Evidence showing that the two-chain form of vitronectin is produced in the liver by a selective furin cleavage

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Abstract The adhesive protein vitronectin (75 kDa) occurs in human blood fluid in a one-chain (Vn_{75}) or a two-chain form (Vn_{65+10}), and is produced by a specific cleavage (at Arg^{379}–Ala^{380}), by a proteinase not identified hitherto. These two forms were shown to be functionally different and therefore, this cleavage may have a regulatory significance in vivo. Here, we report the use of a tailored one-chain recombinant Vn, a specific protein kinase A phosphorylation at Ser^{378}, and sequence analysis to show: (1) that none of the proteinases originating from blood, previously thought to be the endogenous proteinase (plasmin, thrombin, tPA, and uPA), is indeed the in vivo convertase; and (2) that furin, a serine endoproteinase residing in the secretory pathway of hepatocytes, where Vn is synthesized, specifically cleaves Vn at the endogenous cleavage site. Consequently, we propose that the Vn_{75} to Vn_{65+10} conversion takes place in the liver (not in blood) and is carried out by furin. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Vitronectin (Vn) was discovered as a 'serum spreading factor' [1]; however, it is now considered to be not only an important adhesive glycoprotein in the extracellular matrix, and in circulating blood, but also an important participant in a large variety of other biological functions. These include cell attachment, spreading and migration, blood coagulation, plasminogen activation, fibrinolysis, and the regulation of complement function ([2–5] and references therein).

Vn is present in human blood fluid at concentrations of 200–400 μg/ml, and therefore constitutes 0.2–0.5% of the total plasma proteins. Reduced plasma levels of Vn have been reported in patients with severe liver failure, suggesting that the liver is the major source of Vn in plasma [6,7].

In circulating blood, Vn is found in two molecular forms: a one-chain form of 75 kDa (Vn_{75}) and a clipped form (Vn_{65+10}) composed of two chains (65 and 10 kDa) held together by a disulfide bridge [8]. The two forms seem to be the product of distinct genes, that differ in a single amino acid at position 381. Methionine at this position gives rise to the V_{75} form, whereas threonine gives rise to a cleavage in vivo (at Arg^{379}–Ala^{380}), and yields the Vn_{65+10} form [9].

Since evidence was presented to show that Vn is secreted from Hep G2 cells as a single chain glycoprotein [10], it seemed reasonable to assume that this cleavage takes place in blood fluid. Several blood proteinases were proposed to carry out the Arg^{379}–Ala^{380} cleavage, however, none of them was rigorously demonstrated (by sequence analysis) to be the endogenous proteinase that carries out the cleavage in vivo. Consequently, the locus of the endogenous cleavage was not established unequivocally.

Our interest in establishing the identity of this endogenous proteinase stemmed from our finding that this endogenous cleavage yields a functionally distinct form of Vn. For example, we found that under physiological conditions, Vn_{65+10} has a higher affinity for heparin [11], and a different heparin dependence in its phosphorylation by protein kinase A (PKA) [12].

Here we show that furin, a serine endoproteinase which resides in the secretory pathway of hepatocytes selectively cleaves this bond, and propose that the conversion of Vn_{75} to Vn_{65+10} takes place in the liver (prior to secretion) and not in blood, as suggested hitherto.

2. Materials and methods

2.1. Chemicals and enzymes

The following materials were purchased from the commercial sources: heparin Sepharose, plasmin, and α-thrombin from Sigma; [γ-^{32}P]ATP (3000 Ci/mmol), and [^{35}S]methionine from Amersham; nitrocellulose membranes from Schleicher and Schuell; t-PA, and u-PA were purchased from Calbiochem; recombinant *N*-glycanase from Genzyme diagnostics, furin was purchased from Affinity Bioreagents (ABR).

2.2. Other proteins and enzymes

Vn was purified from freshly frozen human plasma as described previously [8] with the modifications used routinely in our laboratory [11,13]. The catalytic subunit of PKA was purified according to the method described by Beavo [14].

