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Plasmin cleavage of vitronectin

Identification of the site and consequent attenuation in binding plasminogen activator inhibitor-1

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Plasmin is shown to specifically cleave vitronectin at the Arg^{361}–Ser^{362} bond, 18 amino acid residues upstream from the site of the endogenous cleavage which gives rise to the two-chain form of vitronectin in plasma. The cleavage site is established using the exclusive phosphorylation of Ser^{378} with protein kinase A. As a result of the plasmin cleavage, the affinity between vitronectin and the type-1 inhibitor of plasminogen activator (PAI-1) is significantly reduced. This cleavage is stimulated by glycosaminoglycans, which are known to anchor vitronectin to the extracellular matrix. A mechanism is proposed through which plasmin can arrest its own production by feedback signalling, unleashing PAI-1 from the immobilized vitronectin found in the vascular subendothelium, which becomes exposed at the locus of a hemostatic event.

Fibrinolysis; PAI-1; Protein kinase-A; Heparin; Extracellular matrix

1. INTRODUCTION

The activation of plasminogen is known to be involved in major physiological and pathological processes such as fibrinolysis and thrombolysis, inflammation, ovulation, tissue remodeling and development, malignant transformation and tumor cell invasion [1,2]. This activation is under the concerted control of plasminogen activators and their inhibitor PAI-1 [3,4], which was recently shown to be bound to vitronectin in the extracellular matrix [5–8].

Originally discovered as a serum spreading factor [9], vitronectin is now recognized as a multifunctional regulatory protein [10] involved in a variety of extracellular processes such as the attachment and spreading of normal and neoplastic cells [11] and the function of the complement [12,13] and coagulation pathways [12,14,15]. In circulating blood, vitronectin occurs in 2 molecular forms: a single chain 75 kDa form (V_{75}) and a clipped form (V_{65+10}) composed of two chains (65 kDa and 10 kDa) which are held together by a disulfide bridge [12,16]. V_{75} was previously shown to be phosphorylated at Ser^{378} by PKA, released upon thrombin stimulation of platelets [17–21]. By contrast, at physiological pH, V_{65+10} is stoichiometrically phosphorylated only in the presence of glycosaminoglycans [18] (heparin or heparan sulfate) which were shown to expose its otherwise buried Ser^{378} [19]. V_{65+10} was also shown to possess a higher affinity for heparin [15,18]. The endogenous proteinase that converts V_{75} into V_{65+10} was not identified so far, but this cleavage was shown to occur at the Arg^{379}–Ala^{380} bond [12,19].

Until recently it was assumed that there are 'no major functional differences' between V_{75} and V_{65+10} [22]. However, the distinctly different behaviour in their phosphorylation by PKA [18,19] raised the possibility that the $V_{75} \rightarrow V_{65+10}$ conversion may be physiologically important. We therefore set out to study this conversion. The enzymes which were reported to simulate the endogenous cleavage were trypsin, elastase and plasmin [10]. Among these, plasmin seemed to be the most interesting from the physiological point of view. This paper shows that the plasmin cleavage site is actually 18 amino acid residues upstream from the endogenous cleavage site, and that this plasmin cleavage has distinct functional consequences, attenuating the binding of PAI-1 to vitronectin.

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Abbreviations: PAI-1, plasminogen activator inhibitor-1; PKA, cAMP dependent protein kinase (catalytic subunit); V, vitronectin (M_r in kDa indicated by subscript); Vn, pure vitronectin as isolated from plasma (containing V_{75} and V_{65+10}); Vn', plasmin-clipped Vn.

2. MATERIALS AND METHODS

2.1. *Materials purchased*

Freshly-frozen human plasma was obtained from the Tel Hashomer Medical Center, Ramat Gan, Israel. Heparin and polyethylene glycol (M_r = 3350) were purchased from Sigma. Rabbit anti-human vitronectin polyclonal antibodies were from Calbiochem. Alkaline phosphatase-labeled antibodies to rabbit IgG were obtained from Dako, Copenhagen. Plasmin, PAI-1 and rabbit anti human PAI-1 were purchased from American Diagnostica Inc., New York. [γ^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham. Im-

mobilon (PVDF) membranes were supplied by Millipore Corp. Nitrocellulose paper (0.45 μm) was from Schleicher and Schuell.

