Hydrolysis of Platelet Vitronectin by Calpain*

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Vitronectin (Vn), an adhesive glycoprotein present in the circulation and in a variety of tissues, appears to have a number of biological functions. It belongs to a group of adhesion molecules that mediate attachment of cells by binding to specific cell surface receptors of the integrin family (1, 2). Vn also exerts several regulatory functions in cell-associated proteolytic enzyme cascades, including the complement, coagulation, and fibrinolytic systems. For example, Vn binds to and stabilizes the biological activity of type 1 plasminogen activator inhibitor (PAI-1), the physiological inhibitor of both tissue-type and urinary-type plasminogen activators (3). Binding sites for PAI-1 in Vn have been mapped to both the N-terminal somatomedin B domain of Vn (4–6) and the C-terminal heparin binding domain (7–10). The relative importance of each single site for the binding of PAI-1 has not been established.

Plasma contains the majority of Vn present in vivo, and plasma Vn concentrations have been reported to be 200–400 μg/ml (2). A second circulatory pool of Vn is contained within platelets (11–13), and the concentration of Vn in platelets has been estimated to be 2–8 μg/10⁹ platelets (12, 13). Thus, platelets contain approximately 0.8% of the circulating pool of Vn, and platelet Vn accounts for 1% of total platelet proteins. Platelet Vn is compartmentalized in the α-granules and released upon stimulation with physiological agonists (13). In contrast to plasma Vn, the released platelet Vn is present in a high molecular weight form and, at least in part, in complex with PAI-1 (13, 14). Moreover, Vn released from stimulated platelets expresses epitopes for a conformationally sensitive monoclonal antibody (mAb) that is not expressed in the native plasma form (14). Taken together, these observations have led to the concept that the circulation contains two structurally and functionally distinct pools of Vn.

In studying structure-function relationships of platelet Vn, it became apparent that platelet Vn is rapidly degraded in lysates prepared under nonenaturing conditions. Evidence is provided that Vn is susceptible to proteolytic modification by platelet calpain, resulting in the dissociation of PAI-1, heparin, and cell binding functions of the adhesive glycoprotein, suggesting that the principal PAI-1 binding site in Vn is located in N-terminal Vn fragments. These results suggest that calpains released upon platelet membrane damage or upon tissue injury and necrosis differentially regulate functional domains of the Vn molecule.

Vitronectin (Vn), an adhesive glycoprotein present in platelets but also regulates proteolytic enzyme cascades, including the blood coagulation, fibrinolytic, and complement systems. In human platelet lysates prepared by freeze-thawing or by the addition of nonionic detergent, the Vn antigen content was drastically reduced in comparison with lysates prepared in the presence of SDS, suggesting that Vn is hydrolyzed by platelet-associated enzymes. Exogenously added purified human Vn and Vn present in plasma were also cleaved by these enzyme systems. Degradation was mediated by a nonsecreted or membrane-associated protease system that was inhibited by E-64, EDTA, and leupeptin but not inhibitors of serine and aspartic proteases, suggesting an involvement of calcium-dependent cysteine proteases. Consistently, calpastatin inhibited the hydrolysis of Vn, suggesting that Vn is a substrate for calpain. This was confirmed in a purified system. Vn was cleaved by calpains I and II in a dose- and time-dependent manner, resulting in defined Vn fragments with similar electrophoretic mobility in comparison with those detected in platelet lysates. Functional characterization of the calpain-hydrolyzed Vn revealed that while the type 1 plasminogen activator inhibitor binding activity was unchanged, the heparin and cell binding functions were destroyed. These results suggest that calpains released upon platelet membrane damage or upon tissue injury and necrosis differentially regulate functional domains of the Vn molecule.

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1The abbreviations used are: Vn, vitronectin; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PAI-1, type 1 plasminogen activator inhibitor; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline.

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sodium dodecyl sulfate (1% v/v), by freeze-thawing (5 min in liquid nitrogen, 10 min at 37 °C; 3 cycles), or by treatment with Triton X-100 (0.5% v/v). In some experiments, the addition of PMA prior to lysis was omitted.