2.3. Expression and purification recombinant Vn

The Vn(Thr-381) was expressed as described previously [15]. The expressed protein was collected from medium of High-5 infected cells and loaded on a heparin agarose column previously equilibrated with 150 mM NaCl/50 mM Tris–HCl (pH 7.5), all at 4°C. The column was washed with the same buffer and fractions were eluted by a gradient of 150 mM to 1 M NaCl in Tris–HCl (pH 7.5). Eluted fractions were analyzed by immunoblotting and by silver staining. Samples of the peak were pooled and concentrated on centrifugal membrane filter device (Millipore), yielding a pure and concentrated Vn(Thr-381) (0.5–1 mg/ml, as determined by the Pierce protein assay).

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2.4. Cleavage of Vn(Thr-381) by various blood proteinases (thrombin, plasmin, u-PA, or by t-PA)

The reaction mixture (180 μl) contained the following constituents at the indicated final concentrations: Vn (27 μg/ml), HEPES buffer (50 mM), pH 7.5, and one of the proteinases as follows; thrombin (17.5 U/ml), plasmin (1.7 μg/ml), u-PA, t-PA (200 IU in 240 μl). Each reaction was allowed to proceed at 37°C for the time periods indicated in the appropriate figure legend.

2.5. Cleavage of Vn(Thr-381) by furin

The reaction mixture (total volume 100 μl) contained the following constituents at the indicated final concentrations: Vn(Thr-381) (20 μg/ml), HEPES buffer (100 mM), pH 7.5, CaCl$_2$ (2 mM), β-mercaptoethanol (1 mM), octyl-β-D-glucopyranoside (0.1%), and furin (100 U/ml). The reaction was allowed to proceed at 30°C for 20 h. Aliquots were removed and analyzed by PKA phosphorylation (which occurs specifically at Ser[378]) and SDS–PAGE with Coomassie blue staining in order to identify the cleavage products. The experimental conditions used for the cleavage were adopted from [16]. It should be noted that the prolonged incubation with Vn was necessary to obtain the extent of cleavage needed for analysis of the cleavage products, presumably due to the fact that the enzyme we used is a recombinant protein, and, as such, it might not be optimally folded. It might also be due to the fact that furin resides in the cell in a membranal milieu which we might not adequately simulate in aqueous solution.

2.6. Phosphorylation of Vn(Thr-381) by PKA

The phosphorylation assay mixture (50 μl) contained the following constituents at the indicated final concentrations: Vn(Thr-381) (11 μg/ml), the C-subunit of PKA (3 μg/ml), magnesium acetate (20 mM), [γ^{32}P]ATP (10 μM; 6 Ci/mmol), HEPES buffer (20 mM, pH 7.5), and heparin (10 μg/ml). The reaction was allowed to proceed at room temperature for 30 min and was arrested by the addition of 17 μl of 4× concentrated Laemmli's sample buffer and boiling for 3 min. Thereafter, the samples were subjected to SDS–PAGE, the gels were then dried and exposed to radiography.

2.7. Western blots analysis of Vn(Thr-381)

The blots were probed with polyclonal α-Vn antiserum, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase. The bands were visualized by ECL.

2.8. Sequencing the 10-kDa band produced by furin cleavage of Vn(Thr-381)

Furin-cleaved Vn(Thr-381) was run on 12.5% PAGE previously pre-run for 10 min using 5 mM sodium thioglycolate in the running buffer. The gel was transferred to a PVDF membrane using a Bio-Rad wet transfer apparatus. The bands were visualized by light Coomassie blue staining (in 50% methanol and 5% acetic acid, for 5–10 min) followed by destaining in 50% methanol. The 10-kDa band, excised from the dried membrane, was sequenced in a Procise protein sequencer 491, from Applied Biosystems.

2.9. Preparation of the Vn(1–379) mutant, and of the Vn(S378A) mutant

The Vn(1–379) mutant was generated by inserting a stop codon after R[379]. Taking advantage of the two Msc1 sites (332 base pair apart) a PCR fragment was generated using a sense oligonucleotide upstream from the first Msc1 site and an antisense oligonucleotide downstream the second Msc1 site which contained a TAA stop codon insertion (underlined). The antisense oligonucleotide sequence was: 5′-GGA TGG CCA CGT <u>TTA</u> GCG GGA TGG CCG-3′. The PCR product was digested with Msc1 and subcloned into Msc1 digested Vn(Thr-381)-pRSET.

