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Thrombin-Independent Activation of Platelet Factor XIII by Endogenous Platelet Acid Protease

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Key words

Platelet factor XIII – Transglutaminase – Acid protease – Cathepsin

Summary

Platelets contain factor XIII, an A subunit zymogen form of transglutaminase (TGase), that is activated by thrombin. In addition a thrombin-independent TGase (A^*) was observed. A^* was formed in platelet preparations lysed at acid pH, and its generation inhibited by protease inhibitors and alkaline pH. When maximal A^* activity was generated in acidified lysates no further TGase activity could be induced by subsequent treatment with thrombin. Both FXIII zymogen and A^* copurified as for FXIII, from either alkaline or from acidified platelet lysates respectively, by the conventional procedure. The pH optima, Km's for NN dimethyl casein, molecular weights, heat lability of active forms, requirements for calcium and reducing agents, and immunological characteristics of both TGases were the same. Studies with inhibitor substrates suggested that a thrombin-like cathepsin C or carboxypeptidase was responsible for A^* formation. Purified FXIII zymogen could be activated directly by cathepsin C. Thus, the predominant, and probably only, TGase of platelets is factor XIII, which may be activated either by thrombin or by endogenous platelet acid protease(s).

Introduction

Platelets contain coagulation factor XIII protransglutaminase (1, 2). The structure, composition and behaviour of platelet factor XIII is identical to the dimeric A subunits of plasma factor XIII (3–7), and like plasma factor XIII is activated by thrombin by the limited proteolysis of a 4,000 dalton peptide (4–6, 8). Platelets have also been reported to contain a thrombin independent transglutaminase (TGase) (9–12), that catalyses ε-(γ-glutaminyl)-lysine crosslink formation.

McDonagh and McDonagh (9) reported that of the total platelet TGase activity, 56% was in the thrombin-independent form, and by definition, did not require thrombin activation for its activity. Furthermore, 79% of this activity could be removed by heat treatment at 56° C, conditions under which the zymogenic form remained stable. Tsukada (11), suggested that the heat stability, optimal pH and calcium requirement for this tissue-like, thrombin-independent TGase (termed here A^*) was different from that of the platelet factor XIII, although this enzyme was not purified. The failure of synthetic inhibitors of thrombin and factor Xa, such as TAME and OM-189, to alter this TGase activity suggested to them, that it did not arise as a result of activation of platelet factor XIII by thrombin or factor Xa during the isolation procedures.

We have examined the factors leading to generation of A^* and the properties of purified A^* and of factor XIII zymogen. Results indicate that A^* arises *in vitro* from the activation of factor XIII by endogenous acid protease(s) on platelet lysis.

Materials and Methods

Hammerstein casein was purchased from Merck (Darmstadt, West Germany) and was NN-dimethylated according to the procedure in Lin et al. (13). Human α-thrombin (USA standard thrombin lot. HI) was kindly supplied by Dr. David Aronson (Bureau of Biologics Standards, Bethesda, Md, USA). The α-thrombin migrated as a single protein band on SDS-PAGE. Bovine thrombin (Thrombin, topical) was obtained from Parke, Davis & Co. (Detroit, Mich, USA). Trasylol (aprotinin) from Bayer Co. (Leverkusen, Germany) and N-ethylmaleimide from Koch-light Laboratories Ltd. (Colinbrook, Bucks, England). Cathepsin C (dipeptidyl-peptidase), from beef spleen, was from Mannheim (GmbH, West Germany).

Chromogenic substrates S2238 and S2222 were obtained from Kabi Diagnostica (Stockholm, Sweden). Peptide enzyme substrates and inhibitors, HEPES and Dithiothreitol (DTT) were obtained from Sigma (St. Louis, Mo, USA). Dimethyl PoPoP [1.4-bis.2-(4-methyl-5-phenyl-oxazolyl-oxazole)benzene] was from Packard Instrument Co. Inc. (Ill, USA), PPO (2.5-diphenyl-oxazole) from Koch-Light laboratories and Toluene from Mallinckrodt Chemical works (St. Louis, Mo., USA). [^{14}C] putrescine (116 mCi/mMol) and [^{3}H] putrescine (19 Ci/mMol) were from the Radiochemical Centre Ltd. Amersham (Buckinghamshire, England).