2.2. *Vitronectin*

Vitronectin was prepared from freshly-frozen human plasma using a purification procedure described elsewhere [21]. Our preparations usually contained the 2 molecular forms of this protein: V_{75} – a one-chain (75 kDa) form, and V_{65+10} – a proteolytically clipped, two-chain protein (65 kDa and 10 kDa) held together by a disulfide bridge [12,13].

2.3. *The catalytic subunit of PKA*

Pure catalytic subunit of PKA was prepared according to Beavo et al. [23] and assayed as described elsewhere [24].

2.4. *Identification and assay of vitronectin by phosphorylation with PKA*

Aliquots of the samples to be assayed were added to a reaction mixture (final volume 50 μl) which contained the following constituents at the indicated final concentrations: PKA (2.5 μg/ml); $Mg(CH_3COO)_2$ (10 mM); [γ^{32}P]ATP (10 μM, 50 Ci/mmol), HEPES (50 mM, pH 7.5). Phosphorylation was allowed to proceed for 15 min at 30°C and arrested by addition of 12 μl of sample buffer to yield the following final composition: SDS (2%); 2-mercaptoethanol (0.75 M); glycerol (10%); Tris-HCl buffer (25 mM, pH 6.7); bromophenol blue (0.1 mg/ml). The samples were subjected to SDS-PAGE and autoradiography or counting.

2.5. *Procedures carried out as described in the literature*

Protein concentration was determined by the method of Bradford [25]. SDS-PAGE was carried out according to the procedure described by Laemmli [26]. The gels were fixed and stained with a Coomassie blue solution (0.25%) in 50% methanol and 7% acetic acid. Destaining was carried out with a 20% isopropanol solution containing acetic acid (7%). Gels assayed for radioactively labeled proteins were dried under vacuum and autoradiographed at – 20°C using Agfa X-ray film. Alternatively, the lanes of such gels were cut out and their radioactivity was counted in a scintillation counter using a toluene-based scintillant. Immunoblotting was carried out as described by Towbin et al. [27]. The binding of Vn or Vn' to immobilized PAI-1 was determined as described by Salonen et al. [6]. The activation of PAI-1 by boiling was carried out as described by Katagiri et al. [28].

2.6. *Amino acid sequencing*

Sequence analysis of vitronectin or its fragments was performed after resolution by SDS-PAGE (using a 7–20% linear gradient of polyacrylamide) and transfer by Western blotting onto an Immobilon membrane [29] in 50 mM HEPES, pH 7.5 and 20% methanol. A constant current of 300 mAmp was applied for 3 h with cooling. The Immobilon paper was washed extensively with distilled water, stained with Coomassie blue (0.25%) in 50% methanol and 7% acetic acid and washed with a 1:1 mixture of methanol and 20% acetic acid to remove the excess dye. The stained bands of interest were cut out from the paper and stored desiccated at – 20°C until sequencing by an Applied Biosystem 475A sequencer.

3. RESULTS AND DISCUSSION

3.1. *Identifying the plasmin cleavage site*

When plasmin is allowed to cleave a vitronectin preparation composed of V_{75} and V_{65+10}, monitoring the cleavage of SDS-PAGE and either protein staining (Fig. 1A) or immunoblotting with anti-Vn (Fig. 1B), one may initially be led to believe that V_{75} is converted into V_{65+10}, i.e. that plasmin simulates this endogenous cleavage [10]. However, if the plasmin cleavage is monitored by PKA phosphorylation of the cleavage products with [γ^{32}P]ATP, SDS-PAGE and autoradiography, it becomes clear that the plasmin cleavage site must be upstream from the endogenous