The S378A point mutation in Vn was generated by PCR amplification using Vn(Thr-381) cDNA (in pGEX 2T) as a template. The sense oligonucleotide was: 5′-TCC CGC CGG CCA GCC CGC GCC-3′. The antisense oligonucleotide designed from the vector-pGEX 2T was: 5′-CGT CAG TCA GTC ACG ATG AAT TC-3′. Purified PCR-amplified fragments digested with NaeI and EcoRI were ligated into Vn(Thr-381)-pGEX-2T digested with NaeI and EcoRI. Both constructs were sequenced in order to confirm that the amplification was correct, before further subcloning into the PVL 1393 transfer vector (BamHI and EcoRI sites). Forced recombination into the viral DNA and further procedures for the expression were carried out as described earlier [15].
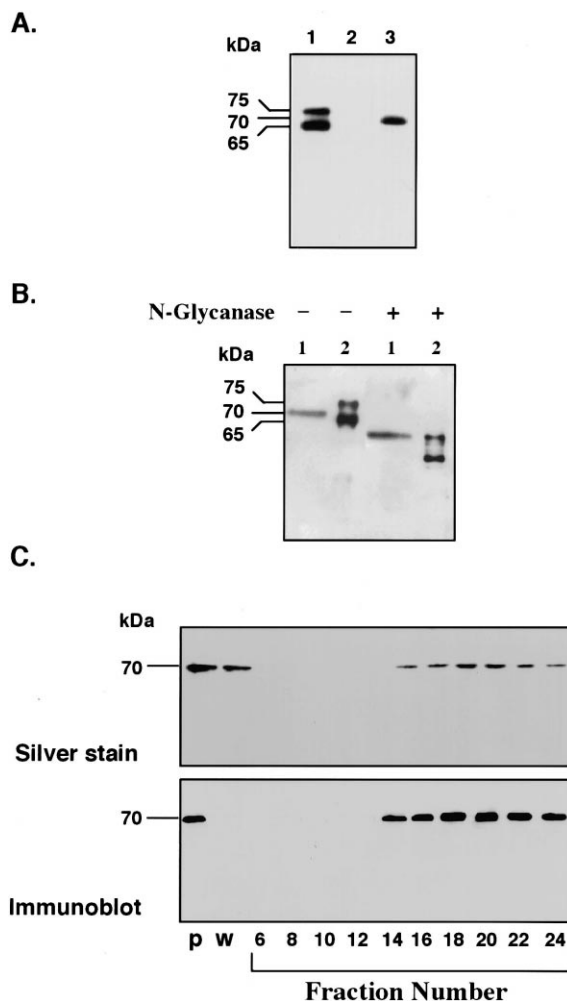

Fig. 1. Purification and characterization of Vn(Thr-381) expressed in a baculovirus system. (A) Expression of Vn(Thr-381) in High-5 insect cells: Vn was expressed in baculovirus infected High-5 cells. Samples of medium containing secreted Vn(Thr-381) were analyzed by immunoblot using anti-human Vn polyclonal antibodies. Vn(Thr-381) (lane 3); medium of non-infected High-5 cells (lane 2); and Vn purified from human plasma (lane 1). (B) Deglycosylation of plasma Vn, and of Vn(Thr-381) expressed in High-5 cells. Medium of a sample containing secreted Vn(Thr-381) (lane 1), or a sample of Vn (lane 2), purified from plasma. Samples (0.5 μg of each) were denatured at 56°C for 15 min then treated with 1 U of N-glycanase in 100 mM Tris buffer, pH 8.6 (total reaction mixture 100 μl) for 2 h at 37°C. The samples were analyzed by immunoblotting using anti-human Vn polyclonal antibodies. (C) Purification of Vn(Thr-381) expressed in High-5 insect cells. Vn(Thr-381) collected from medium of High-5 infected cells was loaded on a heparin agarose column previously equilibrated with 150 mM NaCl/50 mM Tris–HCl (pH 7.5), all at 4°C. Thereafter the column was washed with the same buffer and fractions were eluted by a gradient of 150 mM to 1 M NaCl in a Tris–HCl buffer (pH 7.5). Aliquots from the indicated fractions were boiled (5 min in Laemmli's sample buffer) and loaded on SDS–PAGE (10% acrylamide). The gels were analyzed by silver staining (top gel), and by immunoblotting with anti-Vn polyclonal antibodies (bottom gel). p, put on; w, wash through.