Platelets were obtained from freshly drawn blood of volunteer donors or from outdated platelet concentrates from the Australian Red Cross Blood Bank. Rabbit antisera to factor XIII A subunits were obtained from Behringwerke (Marburg, West Germany); K./lot No. 105812 C and Ch.-B./lot No. 105817 B.

Platelet Preparation

Blood was collected from normal volunteers by venipuncture into 3.8% trisodium citrate, and platelet rich plasma (PRP) obtained by centrifugation at 180 × g for 20 minutes at 25° C. The pH was lowered to 6.6, and the platelets washed by differential centrifugation at 2,200 g at 25° C (14–15), once in buffer A (16 mM sodium citrate; 28 mM glucose; 124 mM sodium chloride; 10 mM HEPES pH 6.6), then in buffer B (20 mM glucose; 107 mM NaCl; 3 mM KCl; 40 mM HEPES pH 6.6), and once in buffer B (pH 7.9). The final pellet was resuspended in buffer C (20 mM glucose; 90 mM NaCl; 40 mM HEPES; 20 mM Glycine pH 7.9) to 0.1–5 × 10^9 platelets/ml. Washed platelets were lysed by sonication (4 × 15 sec bursts at 98 watts) using a labsonic 1510 ultrasonic homogeniser (Braun, Germany).

Purification of Platelet Factor XIII Zymogen

Platelet factor XIII A subunit zymogen was purified as described by Schwartz et al. (4). Platelet lysate was prepared from 130 ml of washed platelet concentrate (5 × 10^9 platelets/ml) by sonication at pH 7.9 in 0.05 M Tris/0.002 M EDTA buffer, and purification performed using this buffer. Protein concentrations were measured according to Lowry (16).

Abbreviations:
TGase: Transglutaminase; A': Factor XIII A subunit activated by thrombin; A^*: Factor XIII A subunit activated independently of added thrombin; SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; EDTA: Ethylene Diamine Tetra-acetic Acid; TAME: Tosyl Arginine Methyl Ester; SBTI: SoyBean Trypsin Inhibitor.

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Purification of Thrombin-Independent Platelet A TGase*

A* protein was purified as for factor XIII zymogen using washed platelets which had been dialysed against buffer B, pH 6.6 overnight, and then against 0.05 M Tris/0.001 M EDTA buffer pH 7, for 24 hours. Platelet concentrate (102 ml of 8.7×10^9 platelets/ml) was sonicated for 5×30 seconds and the lysate dialysed against Tris/EDTA buffer pH 7.5. After centrifugation at $100{,}000 \times g$, the supernatants were processed as for the purification of factor XIII zymogen, except that 0.05 M Tris/0.001 M EDTA buffer pH 7.5 was used throughout. N terminal amino acid analysis of purified A* protein was performed as described in (17).

Antibody Preparation

Specific antibody to purified factor XIII zymogen or A* proteins (prepared and purified as above) were raised in New Zealand white male rabbits by immunizing both intramuscularly and intradermally with 25.5 µg of the respective antigen. For immunoblotting, 450 µl of heat treated serum (i. e., serum incubated at 56° C for 30 min, 2° C for 30 min, and the denatured fibrinogen removed by centrifugation at $10{,}000 \times g$) was diluted in 30 ml blotto solution (described below) prior to addition to nitrocellulose transfer sheets.

Measurement of TGase Activity

Platelet factor XIII was measured by an analytical radio-enzymic assay as previously described (2, 18–19). This measured the covalent incorporation of amine ([^{14}C] or [^{3}H] putrescine) into glutamine acyl donor protein (Hammerstein casein or its NN dimethylated form) (20). In each assay, 50 µl of sample (platelet lysate or purified platelet factor XIII) was mixed with 50 µl of the relevant assay mixture (see below) and incubated for 30 minutes at 37° C.

