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PROTEASE NEXIN-1 COMPLEXES AND INHIBITS T CELL SERINE PROTEINASE-1

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The T cell serine proteinase-1 (TSP-1) which most probably is involved in cell killing by cytotoxic T cells is inhibited by protease nexin-1 (PN-1), an extravascular serine protease inhibitor. The inhibition is irreversible and correlates with formation of SDS-stable complexes between the two proteins. Two distinct species of complexes (91 and 122 kDa) are observed upon SDS-PAGE analysis of the reacted proteins, indicating that PN-1 is capable of complexing and inhibiting both subunits of the homodimeric TSP-1 molecule. Heparin (2 ug/ml) increases the association rate constant from 4.2×10^4 M^{-1} sec^{-1} to 4.8×10^5 M^{-1} sec^{-1}. These observations suggest that PN-1 may function as a major extravascular inhibitor of TSP-1 released from cytotoxic T lymphocytes.

T cell serine proteinase-1 (TSP-1; also known as granzyme A and BLT-esterase) is a serine proteinase made predominantly by $CD8^+$ cytolytic T cells and suggested to play a role in T cell-mediated cytotoxicity (1-4). It is a disulfide-linked homodimer of 60 kDa stored inside cytoplasmic granules of cytolytic T cell lines (2,5) and released upon their cytolytic attack on target cells (2). The enzyme is not expressed in thymocytes, resting T cells (6) or non-cytolytic T cell lines (2). It was shown to degrade fibrin and casein (3) and extracellular matrix (ECM) proteins (7), although its specific substrate(s) involved in cell killing remains obscure. It seems likely that a physiological mechanism would exist to inactivate this protease following its release, so that damage to surrounding tissue during T cell-mediated cytotoxicity is controlled.

Protease nexin-1 (PN-1) is a serine protease inhibitor which controls several serine proteases in the extracellular environment (8). It is made by several cell types, including fibroblasts (8); it is localized to the ECM of these cells (9). PN-1 inhibits serine proteases by forming covalent complexes with their catalytic site serine residues (8,10). Such complexes then bind to the cell membrane via discrete PN-1 receptors and are rapidly internalized and degraded (11). Here we show that PN-1 is a prominent candidate for clearing released TSP-1.

MATERIALS AND METHODS

Mouse TSP-1 and human PN-1 were purified from lysates of cultured mouse CTLL 1.3E6 cells (12) and culture medium of human fibroblasts (13), respectively, as described. Both

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preparations were apparently homogeneous as judged by silver-staining of SDS-PAGE acrylamide gels. The chromogenic substrate H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride (S-2302) (12) was from Helena Labs. Heparin (porcine, 168 units/mg) and dithiothreitol (DTT) were from Sigma. $Na^{125}I$ was from New England Nuclear. TSP-1 was radioiodinated to a specific activity of 6650 cpm/ng by using Iodogen (Pierce) (8) without detectable loss of activity. Conditions for complex formation between TSP-1 and PN-1 are given in the Figure legends. The association rate constants for complex formation were calculated according to

$$K_{cal} = \frac{\ln \frac{P(I-PI)}{I(P-PI)}}{(I-P)t} \qquad \text{eq. (1)}$$

where P and I are the initial concentrations of the protease and the inhibitor, and PI is the total concentration of complexes at time t (14).

RESULTS

Preincubation of purified TSP-1 with PN-1 inhibited its ability to hydrolyze the synthetic substrate S-2302 (Table 1). Notably, heparin increased both the activity of TSP-1 and its inhibition by PN-1. The association rate constants calculated from these data using eq. (1) are given in Table 1. These constants, however, may not precisely define the inhibition of TSP-1 since the enzyme, inhibitor and substrate may interact during measurement of the hydrolysis of the latter. This is especially true for the homodimeric TSP-1, where complexing of an inhibitor molecule to one subunit may affect the activity of the remaining free subunit.

We therefore analyzed complex formation between TSP-1 and PN-1 using radiolabeled TSP-1 and SDS-PAGE (Figures 1&2). As observed previously for complexes between PN-1 and thrombin or urokinase (8,10,11), the complexes between PN-1 and TSP-1 were stable after SDS-PAGE. Moreover, this analysis enabled the detection of two distinct species of complexes (indicated by arrows in Figures 1A and 2A); judged from their apparent molecular weights of 91 and 122 kDa they represent TSP-1:PN-1 and TSP-1:$(PN\text{-}1)_2$, respectively. As the size of TSP-1 and PN-1 deduced from SDS-PAGE is 60 and 43 kDa, respectively, this indicates that a peptide of about 12 kDa was clipped from PN-1 during complex formation, in accord with previous observations on PN-1 complex formation with thrombin or urokinase (8,10). Notably, only a

TABLE 1

additions	S-2302 hydrolized (nmol min^{-1})	K_{cal}
none	285	
46nM PN-1	187	3.5×10^4 $M^{-1}sec^{-1}$
50ug/ml heparin	486	
46nM PN-1 + 50ug/ml heparin	173	1.0×10^5 $M^{-1}sec^{-1}$

Purified TSP-1 (31 nM) was preincubated with the indicated additions for 5 min at 37° C in a final volume of 0.1ml phosphate-buffered saline (PBS) containing 1mg/ml BSA (Sigma, RIA grade). The reaction was quenched by diluting 10-fold into PBS containing the chromogenic substrate S-2302 (0.5mM); its hydrolysis was immediately analyzed by measuring OD at 405nm. K_{cal} is the association rate constant calculated as described in Materials and Methods from the inhibition of TSP-1 activity following preincubation with PN-1.