3. Results and discussion

3.1. Expression, characterization and purification of the Vn(Thr-381) used in this study

To identify the endogenous proteinase that clips Vn at the Arg[379]–Ala[380] bond, we prepared a recombinant Vn contain-
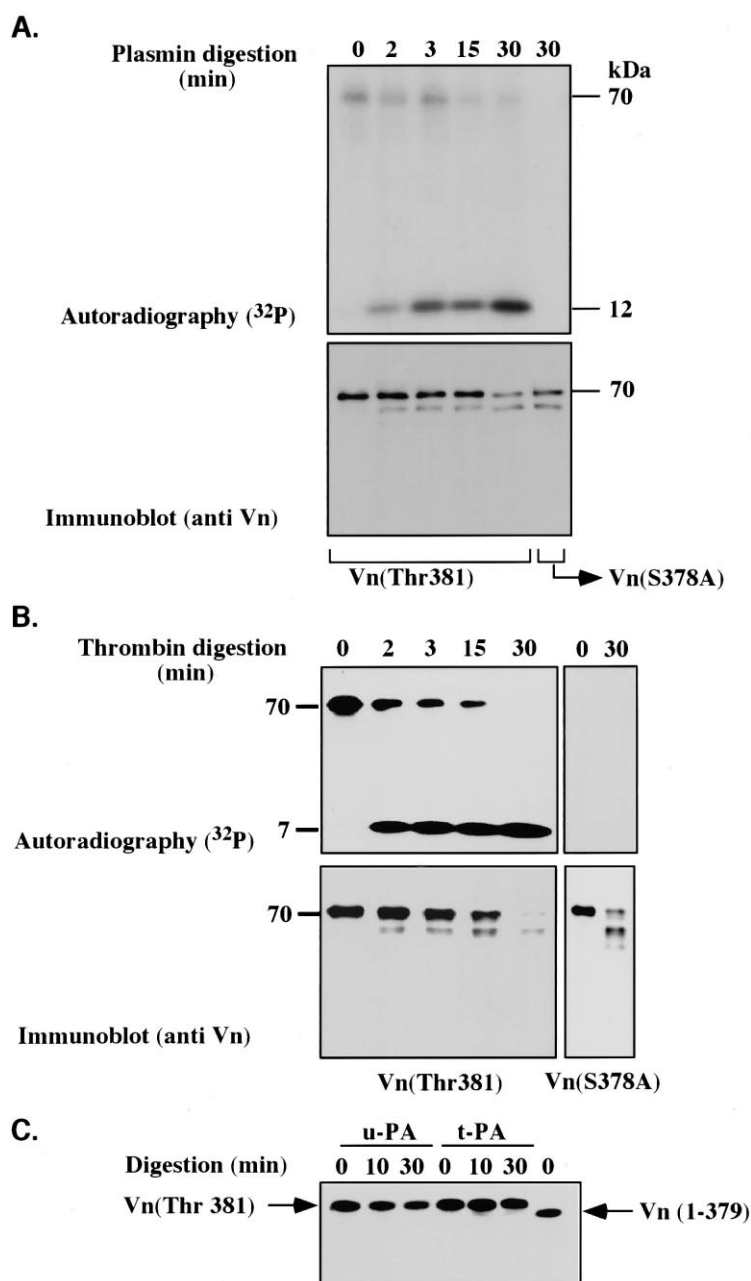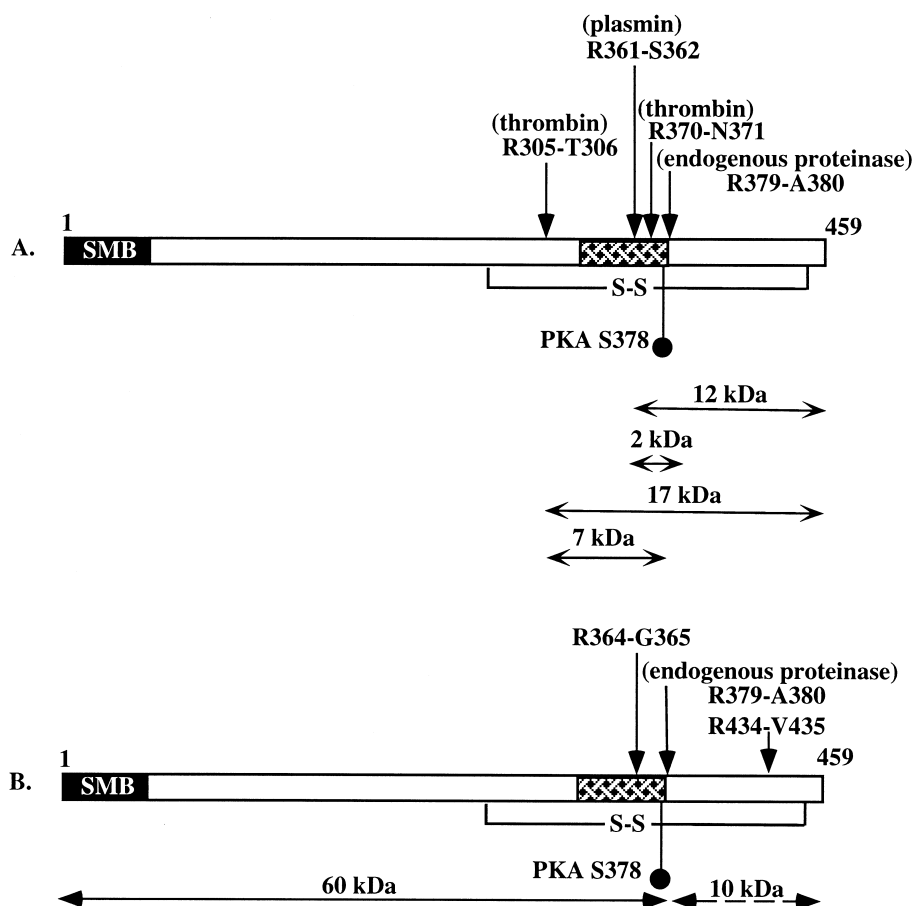
Fig. 2. Identification of the Vn cleavage products following digestion by proteinases originating from blood fluid. (A) Monitoring the plasmin cleavage. Vn(Thr-381), or Vn(S378A) mutant were digested with plasmin (for details, see Section 2). The cleavage products were revealed by PKA phosphorylation (one site in Vn at Ser^{378}) which was carried out at 22°C for 30 min in the presence of $[\gamma^{32}P]ATP$. The reaction was terminated by boiling for 5 min in Laemmli's sample buffer and loading on SDS–PAGE (10% acrylamide). The gels were dried and exposed to autoradiography (top gel) or transferred to nitrocellulose membranes and blotted (bottom gel) using human anti-Vn polyclonal antibodies. (B) Monitoring the thrombin digestion. Vn(Thr-381) was digested by thrombin (for details, see Section 2). The cleavage products were revealed as described in A. (C) Monitoring the u-PA and t-PA digestion. Vn(Thr-381) was digested by u-PA or t-PA (for details, see Section 2). The cleavage products were revealed as described in A.