1. In the thrombin-dependent assay for A' activity the following were used: calcium (7.5 mM), casein or NN dimethylated casein (0.2%), [^{14}C] putrescine (17.4 mCi/mMol) or [^{3}H] putrescine (202.5 mCi/mMol), putrescine (0.15 mM), thrombin (bovine or human, 10 NIH U/ml) and DTT (10 mM) in buffer C, pH 7.9. In some cases, casein or NN dimethyl casein was omitted and incorporation of labelled amine into endogenous platelet proteins (per 0.5×10^9 platelets/ml platelet lysate) was measured.

2. For A* activity assays were performed as for 1, but thrombin was omitted.

Unless otherwise stated, dithiothreitol was omitted from the mixture when platelet lysates were examined. Assays were terminated by transfer of 50 µl of incubation mix onto a Whatman GF/C 2.1 cm glasss fibre disc (Whatman, Ltd. England) which was immersed in ice cold 10% trichloroacetic acid (TCA). Discs were washed in TCA solutions (0.2–10%), dried and counted for beta emissions. Net activities were expressed following subtraction of background blanks without enzyme or containing EDTA.

Thrombin Assay

Thrombin and factor Xa were assayed using chromogenic substrates S2238 and S2222 respectively, as previously described (21–22). Human thrombin was also assayed by solid phase-radioimmunoassay (23), using thrombin inactivated with phenyl methyl sulphonyl fluoride.

Gel Electrophoresis

Samples for electro-transfer and immunoblotting were electrophoresed on SDS-PAGE by the procedure of Laemmli (24), using a 5–15% acrylamide gradient, with a 3% stacking gel, for 17 hours at 22 volts. Samples were added with an equal volume of a solution containing 0.003% bromophenol blue, 1.6% sodium dodecyl sulphate, 4.1% dithiothreitol, 6.5 M urea in 0.12 M Tris HCl, pH 6.8 and boiled for 10 minutes prior to loading. The relative mobilities of purified samples were determined following SDS-PAGE on 7.5% acrylamide gels. Protein standards were, Glutamic dehydrogenase (250 and 50 Kd), platelet Myosin (200 Kd), platelet Actin (43 Kd), β Galactosidase (130 and 40 Kd), Gamma Globulin (150 and 50 Kd), Bovine Serum Albumin (68 Kd), and Transferrin (90 Kd), or the Pharmacia high molecular weight kit.

Immunoblotting

Proteins were transfered to nitrocellulose (Biorad, Richmond, Ca USA) as described by Towbin et al. (25). The lanes carrying the standards were cut off and stained with colloidal gold (26), and the remainder placed in "Blotto" solution (i. e., skimmed milk powder (50 mg/ml), 0.015 M NaCl, 0.075 M $CaCl_2$, sodium azide (1 µg/ml), 0.05% Tween 20 (vol/vol) and 0.025 M Tris/HCl, pH 8.1), overnight at 22° C. The papers were then treated with rabbit antiserum (15 µg/ml "Blotto") for 1.5 hours, washed, and specific rabbit IgG binding visualised with gold-conjugated rabbit anti-human IgG as modified from Hsu (27). Goat anti-rabbit immunoglobulin (Silenus Laboratories Pty Ltd, Melbourne, Vic, Australia) was conjugated with colloidal gold according to Lin and Langenberg (28), modified to use 10 fold the amount of IgG. Pink bands were visualised after 1–3 hrs and maximum intensity was reached in 18 hrs.

Results

TGase in Platelet Lysate Active in the Absence of Added Thrombin

Thrombin treatment of platelet lysates (0.5×10^9 platelets/ml) at pH 7.5, resulted in the activation of platelet factor XIII and expression of A' TGase activity (i. e. $56.8 \pm 5.7 \times 10^{-12}$ M), when assayed for amine ([^{14}C]-putrescine) incorporation into endogenous platelet proteins. Significant activity was also observed when lysates were assayed without thrombin. However, this activity (termed A$^{\#}$) varied from one platelet preparation to another and ranged from 10–65% of the total TGase measurable with thrombin, using either endogenous platelet protein or exogenous casein substrates.