Susceptibility of Exogenously Added Vn to Degradation by Proteases Present in Hep 3B Cells and Washed Platelets—Hep 3B human hepatoma cells were obtained from ATCC and maintained as described (15). Confluent cells were washed three times with PBS and detached with trypsin/EDTA (Biowhittaker, Inc.). Trypsin was neutralized by the addition of complete medium, and the cells were washed three times with platelet wash buffer as above for the platelet isolation. Purified denatured Vn (1 μg; see below) was added to 1.3 × 10^5 cells resuspended in platelet wash buffer, and the cells were lysed either in SDS or by freeze-thawing. In similar experiments, washed platelets (2.5 × 10^7) were supplemented with either purified Vn (1 μg) or human plasma (1 μl) anticoagulated with sodium citrate (0.38% w/v). Again, cells were lysed either in SDS or by freeze-thawing. For some experiments, a constant amount of Vn (4 μg) was added to the indicated number of platelets in platelet wash buffer containing 1 mM Ca^2+^, and the platelets were lysed in 0.5% Triton X-100.

Purification of Vn and Digestion with Calpain—Vn was purified by heparin affinity chromatography in the presence of 8 μm urea (16) or in the absence of protein denaturants as described (17). The latter form of Vn was designated as native Vn. Purified Vn (1 μg) was dissolved in 40 μl of platelet wash buffer containing the indicated concentration of Ca^2+. The samples were incubated (37 °C) for 1 h, fractionated by SDS-PAGE, and analyzed either by immunoblotting using mAb 1244 or rabbit anti-human Vn (see below) or by staining with silver nitrate. For the functional characterization of the calpain-digested Vn, the enzymatic reaction was stopped by the addition of EDTA (10 mM) or E-64 (125 μg/ml).

Functional Characterization of Calpain-digested Vn—The PAI-1 binding activity of the calpain-derived Vn fragments was tested in competitive binding essays as described (4). Heparin binding of Vn fragments was determined by binding to immobilized heparin (18). The ability of calpain-derived Vn fragments to promote cell adhesion was tested essentially as described (19). The human fibroscarcoma cell line HT 1080 was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal calf serum. Semiconfluent cultures were labeled for 16 h with [35S]methionine (Amersham Corp.; 100 μCi/ml in 80% methionine-deficient medium, 20% complete growth medium). After washing with PBS, the cells were detached with trypsin/EDTA (BioWhittaker, Inc.). Trypsin was neutralized by the addition of complete medium, and the cells were washed three times with adhesion buffer (1 × Hanks' balanced salt solution, 50 mM HEPES, 1 mg/ml bovine serum albumin, 1 mM CaCl_2_, 1 mM MgCl_2_, pH 7.4) and resuspended to 1 × 10^6 cell/ml in adhesion buffer. Flat-bottomed microtiter wells were coated with the indicated concentration of purified Vn or Vn fragments. Following an 18-h coating period at room temperature, the plates were blocked with PBS containing 10 mg/ml bovine serum albumin. Cells (1 × 10^6) were allowed to adhere for 1 h. The wells were washed gently three times in PBS to remove nonadherent cells, and the bound cells were solubilized in 1% SDS and quantitated by β-counting. The radioactivity associated with bovine serum albumin-coated wells was subtracted from that of the Vn-coated wells. In control experiments, the coating efficiency of the Vn and Vn fragments was tested in a solid phase binding assay using rabbit anti-Vn IgG (10 μg/ml), followed by biotin-labeled goat anti-rabbit IgG (Zymed Laboratories, Inc.), streptavidin alkaline phosphatase (Zymed), and p-nitrophenyl phosphate (Zymed).

Miscellaneous—mAb 1244 was obtained by standard hybridoma techniques (5), and the hybridoma clone producing mAb BE 6 was kindly provided by Dr. D. Mosher (University of Wisconsin). mAbs were produced as ascites and employed forimmunoblotting analysis without further purification. Antibodies to Vn were also raised in rabbits, and the IgG fraction was purified using protein A-agarose. IgG was further affinity-purified on immobilized Vn as described (15). SDS-PAGE was performed on 4% acrylamide, stacking gel, and 9 or 15% acrylamide, resolving gel, according to Laemmli (20). After electrophoresis, the proteins in the gel were transferred onto nitrocellulose membranes and analyzed by immunoblotting essentially as described (15) using either radiolabeled secondary antibodies followed by autoradiography or peroxidase-labeled secondary antibodies followed by the ECL detection system.