ing Thr at position 381 (Vn(Thr-381)), as in the physiological precursor of the two-chain form of Vn. This recombinant Vn (MW $\sim$ 70 kDa (Fig. 1A)) was expressed in High-5 insect cells and secreted into the growing medium using its own signal peptide. The secreted protein was found to be a one-chain protein, with a reduced molecular size (70 instead of 75 kDa) most likely due to an incomplete glycosylation in the insect cells. Indeed, upon enzymatic deglycosylation, both the Vn(Thr-381) we prepared, and the one-chain form of Vn (75 kDa) isolated from plasma, were found to co-migrate when

run alongside (Fig. 1B). Therefore, the recombinant Vn that contains a Thr residue at position 381 and is not clipped in the High-5 cells is a suitable substrate for the identification of the endogenous proteinase that cleaves Vn at its Arg^{379}–Ala^{380} bond. The Vn(Thr-381) we collected from infected high-5 cells medium was purified on a heparin agarose column, and the eluted fractions were analyzed by immunoblotting and by silver staining and shown to be pure (Fig. 1C). The samples from the peak fractions were pooled and concentrated to 0.5–1 mg/ml as described in Section 2.

Scheme 1. Schematic presentation of the cleavage sites and localization of the proteolytic fragments generated following exposure to blood-fluid proteinases and subsequent phosphorylation with PKA at Ser^{378}. (A) The Vn cleavage products generated following plasmin or thrombin cleavage. Based on the already known sites of cleavage by plasmin [17] and by thrombin [18], we predicted that the radiolabeled fragments of cleaved Vn(Thr-381) used in this study should be: a 12-kDa radiolabeled fragment containing residues 362–459 if plasmin does not cleave at the endogenous cleavage site (Arg^{379}–Ala^{380}); and a 2-kDa radiolabeled fragment corresponding to residues 362–379 if plasmin also cleaves at this site. Similarly, based on the known sites of thrombin cleavage in Vn, we expected to get a 17-kDa radiolabeled fragment originating from the 305–459 segment in Vn, if thrombin does not cleave the endogenous cleavage site (i.e. the Arg^{379}–Ala^{380} bond), and only a 7-kDa fragment corresponding to residues 305–379 in Vn, if thrombin cleaves this site. (B) The Vn cleavage products generated following its digestion with furin. If furin cleaves Vn at the Arg^{379}–Ala^{380} bond we expect to generate a 60-kDa radiolabled fragment corresponding to the 1–379 segment, which should contain the PKA phosphorylated Ser^{378}.

3.2. Vn(Thr-381) is not cleaved by proteinases found in blood

Based on the approximate MW of the cleavage products obtained from plasma Vn by some basophilic proteinases it was assumed that either plasmin, tPA, or uPA might be the natural endogenous proteinase responsible for the cleavage of the Arg^{379}–Ala^{380} bond in Vn. The identification and characterization of this endogenous proteinase further attracted our attention when we found that the one-chain and the two-chain forms of Vn differ in their properties in a way that might imply a difference in their physiological function [11,12] and have a distinct regulatory role in blood. If so, the proteinase might even constitute an interesting pharmacological target. The determination of the locus in which this cleavage occurs (blood itself as assumed hitherto, or liver where the biosynthesis of Vn is known to take place) is also important in that respect.

In our search for the endogenous proteinase, we used: (1) a one-chain recombinant Vn containing Thr at position 381 (Vn(Thr-381)) as in the natural precursor of the two-chain Vn form, as well as a truncated Vn at the endogenous site (Vn(1–379) and a single-site mutated Vn (Vn(S378A)); (2) the

selective phosphorylation of Ser-378 with PKA; (3) the consensus sequence for the cleavage site of each proteinase; and (4) a rigorous determination of the exact cleavage site of each suspected proteinase by amino acid sequencing or by mass spectrometry of the cleavage products, and not only by determining the approximate molecular size of the cleavage product(s) using SDS–PAGE.

We have previously shown that plasmin cleaves the Vn found in blood fluid at the Arg^{361}–Ser^{362} bond yielding a 12-kDa radiolabeled fragment [17]. However, we considered the possibility that all the cleavable Vn in blood with a Thr at position 381 had already been clipped and that the plasmin cleavage at the Arg^{361}–Ser^{362} bond that yielded the 12-kDa radiolabeled fragment represents the plasmin cleavage of the Vn molecules having a Met at position 381 (to investigate possibility that plasmin also cleaves at Arg^{379}–Ala^{380}, we needed a one chain Vn having a Thr at position 381 (Vn(Thr-381)). If plasmin cleaves this Vn at Arg^{379}–Ala^{380}, we should get a 2-kDa radiolabled band of the phosphopeptide 361–379, rather than the 12-kDa fragment of the segment 362–459 (see Scheme 1A for size prediction).

