), and the previously reported 70-kDa degradation product of the native complex [13], we observed a 22-kDa band not previously described (Fig. 1, lane 3). All the bands were acid-elutable, and therefore they were exposed on the cellular surface. The first important consequence of this observation is that the degradation process of the uPA–PAI-2 complex, already described in THP-1 cells [13], does not involve, as suggested, the PAI-2 molecule, but rather uPA itself. The complexed enzyme, in fact, is cleaved into two pieces: the 22-kDa fragment and another fragment, which is part of the 70-kDa degradation product. The sum of the



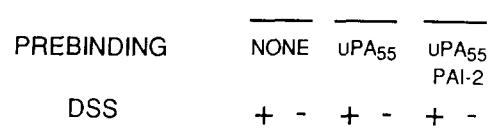


Fig. 4. Effect of the degradation process of uPA–PAI-2 complexes on the uPA receptor. THP-1 cells, after incubation with [^{125}I]uPA–PAI-2 complexes (lanes 5 and 6), [^{125}I]uPA alone (lanes 3 and 4) or medium (lanes 1 and 2), were acid-treated and re-incubated with fresh [^{125}I]uPA. Finally, cells were treated with DSS (lanes 1, 3 and 5) or without (lanes 2, 4 and 6), lysed and analyzed by SDS-12% PAGE and autoradiography.

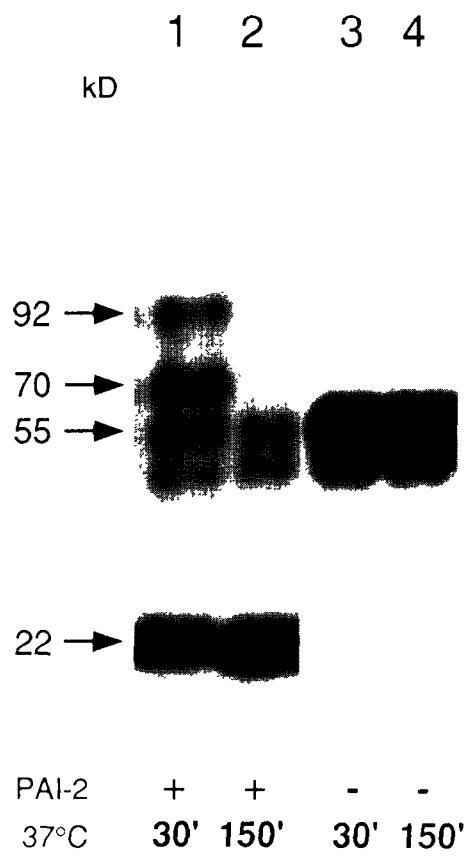


Fig. 5. Fate of uPA–PAI-2 complexes bound to THP-1 cells, at 37°C. THP-1 cells were incubated at 37°C with [^{125}I]uPA–PAI-2 complexes (lanes 1 and 2) or [^{125}I]uPA alone (lanes 3 and 4), for the indicated times. Cells were then washed, treated with acid and the eluates were analyzed by SDS-12% PAGE and autoradiography.

molecular masses of the two molecules equals the molecular mass of the native complex (92 kDa). The cleavage of uPA takes place only when the enzyme is complexed to its inhibitor. The 22-kDa band has probably eluded detection because in the generally used 10% SDS-PAGE it migrates with the front. Immunoprecipitation with monoclonal anti-ATF antibodies and zymography proved that the 70-kDa degradation product contained the active site of the enzyme, i.e. the B chain, while the 22-kDa molecule consisted of the A chain, which is devoid of activity (Figs. 2 and 3).

The cleavage of the bound complex does not involve the uPA receptor, because, after acid elution of native complexes and of their degradation products, cells bound fresh labelled uPA to the same extent as control cells (Fig. 4). We cannot exclude some degradation of the PAI-2 molecule, although no major degradation products were observed.

Whereas the 22-kDa molecule most probably binds to the cellular surface by the canonic receptor, the 70-kDa molecule could not, because it lacks the ATF. This suggested the existence of a different receptor for the larger fragment or of a novel subunit (or binding site) of the canonic uPA receptor, which could bind the B chain of uPA or PAI-2 or both. However, uPA33–PAI-2 complexes do not bind THP-1 cells ([13] and our unpublished observation), thus suggesting that the second site is not available on the cell surface, unless the native uPA–PAI-2 complex is bound by the canonic site for ATF.

The specificity of the second binding site remains to be established. We performed competition experiments (not shown) between preformed, labelled uPA–PAI-2 complexes and cold competitors, such as uPA, PAI-2, uPA33, uPA–PAI-2 and uPA33–PAI-2 complexes. The results were not conclusive and although they suggested a low affinity of the novel site for PAI-2, since PAI-2 alone and uPA33–PAI-2 complexes weakly inhibited (20– 30%) the binding of intact uPA–PAI-2 complexes, we cannot exclude that the second binding involves also, or only, uPA. Experiments with phospholipase C (PLC), which cleaves the GPI anchor of the uPA receptor, might be relevant in this regard; if the receptor is occupied by the uPA–PAI-2 complex and not by uPA alone, it is somehow protected from the PLC action, probably because of a physical contact between the receptor and the inhibitor [20].

Cross-linking experiments performed with DSS on THP-1 cells incubated with uPA–PAI-2 complexes did not provide useful information: no new and unexpected band, that could not be accounted for by adding the molecular weight of the enzyme, the inhibitor and the receptor, was observed (not shown). On the basis of this observation, we cannot exclude the presence of a different receptor, i.e. the low density lipoprotein receptor related protein (LRP)/2 macroglobulin receptor (2MR) [21], or of a different subunit of uPAR, which could have been inaccessible or not amenable to cross-linking by DSS.

In conclusion, there are several steps that precede the endocytosis of the uPA–PAI-2 complex. These steps can be examined by blocking the whole process at 4°C, just before endocytosis. On the basis of our observations, we propose the following model of the interaction between uPA, PAI-2 and uPAR:

(1) The uPA–PAI-2 complex binds with high affinity to the canonic receptor via the ATF region. The binding could determine a conformational modification of the receptor, thus exposing a new binding site, possibly for PAI-2; alternatively, it could somehow allow the binding of the complex to a different receptor, as described for the uPA–PAI-1 complex [21].

(2) These two almost contemporaneous bindings could stretch the complex, causing it to cleave in two fragments of 70 and 22 kDa bound, respectively, to the novel binding site and the binding site for ATF.

(3) At 37°C the 70-kDa species is endocytosed or released; the 22-kDa fragment would remain outside the cell, on its own binding site, since it is still acid-elutable after 150 min at 37°C. Therefore, the final result of this

process is that the concentrated proteolytic activity localized on the cell membrane is completely neutralized; in fact, the B chain is first inhibited by PAI-2 and then endocytosed or released, while the A chain, catalytically inactive, continues to occupy the uPA receptor on the cell membrane, thus preventing the binding of new active uPA.

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