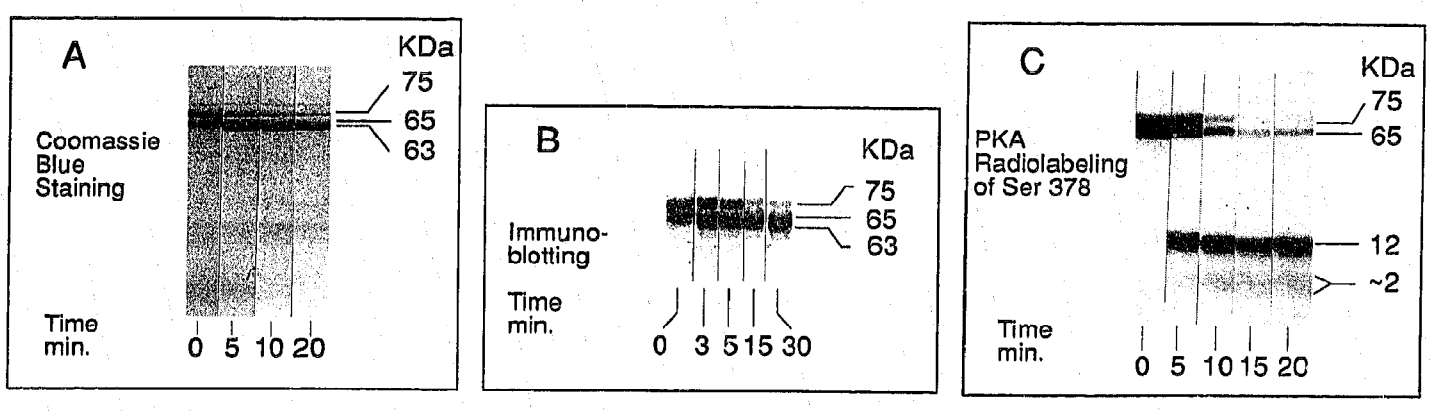


Fig. 1. Plasmin cleavage of vitronectin monitored (A) by protein staining; (B) by immunoreactivity; (C) by PKA radiolabeling of Ser^{378}. *Panel A:* the reaction mixtures (50 μl each) contained vitronectin (140 μg/ml), plasmin (1 μg/ml) and HEPES buffer, pH 7.5 (50 mM) (all final concentrations). Cleavage was allowed to proceed at 37°C and arrested at the times indicated by boiling (3 min at 95°C) with 12 μl of an 'arresting buffer' composed of SDS (10% w/v), 2-mercaptoethanol (3.75 M), glycerol (50%), Tris-HCl buffer (125 mM, pH 6.7) and Bromophenol blue (0.1 mg/ml). The samples were then analyzed by SDS/PAGE (7–20% gels) under reducing conditions [26], then stained with Coomassie blue. *Panel B:* experimental details as described in panel A except for the fact that the analysis was carried out with 7% gels and immunoblotting [27] with polyclonal anti-human vitronectin antibodies. *Panel C:* the reaction mixtures were of 30 μl (concentration as in panel A), and the cleavage was arrested by boiling (5 min at 95°C) only. Vitronectin phosphorylation was carried out with PKA by addition of 20 μl of a solution containing the following phosphorylation ingredients (final concentrations given): C subunit of PKA (25 nM), [γ^{32}P]ATP (50 μM, specific radioactivity 0.5 Ci/mmol), $Mg(CH_3COO)_2$ (10 mM). Phosphorylation was allowed to proceed for 30 min, then stopped by boiling with 12 μl of the 'arresting buffer'. After SDS/PAGE under reducing conditions (7–12% gels) the gels were subjected to autoradiography. Each of the experiments described above is representative of at least 3 experiments carried out with different preparations of vitronectin and plasmin. Note that in panel (C) both the 75 and 65 kDa bands are labeled, since the phosphorylation is carried out on denatured (boiled) vitronectin in which Ser^{378} is exposed [18,19] in both V_{75} and V_{65+10}. Also note that the amount of [^{32}P]V_2 seen in this panel does not represent its relative concentration in the reaction mixture. Most of it diffuses out of the gel during washing, since V_2 is a very basic and hydrophilic peptide (cf. legend to Fig. 2, lower panel).