RESULTS

Degradation of Vn in Lysates of Nonstimulated Platelets—Human platelets were isolated by differential centrifugation and washed, and the extent of cross-contamination with plasma Vn was assessed in the supernatant of nonstimulated platelets by immunoblotting using mAb 1244 raised against plasma Vn (Fig. 1A, lane 2). When the membranes were disrupted with the nonionic detergent SDS contained two Vn-related polypeptides of Mr standards indicated. In addition, fractionation of platelet lysates on a 15% PAGE gel followed by immunoblotting with mAb 1244 revealed no evidence for low molecular weight immunoreactive Vn fragments (Fig. 1A, lanes 5 and 6).

The apparent lack of Vn immunoreactivity upon freeze-thawing of platelets raised the possibility that the mAb 1244 epitope was destroyed by limited proteolysis. To explore this possibility, platelet lysates were analyzed by a second mAb with an epitope distinct from that of mAb 1244 (Fig. 1B, lanes 1 and 2) and by a polyclonal antibody (Fig. 1B, lanes 3 and 4). Using these antibodies, Vn immunoreactivity was again drastically reduced by freeze-thawing in comparison with the SDS lysates. These results raised the possibility that the degradation of Vn
Evidence that Vn released upon platelet stimulation is not susceptible to degradation. Washed platelets were stimulated with PMA for 10 min. The platelet releasates (lanes 1 and 2) and platelet pellets (lanes 3 and 4) were separated by centrifugation and harvested in SDS (lanes 1 and 3) or by freeze-thawing (lanes 2 and 4). Alternatively, the stimulated platelets were directly harvested in SDS (lane 5) or by freeze-thawing (lane 6). The proteins were fractionated by SDS-PAGE (9%) and analyzed by immunoblotting using mAb 1244. The migration of M₀ standards is indicated.

Evidence That Vn Is Degraded by a Nonreleased Platelet Enzyme System—Platelets contain a number of proteases that are released upon stimulation with platelet agonists and may hydrolyze proteins in the platelet releasates after separation from the platelet extracts (21). Experiments were performed to localize within platelets the protease systems responsible for the hydrolysis of Vn (Fig. 2). To test the involvement of released proteases, washed platelets were stimulated with PMA and Vn content was determined in the platelet releasates and platelet extracts after separation by centrifugation (Fig. 2). While the Vn concentration in the supernatant was unchanged upon freeze-thawing in comparison with the SDS-harvested sample (Fig. 2, lanes 1 and 2), the nonreleased or possibly re-bound Vn fraction was significantly reduced upon freeze-thawing (Fig. 2, lanes 3 and 4). It should be noted that under these experimental conditions, the majority of platelet-associated Vn was released into the supernatant and was not susceptible to proteolytic cleavage. When platelets were stimulated with PMA and the platelet releasates were not separated from the pellet, Vn immunoreactivity was lost upon freeze-thawing (Fig. 2, lanes 5 and 6). Similar results were obtained when platelets were stimulated with thrombin (1 unit/ml; not shown) or calcium ionophore A23187 (1 μM; not shown). These results indicate that the Vn-degrading protease system is not released upon stimulation.