Fig. 2 depicts the exposure of Vn(Thr-381) to four proteinases which are activated in blood. It is clearly evident that while plasmin and thrombin cleave Vn(Thr-381) (Fig. 2A,B), u-PA and t-PA do not cleave it (Fig. 2C). When Vn(Thr-381) is exposed to plasmin for increasing time periods, and then subjected to phosphorylation by PKA, a $\sim$12-kDa clipped product is formed that was found to be radiolabeled by radioactively labeled ATP and PKA (Fig. 2A, upper panel). The phosphorylation of Vn by PKA was previously shown to be site specific with Vn isolated from plasma [12]. Nevertheless, this was reconfirmed here with the recombinant substrate used in our study, by the demonstration that when Ser-378 is mutated to Ala, it is not phosphorylated at all by PKA (Fig. 2A,B). Since upon cleavage with plasmin the radiolabel is found in the small fragment (12 kDa) we could conclude that plasmin does not cleave Vn at the Arg^{379}–Ala^{380} bond since the Ser^{378} phosphorylation site precedes this bond (cf. Scheme 1).

We have previously shown that thrombin cleaves Vn at Arg^{305}–Thr^{306}, and at Arg^{370}–Asn^{371} [18]. If thrombin cleaves Vn also at the endogenous cleavage site (Arg^{379}–Ala^{380}), we would expect to get a 7-kDa radiolabled band originating from the 305–379 segment (see Scheme 1A). Indeed, such a labeled peptide was formed (Fig. 2B, upper panel). However, mass-spectrometry analysis of the cleaved fragments indicated that this peptide was formed as a result of an additional thrombin cleavage site (at Arg^{444}–Ser^{445}, not reported previously), that yields a 371–444 radiolabeled peptide.

3.3. Furin selectively cleaves the Arg^{379}–Ala^{380} bond in Vn

In view of the fact that none of the plasma proteases tested was able to cleave Vn at the endogenous cleavage site, we considered the possibility that the proteinase might cleave Vn already during its production in the liver. We considered this possibility, in spite of the fact that previous reports indicated that Vn is secreted from hepatoma cells in its single chain form (MW of 75 kDa [10]). This reconsideration was based on another report from which one could deduce that during Vn production in Hep G2, both forms of Vn (the one chain (Vn_{75}), and the two chain form (Vn_{65+10})) are secreted [19]. In addition, a similar observation was also made in our laboratory (Z. Gechtman and S. Shaltiel, unpublished results).

In view of the above, we attempted to establish whether the cleavage of Vn at Arg^{379}–Ala^{380} occurs in the liver prior to secretion, for example by a member of the hepatic family of proenzyme convertases (PCs), that are known to reside in the

Table 1
Sequence analysis of the 10-kDa band resulting from Vn(Thr-381) cleavage by furin

Amino acid position in Vn	Cycle	Amino acid determined	Yield (pmol)
380	1	A	14.0
381	2	T	8.5
382	3	W	0[a]
383	4	L	13.4
384	5	S	13.5
385	6	L	12.3
386	7	F	7.8
387	8	S	13.3
388	9	S	13.8
389	10	E	7.5

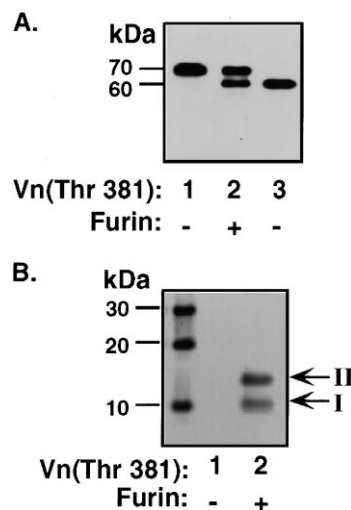[a]The yield was too low for quantitative determination because of the lability of tryptophan during acid hydrolysis.