Neither the length of sonication (5 sec – 2 min) used to lyse platelets, platelet concentration ($0.1–5 \times 10^9$ platelets/ml), nor removal of granules from crude lysate preparations (by centrifugation at $30{,}000 \times g$), altered the amount of A$^{\#}$ recovered. Incubation of lysate at 37° C or at room temperature for 0 to 80 minutes also failed to uncover any further activity.

Mechanism of Generation of A$^{\#}$ Activity

Effect of Protease Inhibitors

The varied expression of A$^{\#}$ in different platelet preparations suggested that A$^{\#}$ might arise as a result of proteolysis during platelet solubilisation. Inclusion of protease inhibitors during platelet lysis decreased A$^{\#}$ generated (Table 1), with most inhibition occuring with TAME and hirudin. In this assay system of zymogen activation followed by enzyme expression (2, 18–19), these agents inhibited only the activation of factor XIII zymogen, and had no effect on the enzymic expression of TGase activity (data not shown).

To examine whether thrombin or factor Xa might be responsible for formation of A$^{\#}$ activity during platelet lysis, levels of thrombin were measured using chromogenic substrates S2238 and S2222, specific for thrombin and factor Xa respectively. Neither thrombin nor factor Xa were detected either when platelets (0.5×10^9 platelets/ml) were lysed by sonication in the presence of the appropriate substrate (0.1–1.3 µMol/ml) at pH 8.2 and incubated for 30 minutes at 37° C, or when platelet lysate was assayed for thrombin or factor Xa by the standard chromogenic procedure (21–22). Solid phase thrombin radioimmunoassay which is sensitive to as little as 1 ng/ml (23), also failed to detect any thrombin in platelet lysate (5×10^9 platelets/ml).

Effect of pH

Lowering the pH of platelet lysate to 5 prior to assay at pH 7.9, resulted in an increase of A$^{\#}$ activity equal to that observed when assays were performed at pH 7.9 in the presence of thrombin (i. e.

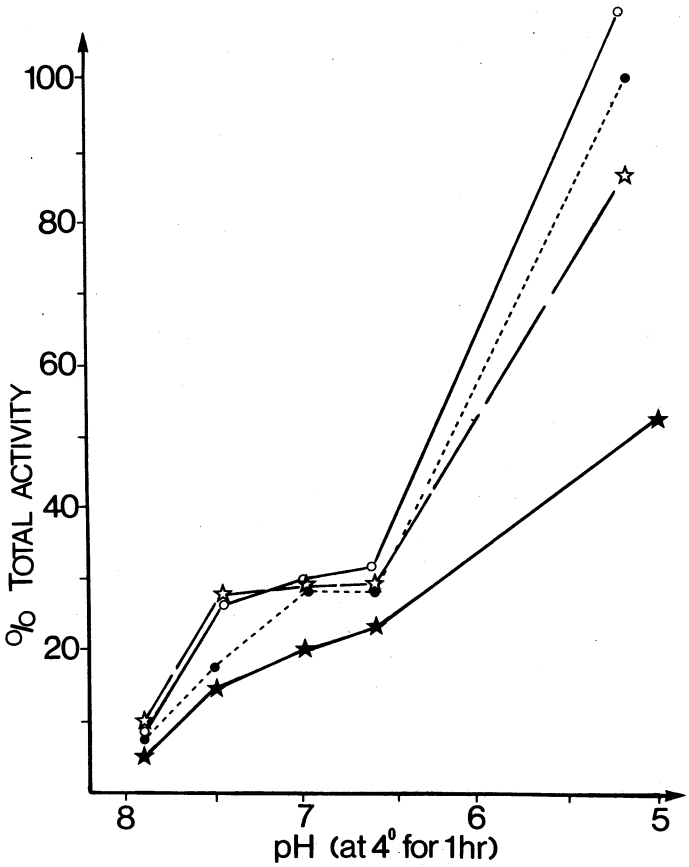

Fig. 1 The effect of pH and protease inhibitors on generation of A* activity in platelet lysates.
Washed platelet lysate (1×10^9 platelets/ml) was obtained following sonication (2 min at 4° C) in the presence of 0.3 mM leupeptin ○----○, 5 mM EDTA ☆----☆, 30 mM TAME ★----★, or buffer ●----●, at pH 7.9. The pH was then lowered to between pH 5 and 7.5 and kept at 4° C for 1 hour. Prior to assay the pH was readjusted to 7.9, and 10 mM calcium was added to the EDTA-containing samples. The TGase activities measured without thrombin are given as percent of total TGase activity obtained in the presence of thrombin (10 U/ml) and are the means of 4–6 determinations