Susceptibility of Plasma and Purified Vn to Hydrolysis by Platelet Proteases—While plasma Vn is present in the native folded conformation, platelet Vn is conformationally altered and present in high molecular weight multimers (14). To test the possibility that the susceptibility to proteolytic cleavage is a specific function of platelet Vn, purified native (Fig. 3, lanes 1–3) denatured Vn (Fig. 3, lanes 4–6), as well as plasma, an abundant source of Vn (1, 2) (Fig. 3, lanes 7–9), were added to nonstimulated platelets prior to lysis. In all three cases, Vn was quantitatively recovered from the SDS lysates, whereas the lysates prepared by freeze-thawing had significantly reduced Vn immunoreactivity (Fig. 3). However, the hydrolysis of plasma Vn was incomplete, suggesting that plasma either contains a subpopulation of Vn not susceptible to cleavage by platelet-associated proteases or that specific protease inhibitors present in plasma prevented the cleavage of Vn (Fig. 3, lanes 3, 6, and 9). These results indicate that the susceptibility to proteolytic cleavage by platelet-associated proteases is not a specific function of platelet Vn. It should be noted that the SDS lysates contained more Vn immunoreactivity in comparison with the exogenously added amount of Vn due to the presence of endogenous platelet Vn. Similar experiments using cell lysates prepared from the human hepatocarcinoma cell line Hep 3B revealed that exogenously added Vn was not susceptible to cleavage (Fig. 3, lanes 10–12). These data raised the possibilities that either the Vn-degrading proteases are not contained in Hep 3B cells or that protease inhibitors exceed the amount of specific proteases.

Identification of the Group(s) of Platelet Proteases Involved in the Degradation of Vn—In these experiments, platelet lysates were prepared in the presence of SDS (Fig. 4A, lane 1) or by freeze-thawing in the absence (Fig. 4A, lane 2) or presence of a number of protease inhibitors (Fig. 4A, lanes 3–8). α₂-Macroglobulin, a broad spectrum protease inhibitor, prevented the degradation of Vn in platelet lysates (lane 3). This observation confirms that proteases are involved in the loss of Vn immunoreactivity. The proteolysis was significantly reduced by the cysteine protease inhibitor E-64 (lane 4), the cysteine and serine protease inhibitor leupeptin (lane 5), and EDTA (lane 8). In contrast, inhibitors of serine and aspartic proteases (lanes 6 and 7) and reducing agents (i.e. 50 mM dithiothreitol; not shown) were ineffective. The inhibition profile raised the possibility that Ca²⁺-dependent cysteine proteases were involved in the degradation of Vn.

Platelets are an abundant source of both calpains I and II, Ca²⁺-dependent cysteine proteases that differ in their Ca²⁺ requirements to hydrolyze substrate proteins (22, 23). While calpain I is active in the presence of micromolar levels of calcium, calpain II requires millimolar concentrations of calcium (22). To more directly test the involvement of these protease systems, the effects of calpastatin, a relatively specific inhibitor of calpains I and II (23), on the degradation of Vn were tested. Calpastatin prevented the hydrolysis of Vn in a dose-dependent manner (Fig. 4B), suggesting that platelet Vn is cleaved by calpain.

Hydrolysis of Vn by Purified Calpain—The susceptibility of
Vn to cleavage by calpains was confirmed in a purified protein system (Figs. 6 and 7). In initial experiments, purified Vn was incubated with calpain II, and after incubation for 1 h at 37 °C, the digest was fractionated by SDS-PAGE and analyzed by immunoblotting or silver staining (Fig. 5). Vn was digested at a relatively low concentration (25 μg/ml) similar to that present in the ex vivo platelet Vn degradation experiments (Figs. 1–4) to allow a comparison of the results. The immunoblotting studies revealed that Vn is hydrolyzed by calpain II in a dose-dependent manner and that digestion with relatively low concentrations of calpain resulted in defined Vn fragments (Fig. 5A, lanes 1–4 (polyclonal antibodies) and lanes 5–7 (mAb 1244)). The mAb 1244 reacted with fragments of M, 45,000 and 60,000 (Fig. 5A, lane 7), whereas the polyclonal antibody in addition detects a fragment of M, 36,000 (Fig. 5A, lane 4). At higher concentrations of calpain II, the immunoreactivity of the polyclonal and monoclonal antibodies was drastically reduced, resembling results obtained with platelets (Fig. 5A, lanes 2 and 5). It should be noted that no smaller fragments were detected upon fractionation on a 15% acrylamide gel followed by immunoblotting using rabbit anti-human Vn (not shown). Identically sized fragments were detected by silver staining of limited digested Vn (Fig. 5B). At higher concentrations of calpain, the Vn fragments upon silver staining could not be discriminated from the autolytic fragments of calpain (not shown). The cleavage of Vn by calpain II was also dose-dependent with respect to the calcium concentration (Fig. 5C). While Vn was deaved, consistent with the reported Ca2+ concentration range required for activity of calpain II, between 5 mM and 500 μM Ca2+ (Fig. 5C, lanes 1–3), reduction of the concentration to 100 μM prevented the cleavage of Vn by calpain II (Fig. 5C, lane 4). These results strongly suggest that Vn is cleaved by calpain II and practically rule out the possibility that contaminating Ca2+-dependent protease potentially present in the calpain preparation is responsible for the observed cleavage. In similar experiments, Vn was also digested at a constant Vn to calpain ratio using Vn concentrations up to 250 μg/ml. Immunological analysis revealed no evidence for new lower molecular weight Vn fragments. However, analysis of the resulting sample by silver staining revealed the presence of lower M, fragments of approximately 22,000 and 18,000 that partially co-migrated with autolytic fragments of calpain (not shown) that were not further characterized in this study.