cleavage site (Arg^{379}-Ala^{380}), since the single phosphorylation site (Ser^{378}) is now found in 2 small-chain cleavage products (~12 kDa and ~2 kDa, Fig. 1C). Such products would be obtained from V_{75} and V_{65+10} respectively, if the plasmin cleavage site occurs about twenty amino acid residues (~2 kDa) before Arg^{379}, leaving the entire phosphorylation consensus sequence of PKA [30] (residues 375–379) in the small fragments. Indeed, a more careful examination of the cleavage in Figs. 1A and B shows that both the V_{75} and the V_{65+10} chains are cleaved to yield a somewhat smaller chain of ~63 kDa. To identify the exact site of the plasmin cleavage, we phosphorylated only the V_{75} form of vitronectin by carrying out the reaction at pH 7.5. Under these conditions, V_{65+10} is not phosphorylated [17–19,21]. The non-labeled V_{63} and the ^{32}P-labeled V_{12} (cf. Fig. 2) were identified, cut out and sequenced. On the basis of the amino acid residues positively identified (Table I), it was established that plasmin cleaves vitronectin at the Arg^{361}-Ser^{362} bond, reducing the molecular mass of the heavy chain of vitronectin to ~63 kDa (Fig. 2). This cleavage clearly occurs in both V_{75} and V_{65+10}, which yield (respectively) V_{63+12} and V_{63+10} + V_2 (Fig. 3B).

3.2. *The plasmin cleavage of vitronectin attenuates the binding of PAI-1*

Since the plasmin cleavage occurs within the heparin binding domain (positions 348–379) [31] and since this domain may probably be involved in the interaction of vitronectin with PAI-1 [5–8,10] we explored the effect of the plasmin cleavage of vitronectin on its binding onto immobilized PAI-1. Using the enzyme immunoassay developed by Salonen et al. [6] we found that the plasmin cleavage of vitronectin significantly reduces its ability to bind PAI-1 (Fig. 3). This attenuation of bin-