A') (Fig. 1). Activation at pH 5 was inhibited 50% by 20 mM TAME, but not by EDTA (Fig. 1) or leupeptin (Fig. 1, Table 2). Lysis at alkaline pH (pH 7.9) reduced A* formation to 10–16% A', whilst the combined lytic conditions of pH 7.9 and 20 mM TAME, reduced this even further to only about 1% of the activity seen on thrombin treatment.

Table 1 The effect of protease inhibitors on generation of A* TGase activity

Protease inhibitor/Substrate	% Inhibition of A* formation
TAME (20 mM)	71.5 (±9.3)
Hirudin (80 U/ml)	59.3 (±6.7)
PMSF (6 mg/ml)	12.0 (±16)
Trasylol (100 KIU)	17.6
SBTI (2 mg/ml)	34.5

Each protease inhibitor was pre-incubated with platelet suspension (0.5×10^9 platelets/ml) at pH 7.9 for 10 minutes prior to platelet lysis by sonication. Samples were then assayed for incorporation of [^{3}H] putrescine into casein in the presence of 10 mM DTT but without thrombin (as described in materials and methods). Values show the percent reduction in A* activity with the respective inhibitors ± SD (n = 4).

Table 2 The effect of synthetic cathepsin substrates and cathepsin inhibitors on the generation of A* at acid pH

pH	Substrate/Inhibitor	A* TGase activity (% thrombin activated A' activity)
7.9	–	10
5.2	–	94
5.2	Tosyl-arginyl-methyl-ester (TAME)	28
5.2	N-CBZ-L-glutamyl-L-tyrosine	63
5.2	α-N-benzoyl-L-argininamide	73
5.2	glycyl-L-phenylalanine amide	45
5.2	N-CBC-L-glutamyl phenylalanine	8
5.2	Antipain	111
5.2	Leupeptin	116
5.2	Pepstatin	96

Washed platelets were lysed at pH 7.9 with or without synthetic peptide (40 mM) or cathepsin inhibitor: antipain (0.1 mM), leupeptin (0.3 mM) or pepstatin (1.5 mM). The pH was adjusted to pH 7.9 or pH 5.2, and the lysates kept for 1 hour at 4° C. The pH was returned to pH 7.9 and assays performed with (i.e. A') or without (i.e. A*) thrombin. Values show percent of total A' activity measured with thrombin but without substrate peptide or protease inhibitor.

Effect of Cathepsin Substrates

All the synthetic cathepsin substrates (29) examined inhibited generation of A* TGase in platelet lysate at pH 5.2 (Table 2). The most marked inhibition (92%) was observed with a substrate (Glutamyl-phenylalanine) for catheptic carboxypeptidase and cathepsin C. Substrates for cathepsin A and cathepsin B, (Glutamyl-L-tyrosine and Benzoyl-L-phenylalanine respectively) were the least effective inhibitors. Microbially derived protease inhibitors of cathepsins A (i.e. antipain), B (antipain, leupeptin) and D (pepstatin) (30), were ineffective in blocking the generation of A*.

Co-Purification of Platelet Thrombin-Dependent and A TGases*

Platelet lysates were prepared at pH 7.9 to avoid the action of acid protease(s), and the purification procedure for factor XIII (5) was performed at this pH. A single protein, the factor XIII zymogen (MW 78,000 ± 3,000 daltons [SD], n = 6) as previously described (4, 5, 7), was obtained. This protein accounted for more than 2.4% of the total soluble protein fraction of platelets (obtained following 100,000 × g centrifugation of membrane and granule components). When assayed for incorporation of ^{3}H-putrescine into casein with 13 mM glutathione, the thrombin-dependent (A') activity had a specific activity of 14.85 (±2.4) μMoles ^{3}H-putrescine/mg (n = 5). This preparation also had an A* component, because when assayed in the absence of thrombin a specific activity of 1.72 (±0.69) μMoles/mg (~12% of A' activity) was also observed. This was consistent with the level of A* activity in the platelet lysate starting material at pH 7.9, and demonstrates the co-purification of both activities in the one preparation.