In related studies, the susceptibility of Vn to hydrolysis by calpain I was tested (Fig. 6). Calpain I hydrolyzed Vn in a Ca2+-dependent manner (Fig. 6A). Limited cleavage was observed in the presence of 10 μM Ca2+ (Fig. 6A, lane 3), and maximal cleavage was observed at approximately 100 μM Ca2+ (Fig. 6A, lane 4). Thus, as expected from the Ca2+ requirements of calpain I, a lower Ca2+ concentration was needed for Vn cleavage in comparison with calpain II (compare with Fig. 5). The electrophoretic mobilities of the resulting initial Vn fragments (i.e., M, 60,000, 45,000, and 36,000; not shown) were similar to those observed using calpain II (compare Figs. 5 and 6). The cleavage of Vn by calpain I was also dose-dependent with respect to the enzyme concentration, and the higher concentrations of calpain I resulted in the loss of Vn immunoreactive fragments (Fig. 6B).

Limited Proteolysis of Vn in a Purified System and ex Vivo Results in Similarly Sized Fragments—Results derived from the cleavage of Vn by calpains in the purified protein system predicted that Vn fragments should be detectable ex vivo in platelet lysates. To test this hypothesis, a constant amount of purified Vn was incubated with a decreasing number of platelets, lysed by the addition of Triton X-100, fractionated by SDS-PAGE, and analyzed by immunoblotting using mAb 1244 (Fig. 7). As predicted from the purified protein system, Vn was hydrolyzed by calpain present in platelets in a dose-dependent manner (Fig. 7, lanes 1–6). At higher concentrations, the Vn immunoreactivity was lost (lane 2), whereas at lower platelet concentrations, defined Vn fragments were detectable (lanes 3–6), and these fragments co-migrated with Vn fragments derived from cleavage by purified calpain II (lane 7). Thus, hy-
Limited proteolysis of Vn in a purified protein system and ex vivo results in similarly sized initial cleavage fragments. Purified denatured Vn (4 μg, lane 1) was added to washed platelets in platelet wash buffer containing 1 mM Ca²⁺; lane 2, 5 × 10⁶ platelets; lane 3, 1 × 10⁶ platelets; lane 4, 5 × 10⁵ platelets; lane 5, 1 × 10⁵ platelets; lane 6, 5 × 10⁴ platelets), and lysates were prepared by the addition of Triton X-100 (0.5% v/v). In lane 7, purified Vn was cleaved with calpain II at a calpain to Vn molar ratio of 1:1. The samples were fractionated by SDS-PAGE and analyzed by immunoblotting using mAb 1244. The mobility of M₇ standards is indicated.