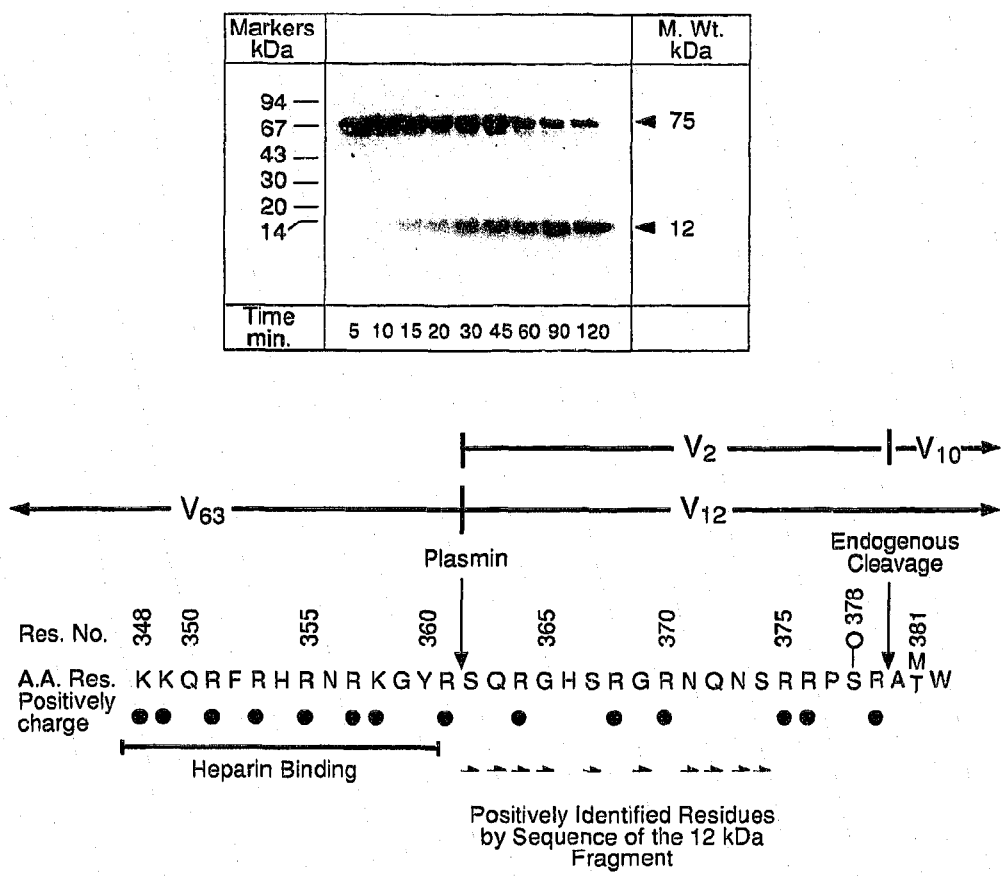


Fig. 2. Determination of the plasmin cleavage site in vitronectin. *Upper panel:* a vitronectin preparation obtained as described earlier [21] was selectively phosphorylated at Ser^{378} with PKA [17,19] and [γ^{32}P]ATP, allowing phosphorylation to take place in V_{75} only [18,19,21]. The Ser^{378}-labeled vitronectin was cleaved with plasmin (cf. legend to Fig. 1) ascertaining cleavage completion by SDS/PAGE and autoradiography. After Western blotting onto an Immobilon PVDF membrane, the N-terminus sequence of V_{12} was determined by the procedure described elsewhere [19]. *Lower panel:* sequence of a portion of vitronectin containing the positively charged cluster of basic amino acids (residues 348–381). This scheme illustrates the relationship between the site of phosphorylation of PKA [17,19] (Ser^{378}), the endogenous cleavage site (Arg^{379}-Ala^{380}, previously assumed to be the site of plasmin cleavage [10], the plasmin cleavage site (Arg^{361}-Ser^{362}) established here on the basis of the N-terminus sequence of V_{12}, and its vicinity to the C-terminus of the heparin binding site [31] (underlined). Note that the numbering of amino acid residues refers to the vitronectin sequence deduced by Jenne and Stanley [13].

Table I

Sequence of the plasmin cleavage products of vitronectin

V_{63}				V_{12}			
Sequence determined		Matching sequence in V_n*		Sequence determined		Matching sequence in V_n*	
Cycle	Residue (yield**)	Position	Residue	Cycle	Residue (yield**)	Position	Residue
1	D (8)	1	D	1	S (12)	362	S
2	Q (20)	2	Q	2	Q (21)	363	Q
3	E (6)	3	E	3	R (3)	364	R
4	S (5)	4	S	4	G (26)	365	G
5	n.d.	5	C	5	–***	366	H
6	K (6)	6	K	6	S (7)	367	S
7	G (11)	7	G	7	–***	368	R
8	R –***	8	R	8	G (19)	369	G
9	n.d.	9	C	9	–***	370	R
10	T (4)	10	T	10	N (9)	371	N
11	E (3)	11	E	11	Q (12)	372	Q
12	G (5)	12	G	12	N (2)	373	N
13	F (3)	13	F	13	S (6)	374	S
14	N (3)	14	N				
15	V (4)	15	V				

*Following the sequence numbering deduced by Jenne and Stanley [13]
**Number of pmols of amino acid-phenylthiohydantoin in derivative recovered
***Yield too low for position identification
n.d., not determined (since the cysteines in V_n were not pre-modified, they were not determined in this analysis).

ding was observed in 4 such experiments. PAI-1 was activated by the 2 different procedures described in the literature [6,28] for the conversion of 'latent' PAI-1 to its 'active' form [3]. The attenuation was less extensive in some of the experiments, but in all 4 experiments the apparent affinity between V_n and PAI-1 was at least 10-fold higher than the affinity between Vn' and PAI-1.

3.3. *Stimulation of the plasmin cleavage by glycosaminoglycans*

The plasmin cleavage of vitronectin was found to be stimulated by heparin (Fig. 4) or heparan-sulfate (results not shown). This stimulation occurred in both the V_{75} and the V_{65+10} forms of vitronectin. It was more pronounced in the case of V_{65+10}, where the rate of cleavage was very slow in the absence of the glycosaminoglycan (Fig. 4). This result would indicate that glycosaminoglycan-bound vitronectin (such as the vitronectin found in the vascular subendothelium [5–8]) might well be preferentially cleaved by plasmin, confining this cleavage to the locus of the hemostatic event. It should be noted that this effect of glycosaminoglycans is in line with previous studies in our laboratory on the heparin-triggered conformational changes in vitronectin, which expose Ser^{378} and make it accessible to PKA phosphorylation [18,19].