When the same purification procedure was conducted on platelet lysate prepared at pH 7.5 and dialysed at pH 6.6, a single protein of 79,000 ± 4,000 daltons (SD, n = 8) which co-migrated with the A subunit zymogen of factor XIII was obtained (see Fig. 3). This A* protein accounted for the total TGase activity of these platelet preparations and represented more than 1.6% of the total platelet protein. Addition of 0–18 U/ml of either human or bovine thrombin failed to uncover any additional activity.

A study of the stability of A* at 56° C was made, to compare it with the thrombin-independent activities observed by others (9, 11). Unlike the factor XIII zymogen, which retained 100% of its

activity after 10 minutes at 56° C, A* was labile, with a 96% loss of activity. The A' activity generated from factor XIII by treatment with thrombin (10 U/ml) was also inactivated at 56° C, with a 100% loss of activity. Instability of active forms but not the zymogen was also observed at 37° C (data not shown).

The A' and A* activities of the two preparations showed the same requirements for calcium (both optimal at 2 mM) and were totally inhibited by 20 mM EDTA. Both activities were maximal at 15 mM glutathione and had the same pH optima (Fig. 2, pH 8.3–9.2) and Km for NN dimethyl casein (Table 3). Moreover, the two preparations were immunologically identical (Fig. 3, see below). Amino acid analysis of the N-terminus of the A* protein indicated the presence of a blocked N-terminus (data not shown).

Activation of Factor XIII Zymogen by Cathepsin C

Purified factor XIII A zymogen (3.12 mg/ml) was incubated with 2 mM DTT and 5% glycerol in the presence or absence of cathepsin C (1.06 U/ml) at pH 7.9, the pH lowered to pH 5.2 and the mixture incubated at 37° C for 10 minutes. The pH was again returned to pH 7.9 for assay. Cathepsin C caused the generation of TGase activity to 89.5% (±21%) (n = 6) of A' activity obtained with thrombin activator (i.e. 10 U/ml thrombin, 10 minutes at 37° C). The acid treatment of samples alone resulted in apparent A* activities of only 6.9% (±13%) (n = 6) of the total A' TGase.

Immunoblotting of Factor XIII Preparations

Both crude and purified preparations of factor XIII A zymogen and A* proteins were analysed by immunoblotting (Fig. 3). Commercial antisera against factor XIII A subunits from placenta identified the A subunit of platelet factor XIII with Mr = 81,000 daltons (Fig. 3. I/II lanes 2,6) and the thrombin activated A' form with Mr = 77,000 daltons (Fig. 3. I/II lanes 3,7) and also recognised both purified A* protein with Mr = 81,000 daltons (Panels I/II lane 5) and a band of protein of identical molecular weight from crude platelet lysates treated at acid pH to generate A* activity (Panels I/II, lane 4). Rabbit antiserum raised against purified A* protein reacted with both the zymogen and A' forms of factor XIII, and an identical band in acid treated platelet lysate (Panel III, lanes 2, 3, 4) as well as with purified A* antigen (Panel III, lane 5) and purified factor XIII A subunits (Panel III, lane 6). The immuno-reactive factor XIII protein of 81,000 daltons also co-electrophoresed with the Coomassie-stained protein band of purified A* protein (lane 9). No comparable banding at these molecular weights was seen when immunoblotting was performed on control red cell preparations (Panel I, lane 1).

One of the commercial antibody preparations (Fig. 3. Panel I) visualised extra bands of higher MW (90,000 and 108,000 daltons). These could have arisen from a contaminating antigen common to both placental and platelet tissues and present in the initial prepration with which this antibody was raised. These extra bands were not seen with the other anti-factor XIII preparation (Panel II) nor with antibody to A* protein (Panel III).

Apparent proteolytic products of lower MW (i.e., 55,000–58,000 daltons) were also observed in lysate preparations (lanes 2, 3, 4) and were detected by all three antibodies. The amounts of this material varied between the different antibody preparations. Proteolytic degradation products of factor XIII of 56,000 daltons have been described previously (5).