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The binding of Vn to glycosaminoglycans is believed to be of importance for the localization of Vn in tissues and for the regulation of heparin-dependent proteolytic enzyme cascades (1, 2). The ability of calpain-hydrolyzed Vn to bind to glycosaminoglycans was tested in solid phase binding assays to immobilized heparin (Fig. 8). While intact Vn and Vn hydrolyzed with low concentrations of calpain bound to heparin in a dose-dependent manner, more extensive cleavage of Vn by calpain resulted in a successive loss of heparin binding. At the highest concentration of calpain, the heparin binding activity of Vn was practically abolished (Fig. 8B). The solid phase heparin binding assay employed polyclonal antibodies to Vn in the detection system, and results presented in Fig. 5A suggested that the immunoreactivity of Vn was reduced upon cleavage with high calpain concentrations. To exclude the possibility that the reduced Vn binding was not due to loss of immunoreactivity but actual reduced heparin binding, the ability of the
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...digest to compete with biotinylated Vn for binding to immobilized heparin was tested. Again, intact and incomplete hydrolyzed Vn competed with the binding of labeled Vn to heparin in a dose-dependent manner, whereas no competition was evident using Vn hydrolyzed by high calpain concentrations (not shown).

The ability of calpain-hydrolyzed Vn to promote cell adhesion was tested in adhesion assays using the human fibrosarcoma cell line HT 1080 (Fig. 8C). Microtiter wells were coated with the indicated concentrations of Vn or Vn digests, and after blocking, prelabeled cells were allowed to adhere for 1 h. After washing, bound cells were solubilized in SDS and quantified by β-counting. While intact Vn and Vn cleaved with low concentrations of calpain II promoted cell adhesion in a dose-dependent manner, the adhesion was drastically reduced using Vn cleaved by intermediate or high concentrations of calpain (Fig. 8C). In control experiments, the coating efficiency of the intact Vn was compared with that of the Vn digests. While the binding of the polyclonal antibodies to the Vn fragment-coated wells was unchanged at the three highest coating concentrations, the binding was slightly reduced (i.e. 3-fold) at the lowest concentrations in comparison with intact Vn (not shown). Thus, the reduced binding of cells to the calpain-digested Vn, at least at the three highest concentrations of Vn employed, was not due to reduced binding of the digest to the microtiter wells but rather to destruction of the cell binding domains of Vn. It should be noted that the functional characterization of the calpain-derived Vn fragments was performed in the presence of 10 mM EDTA (PAI-1 and heparin binding) or that hydrolysis was terminated by the addition of an excess amount of E-64 to both intact and cleaved Vn (cell adhesion) to prevent potential interference of residual calpain activity with the assay systems. Thus, hydrolysis of Vn by calpain dissociates the PAI-1, cell, and glycosaminoglycan binding functions of Vn and suggests that the principal PAI-1 binding site in Vn is distinct from its glycosaminoglycan binding domain.

DISCUSSION

In studies to further characterize the structure-function relationships of platelet Vn, it was noted that the mode of platelet disruption significantly affected the Vn immunoreactivity in platelets (Fig. 1). While platelets lysed by the addition of SDS contained Vn concentrations similar to those reported (12, 13), platelet lysates prepared by freeze-thawing or treatment with Triton X-100 lacked detectable Vn immunoreactivity (Fig. 1). The hydrolysis was not specific for platelet Vn but was also observed when purified Vn preparations and Vn present in plasma were added to platelet prior to lysis (Fig. 3). This degradation was significantly reduced under conditions promoting the inactivation of divalent ion-dependent cysteine proteases (Fig. 4). In addition, Vn degradation was prevented by calpastatin in a dose-dependent manner (Fig. 4), suggesting an involvement of platelet calpains in the hydrolysis of platelet Vn.

Platelets are an abundant source of calpains I and II, which have similar substrate specificity but differ in their divalent ion concentration requirements (23). Platelets are special in comparison with most other cell types in that the amount of calpain inhibitor (i.e. calpastatin) is lower than the amount of calpain (22,23). Accordingly, the proteolytic balance is shifted toward degradation of calpain-sensitive substrates in platelets but not in most other cell types (22). Consistently with these considerations, purified Vn added to Hep 3B cells prior to lysis was not susceptible to cleavage (Fig. 3). It should be noted that the protease inhibitor experiment could only provide circumstantial evidence that calpains are involved in Vn degradation. For example, it appears possible that a limited proteolysis of Vn by calpain may cause destabilization of the structural rigidity, making it more sensitive to proteolytic attack by other cellular proteases. Alternatively, other proteases may be activated by calpain and degrade Vn independent of calpain.