The detailed mechanism through which the plasmin cleavage of vitronectin attenuates its PAI-1 binding remains to be established. In the case of V_{65+10} it may involve the loss of a peptide (Fig. 2), while in the case of both V_{75} and V_{65+10} it may be the result of a conformational change, since the cleavage occurs in a domain which was previously shown to undergo such changes [18,19]. In a very recent publication, Seiffert and Loskutoff [32] have provided evidence that PAI-1 binds to the somatomedin B domain at the N-terminus of vitronectin. Since the latter has been previously proposed to interact with the glycosaminoglycan binding region of this protein [10,18,19], a cleavage-triggered conformational change in vitronectin may also be reflected in its somatomedin B domain.

3.4. *Concluding remarks*

In order to control plasmin action, it is necessary first and foremost to arrest further production of plasmin from plasminogen (by the inhibition of plasminogen activator with PAI-1 [3,4]) and at the same time to inactivate the excess of already-formed plasmin (by α_2-plasmin inhibitor). The detailed molecular events which sense an excessive plasmin level and trigger the action of PAI-1 at the right time and place are not yet known. The simplest and most effective mechanism for that purpose would make use of plasmin itself as a signalling device.

On the basis of the findings presented above regarding the site of the plasmin cleavage of vitronectin, its specificity, the way it is affected by glycosaminoglycans, and the way it affects the binding of PAI-1 to vitronectin, we propose here a mechanism through which plasmin can arrest its own production by feedback signalling (Scheme I). At the initial stage of fibrinolysis, the plasminogen activator converts plasminogen to plasmin. This is made possible since PAI-1 is then anchored (trapped) by the vitronectin molecules immobilized in the extracellular matrix [33], pre-

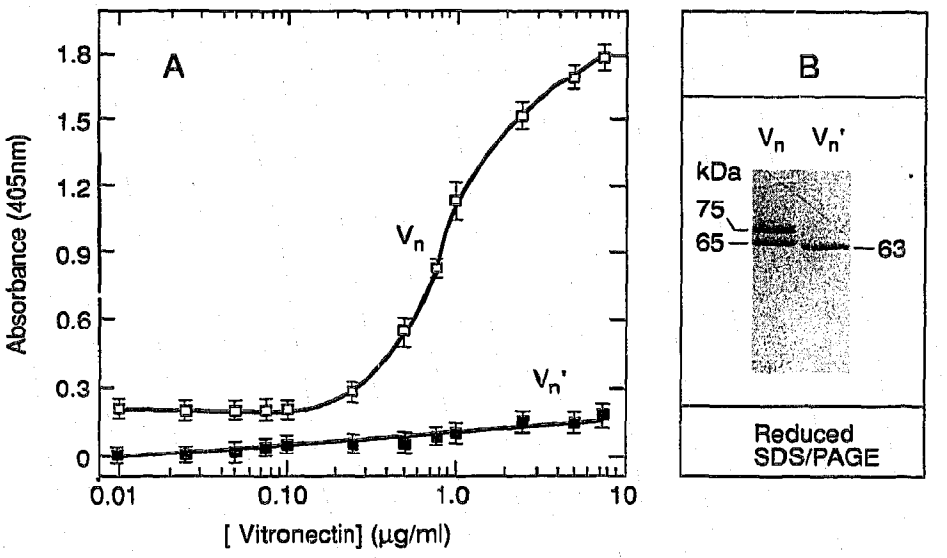


Fig. 3. Effect of the plasmin cleavage of vitronectin on its binding onto immobilized PAI-1. A vitronectin preparation (V_n) containing an approximately equimolar concentration of V_{75} and V_{65+10} was cleaved with plasmin as described in Fig. 1, allowing the $V_{75} \rightarrow V_{63+12}$ cleavage to proceed to completion (see panel B). The native vitronectin (V_n) and its plasmin-clipped product (V_n') were compared with regard to their binding to immobilized activated PAI-1, determined by an enzyme immunoassay as described previously by Salonen et al. [6]. Each point in the graph represents the mean ± SEM of triplicate determinations. The activation of PAI-1 in this experiment was carried out by treatment with SDS [6]. The reduced binding of V_n' was observed in 4 different experiments similar to that presented above. In 2 of these experiments PAI-1 was activated by boiling (rather than by SDS treatment) as described by Katagiri et al. [28]. In all cases there was a distinct (≥ 10-fold) difference in the half-maximal binding of V_n versus V_n'.