Fig. 3. Furin cleaves Vn at the Arg^{379}–Ala^{380} bond, yielding Vn_{65+10}. The recombinant Vn(Thr-381) was digested by furin for 20 h at 30°C. The cleavage products were revealed (A) by PKA phosphorylation (as described in Fig. 2) and (B) by Coomassie blue staining, comparing the low MW bands of Vn(Thr-381). The products of furin digested Vn(Thr-381) (lane 2, A and B) were compared to Vn(Thr-381) incubated under identical conditions, but without furin (lane 1, A and B), and to the Vn(1–379) truncated mutant, in which a stop codon was inserted after the endogenous cleavage site, to yield the polypeptide segment 1–379 (A, lane 3).

secretory pathway of hepatocytes. The candidate proteinase chosen was furin, for the following reasons: (1) it was previously shown to be highly expressed in Hep G2 cells and in normal hepatocytes [20], and to reside in the *trans* Golgi network, where it cleaves a wide variety of proenzymes [21]; (2) it is commercially available in a recombinant form; and most importantly, (3) its specificity is most appropriate to bring about the endogenous cleavage of Vn at $R^{376}PSR\downarrow A^{380}$.

It should be noted that the minimal consensus sequence for cleavage of furin is $RXXR\downarrow$ [16], is found at three different locations in Vn, ($R^{361}SQR\downarrow G^{365}$, at $R^{376}PSR\downarrow A^{380}$, and at $R^{431}TRR\downarrow V^{435}$). However, in a study surveying the sequences of various protein substrates processed constitutively by furin the cleavage at $R\downarrow A$ was found to have a high preference. For example, out of 34 precursor proteins processed by furin, 10 were cleaved at an Arg–Ala site, only two were cleaved at an Arg–Gly site, and none was cleaved at an Arg–Val site [22]. Taking advantage of the specific PKA phosphorylation of Vn at S^{378}, and the fact that the optimal consensus sequence for furin cleavage is at $R^{376}PSR\downarrow A^{380}$, we expected that if furin is indeed the endogenous proteinase, the cleavage of Vn(Thr-381) would yield one radioactively labeled product with a MW of $\sim$60 kDa (the radioactive label originating from S^{378}). As seen in Fig. 3A (lanes 2 and 3), this is indeed the case: the cleavage product obtained co-migrated with the truncated mutant Vn(1–379). To obtain a rigorous identification of the cleavage site we searched for the $\sim$10 kDa (non-labeled) cleavage product which should also be formed as a result of the furin cleavage in order to sequence it and establish unequivocally the cleavage site. For this purpose, we subjected the reaction mixture to SDS–PAGE and staining with Coomassie blue, focusing on the low MW products (<30 kDa). As seen in Fig. 3B after cleavage with furin, we obtained two bands (I and II). Band I had exactly the sequence corresponding to the free N-terminus which is to be formed as

a result of the endogenous cleavage (Table 1). In addition, we often observed an additional band (Band II in Fig. 3B, lane 2). The relative amount of Band II varied from one experiment to another and our attempts to positively identify its structure failed so far. However, we found that it has a blocked N-terminus and, therefore, even if it originates from Vn, it could not originate from the vicinity of the endogenous cleavage site (cf. Scheme 1).

3.4. Concluding remarks

The results presented in this report show that none of the serum proteinases previously proposed to cleave the Arg^{379}–Ala^{380} bond is the endogenous proteinase (they all cleave in the vicinity of this bond, but not at the bond itself). However, furin, a calcium-dependent serine endoprotease which resides in the secretory pathway of hepatocytes specifically cleaves this bond and may thus be the endogenous proteinase responsible for the physiological cleavage of the one-chain into the two-chain form of Vn. On the basis of these findings, we propose that the Vn_{75} to Vn_{65+10} conversion takes place in the liver prior to secretion, not in the blood, as hitherto suggested. This finding sets the stage for getting a deeper insight into the physiological (possibly regulatory) significance of the Vn_{75} to Vn_{65+10} conversion, and identifies a potential target enzyme for pharmacological intervention.

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