To more directly test the involvement of calpains in the degradation of platelet Vn, the susceptibility of Vn to cleavage by calpains was tested in a purified protein system. Purified calpains I and II degraded Vn in a dose-dependent manner, and hydrolysis required calcium ions (Figs. 5 and 6). At relatively high enzyme concentrations, Vn was digested to low molecular weight fragments, resulting in reduction or loss of immunoreactivity for both polyclonal and monoclonal antibodies (Fig. 6). The concentration of enzyme used in these in vitro experiments is in the range expected in vivo since calpains are in at least 10-fold molar excess over Vn present in platelets (23, 24). In the presence of Ca2+ concentrations lower than the requirements for calpains I and II, respectively, no cleavage of Vn was detectable, indicating that the observed cleavage was not due to contaminating calcium-independent proteases (Figs. 5 and 6). The extensive digestion of Vn by calpains was rather surprising, since proteolysis induced by calpain usually produces larger fragments that are not further susceptible to calpain cleavage (25). While the intermediate sized fragments were not observed in the initial degradation experiments of Vn in platelet lysates (Figs. 1–4), dose-response experiments of platelets revealed that identically sized Vn fragments in comparison with those observed in the purified protein system could also be recovered from the platelet lysates (Fig. 7). This observation does not exclude the possibility that lower molecular weight Vn fragments, undetectable by immunoblotting, are generated in platelets that are different from those obtained in a purified protein system. Thus, calpains apparently exert their initial proteolytic action on Vn present in platelets, resulting in the generation of relatively large, immunologically detectable fragments without the involvement of other proteases.

The functional consequences of the degradation of Vn by calpain were further analyzed. In many instances, calpain actually activates biological systems by limited proteolysis, resulting in the gain of function. While we did not observe an increase in biological activities, the modulation of functional activities was domain-specific. For example, the PAI-1 binding activity of the digested Vn was unchanged (Fig. 8), indicating that a high affinity PAI-1 binding site was intact. The observation that the somatomedin B domain, recently identified as a high affinity PAI-1 binding site (5), is extensively disulfide-linked and thus relatively resistant to proteolytic attack (26) is consistent with this result.

The cell binding domain is located immediately C-terminal of the PAI-1 binding site (amino acids 45–47) in the connecting region of the Vn molecule (27, 28). Hydrolysis of Vn by calpain completely abolished the binding of cells to Vn, suggesting that either this domain is destroyed or that the domain is not accessible on the surface of these calpain-derived fragments upon immobilization of microtiter wells. In addition, the heparin binding domain located between amino acids 341 and 379 (1, 2) was destroyed by calpain cleavage. Thus, these results indicate that calpain can dissociate specific Vn functions. The heparin binding domain has been implicated in the binding of Vn to tissues and extracellular matrices but also as a second heparin binding domain has been implicated in the binding of Vn to tissues and extracellular matrices but also as a second

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balance to promote plasminogen activation.

While the data presented here provide a better understanding of the structure-function relationships of the Vn molecule, particularly with respect to the significance of reported PAI-1 binding domains, the physiological significance of the observed degradation of Vn by calpain remains to be elucidated. Within the detection limit of our Vn degradation assay, a biologically active protease was not released upon stimulation with physiological agonists. It should be noted that upon stimulation of platelets with thrombin or platelet-activating factor, approximately 1% of total calpain II immunoreactivity is expressed on the platelet surface (24). It is unknown whether this membrane-associated calpain is biologically active. The observation that Vn was not degraded would rather suggest that this form of calpain is biologically inactive. A number of considerations could account for this observation, including complex formation of calpain with specific protease inhibitors (e.g. calpastatin) on the platelet surface. Alternatively, other released proteins could be more susceptible to degradation and serve as competing substrates for the hydrolysis of Vn by calpain. However, since the majority of calpain is present in the platelet cytoplasm, whereas Vn is contained within the α-granules, both proteins are physically separated unless platelets are stimulated or damaged or platelet membrane integrity is compromised. These considerations suggest that the release of calpains followed by a subsequent degradation of Vn may be important during prolonged storage of platelet concentrates for transfusion, in immunologically induced membrane damage, upon dissolution of aging thrombi, or in tissue injury and necrosis.