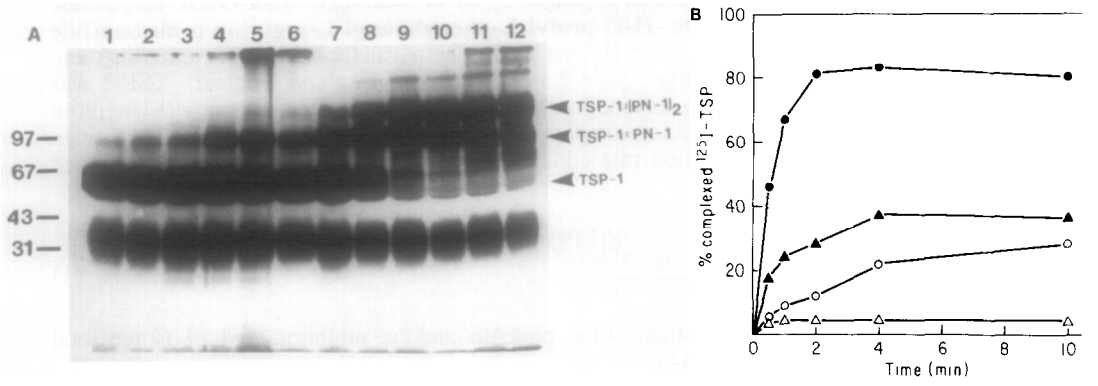

Figure 1
Complex formation between TSP-1 and PN-1. Vials containing ^{125}I-TSP-1 (4nM) were incubated with PN-1 (22nM) in a final volume of 0.1ml PBS containing 1mg/ml BSA at 37° C. At the indicated times the reactions were quenched by adding an equal volume of sample buffer containing 1%SDS and the samples were analyzed by SDS-PAGE, using 10% acrylamide gels. The gels were subsequently stained to visualize the molecular weight standards , dried and exposed to Kodak X-OMAT film. (A) An autoradiogram showing complexes formed using non-reducing conditions following incubations without (lanes 1-6) or with (lanes 7-12) 10ug/ml heparin. Incubation periods were 0.5, 1, 2, 4, 10 and 20 min for lanes 1-6 and 7-12, respectively. Free 125I-TSP-1 and its complexes with PN-1 are indicated with arrows. (B) Similar experiments were done under non-reducing and reducing conditions, using 2 mM DTT for the latter. Following exposure to film, complexes containing 125I-TSP-1 were cut from the gel and their radioactivity was determined using a gamma-counter. The percentage of radioactivity in complexed ^{125}I-TSP-1 is shown as a function of incubation time for the following conditions: control (○), 10 ug/ml heparin (●), 2 mM DTT (Δ), 2 mM DTT + 10 ug/ml heparin(▲).

single complex (61 kDa) was detected when TSP-1 and PN-1 were reacted under reducing conditions, i.e. in the presence of 2 mM DTT (data not shown).

In the absence of heparin the smaller complex form was detected almost exclusively under the experimental conditions depicted in Fig. 1A. In the presence of 10 ug/ml heparin, however, both forms were detected, and it is clear that the smaller complexes were converted to the larger species when longer incubation periods were employed. Data from comparable experiments with the native, homodimeric form of TSP-1 and reduced, monomeric TSP-1 are shown in Fig. 1B. These results show that heparin accelerated the reaction between PN-1 and TSP-1 under both reducing and non-reducing conditions. It is evident, however, that the extent of the reaction was much greater under non-reducing conditions and in the presence of heparin. In these experiments PN-1 was used in large excess over TSP-1. Yet, in the absence of heparin or in the presence of 2 mM DTT the majority of TSP-1 remained uncomplexed within the experimental time frame employed. Longer incubation periods could not be used to further investigate complexing under these conditions due to poor stability of PN-1 at 37° C and complications arising from proteolysis of complexes, presumably by the remaining free or semi-complexed TSP-1 (e.g. lane 12 in Fig.1A). Note, however, that heparin also accelerated the complexing of PN-1 with monomeric TSP-1 (Fig. 1B).