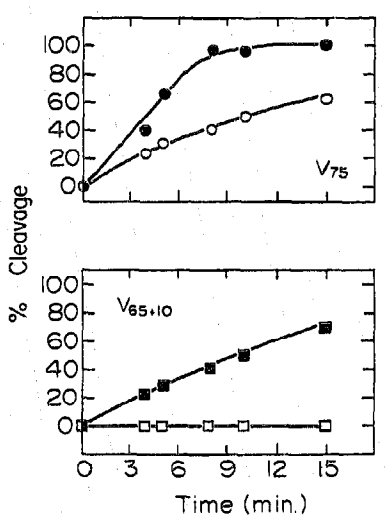
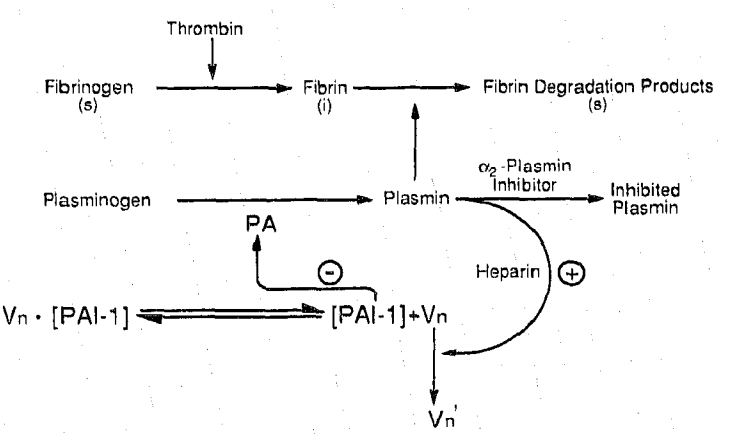


Fig. 4. Effect of heparin on the rate of plasmin cleavage of V_{75} and V_{65+10}. A preparation of vitronectin containing approximately equimolar amounts of V_{75} and V_{65+10} was cleaved with plasmin (0.1%, w/v) in the presence (●,■) and in the absence (○,□) of a 3-fold molar excess of heparin. At the indicated times the reaction was arrested by boiling (3 min at 95°C) and the % cleavage was determined by PKA phosphorylation, SDS/PAGE under reducing conditions, and autoradiography (as described in Fig. 1C), followed by densitometric scanning of the 75 and 65 kDa bands. Note that the 63 kDa band is not phosphorylated (cleavage occurs at Arg^{361} and thus V_{63} does not contain Ser^{378} making it possible to follow the extent of cleavage of V_{65+10} by monitoring the radioactivity of the 65 kDa band without interference from the adjacent 63 kDa band.

sumably through glycosaminoglycans. This anchoring of PAI-1 would locally deplete the inhibitor, preventing it from reaching and inhibiting the plasminogen activator. When plasmin levels become too high, the excess plasmin clips preferentially the vitronectin molecules immobilized in the subendothelium. Con-



Scheme 1. The sequence of molecular events involved in fibrinolysis, showing the mechanism proposed here whereby plasmin can arrest its own production by feedbacking signalling, unleashing PAI-1 from the immobilized vitronectin found in the extracellular matrix (e.g. the vascular subendothelium), which becomes exposed at the locus of a hemostatic event. PA, plasminogen activator; PAI-1, plasminogen activator inhibitor-1; V_n, vitronectin; V_n', plasmin-clipped vitronectin; (s), soluble; (i) insoluble; ⊕, stimulation; ⊖, inhibition.

sequently, the equilibrium between anchored PAI-1 and the detached (mobile) PAI-1 is displaced, thus unleashing PAI-1 to reach and inhibit the plasminogen activator and arrest plasmin production. It should be emphasized that the above mechanism does not specify which species of mobile PAI-1 inhibits the plasminogen activator. It may be either free PAI-1 or PAI-1 attached to plasma vitronectin in a soluble, stable, and active complex [6]. The impairment of this control mechanism of plasmin production may have wider clinical implications, in view of recent findings on the involvement of PAI-1 in angiogenesis [34], malignancy, and tumor invasion [1,35].

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