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REFERENCES
1. Preissner, K. T. (1991) Annu. Rev. Cell Biol. 7, 275–310
2. Tomasini, B. R., and Mosher, D. F. (1990) in Progress in Hemostasis and Thrombosis (Coller, B. S., ed) pp. 269–305, Saunders Co., Philadelphia
3. Loskutoff, D. J. (1991) Fibrinolysis 5, 197–206
4. Seiffert, D., and Loskutoff, D. J. (1991) J. Biol. Chem. 266, 2824–2830
5. Seiffert, D., Ciambrone, G., Wagner, N. V., Binder, B. R., and Loskutoff, D. J. (1994) J. Biol. Chem. 269, 2659–2666
6. Sigurdardottir, O., and Wiman, B. (1994) Biochim. Biophys. Acta 1208, 104–110
7. Preissner, K. T., Grulich-Henn, J., Ehrlich, H. J., Dederck, P., Justus, C., Cidl, D., Pannekoek, H., and Mueller-Berghaus, G. (1990) J. Biol. Chem. 265, 18490–18498
8. Kost, C., Stuber, W., Ehrlich, H. J., Pannekoek, H., and Preissner, K. T. (1992) J. Biol. Chem. 267, 12098–12105
9. Chain, D., Kreizman, T., Shapira, H., and Shaltiel, S. (1991) FEBS Lett. 285, 251–256
10. Gechtman, Z., Sharma, R., Kreizman, T., Fridkin, M., and Shaltiel, S. (1993) FEBS Lett. 315, 293–297
11. Barnes, D. W., Silmutzer, J., See, C., and Shaffer, M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1362–1366
12. Parker, C. J., Stone, O. L., White, V. F., and Bernshaw, N. J. (1989) Br. J. Haematol. 71, 245–252
13. Preissner, K. T., Holzhuetter, S., Justus, C., and Mueller-Berghaus, G. (1989) Blood 74, 1989–1996
14. Stockmann, A., Hess, S., Dederck, P., Timpl, R., and Preissner, K. T. (1993) J. Biol. Chem. 268, 22874–22882
15. Seiffert, D., Wagner, N. N., and Loskutoff, D. J. (1990) J. Cell Biol. 111, 1283–1291
16. Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M. (1988) Cell Struct. Funct. 13, 281–292
17. Dahlbaek, B., and Podack, E. R. (1985) Biochemistry 24, 2368–2374
18. Seiffert, D. (1995) FEBS Lett. 368, 135–159
19. Seiffert, D., Keeton, M., Eguchi, Y., Sawdey, M., and Loskutoff, D. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3402–3406
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Hörnsten, H. (1994) in Hemostasis and Thrombosis: Basic Principles and Clinical Practice (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., eds) pp. 524–545, Lippincott Co., Philadelphia
22. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) FASEB J. 8, 814–822
23. Kambayashi, J.-I., and Sakon, M. (1989) J. Biol. Chem. 264, 251–256
24. Wang, K. K. W., Villalobo, A., and Roufogalis, B. D. (1990) Biochem. J. 262, 693–706
25. Suzuki, S., Pierschbacher, M. D., Hayman, E. G., Nguyen, K., Ohgren, Y., and Ruoslahti, E. (1984) J. Biol. Chem. 259, 15307–15314
26. Zhao, Y., and Sane, D. C. (1993) FASEB J. 7, 2824–2830
27. Seiffert, D., Ciambrone, G., Wagner, N., Loskutoff, D. J., and Loskutoff, D. J. (1990) Br. J. Haematol. 71, 245–252
28. Cherny, R. C., Honan, M. A., and Thia, S. J. (1993) J. Biol. Chem. 268, 9725–9729
29. Sigurdardottir, O., and Wiman, B. (1994) Biochim. Biophys. Acta 1208, 104–110
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