This acceleration was further investigated employing a wide concentration range of heparin (Fig. 2A,B). It is evident that the complexing rate was affected by low heparin concentrations, with half-maximal acceleration observed with ~50 ng/ml heparin for both native and monomeric TSP-1. Moreover, in both instances the acceleration was diminished at high heparin

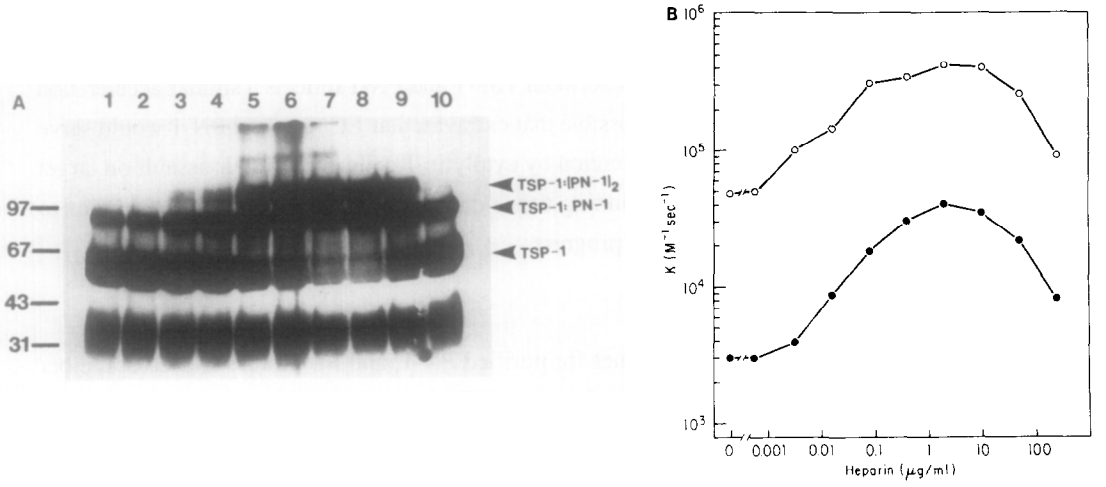

Figure 2
Acceleration of TSP-1:PN-1 complex formation by different heparin concentrations. The same concentrations and conditions given in legend to Fig. 1 were used.
(A) An autoradiogram showing complex formation between TSP-1 and PN-1 in the presence of different heparin concentrations. Samples were incubated for 4 min at 37° C with the following heparin concentrations: 0, 0.0006, 0.003, 0.016, 0.08, 0.4, 2, 10, 50 and 250 ug/ml for lanes 1-10, respectively.
(B) Calculated association rate constants for ^{125}I-TSP-1 and PN-1 as a function of heparin concentration in the absence (○) and presence (●) of 2mM DTT. Concentrations of total complexes were determined by gamma-counting the dried gels, and applied to eq. (1) to yield the corresponding rate constants. Note that in the case of native TSP-1 these rate constants are k_1 in the scheme:

$$\text{TSP-1} + 2(\text{PN-1}) \xrightarrow{k_1} \text{TSP-1:PN-1} + \text{PN-1} \xrightarrow{k_2} \text{TSP-1:(PN-1)}_2$$

Determination of k_2 values requires non-linear regression analysis of a large amount of data and is beyond the scope of the present studies.

concentrations (≥ 50 ug/ml). This suggests that heparin interacts non-cooperatively with both subunits of native TSP-1; i.e. the heparin binding domain is present in each subunit, and does not result from the dimeric tertiary structure.

DISCUSSION

Our data demonstrate that PN-1 inhibits TSP-1 by forming irreversible, SDS-stable complexes with either one or both subunits of this homodimeric serine protease. This indicates that both subunits of TSP-1 are proteolytically active, as PN-1 does not form complexes with serine proteases blocked at their catalytic site serine (8). Moreover, it implies that the TSP-1:PN-1 complex retains the catalytic activity of its free subunit, as it is capable of complexing a second PN-1 molecule. The reaction is accelerated ~10-fold by heparin, regardless whether dimeric or monomeric (i.e. reduced) TSP-1 is used.

These observations not only enlarge the spectrum of serine-proteases known to be complexed by PN-1 (10) but may indicate an important physiological function for this extra-vascular serpin, e.g. protecting surrounding tissue during T cell-mediated cell killing. PN-1 is secreted by a variety of cells, such as fibroblasts (8) and astrocytes (15) and localized to their ECM

(9). Its complex formation with thrombin was shown to be accelerated by ECM heparan sulfate in a manner similar to the acceleration observed with heparin (14). It is plausible that the heparin-induced acceleration of complex formation between TSP-1 and PN-1 reflects a similar acceleration by ECM heparan sulfate. It is therefore possible that extravascular ECM-bound PN-1 would serve as a major agent for inactivating TSP-1 secreted by cytolytic T cells during their assault on target cells , thus limiting the damage to surrounding tissue during inflammation. Detailed studies to address these mechanisms are currently in progress.

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