Thrombin activation of factor XIII involves cleavage of a 4,000 dalton peptide from the A subunit, and results in increased protein migration on SDS-PAGE (Fig. 3. Panel I/II, lanes 3 and 7; to an apparent MW of 77,000 daltons). In contrast, activation of factor XIII of platelet lysates at acid pH (lanes 4), or of factor XIII protein by cathepsin (lane 8), did not result in a detectable change in molecular weight. The purified A* protein (lane 5) also migrated identically with the A subunit zymogen, but not with the thrombin-activated form.

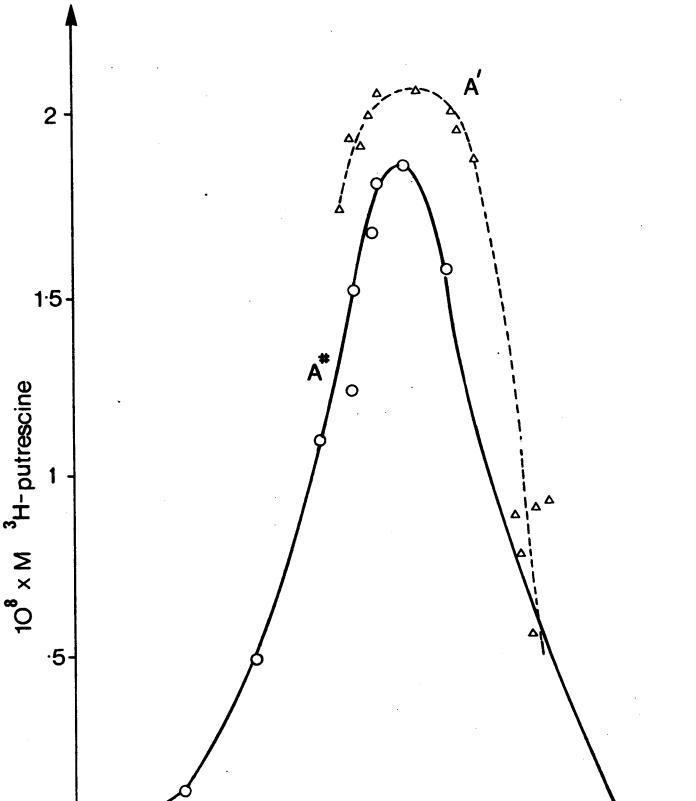

Fig. 2 The effect of pH on the TGase activities of purified platelet factor XIII zymogen (activated by thrombin) (A') and purified A*. Purified factor XIII A (3.9 µg/ml) was pre-activated by thrombin (as described in materials and methods) at pH 7.9 to give A' (△), prior to adjustment of pH and assay. A* (3.3 µg/ml) (○) was assayed without thrombin. Incorporation of [^{3}H]-putrescine into NN dimethyl casein is shown at the respective pH's in constant ionic strength buffer (± 0.003Γ/2) containing: 0.01 M Tris, 0.01 M glycine, 0.01 M HEPES and 0.005 M sodium tartrate

Table 3 Comparison of the affinity constants of purified TGase prepared from acid or alkaline platelet lysates

Activity	pH	$Km_{app.}$ ($\times 10^{-6}$ M)
A'	7.9	0.53
A*	7.9	0.59
A'	8.6	0.59 (± 0.18)
A*	8.6	0.69 (± 0.35)

Apparent Km's for thrombin-dependent factor XIII purified from alkaline platelet lysates were measured following activation by 10 U/ml human thrombin (i.e. A'). The A* protein prepared from acid lysates was assayed in the absence of thrombin. Incorporation of [^{3}H]-putrescine into NN-dimethyl casein (as the varied substrate) at pH 7.9 or pH 8.6 with DTT (5 mM) was measured as described in the materials and methods. The kinetic parameters were determined from Woolf-Augustinsson-Hofstee plots of v versus v/s (47).

Discussion

We have observed, in addition to the well documented thrombin-dependent TGase, a platelet TGase that did not require

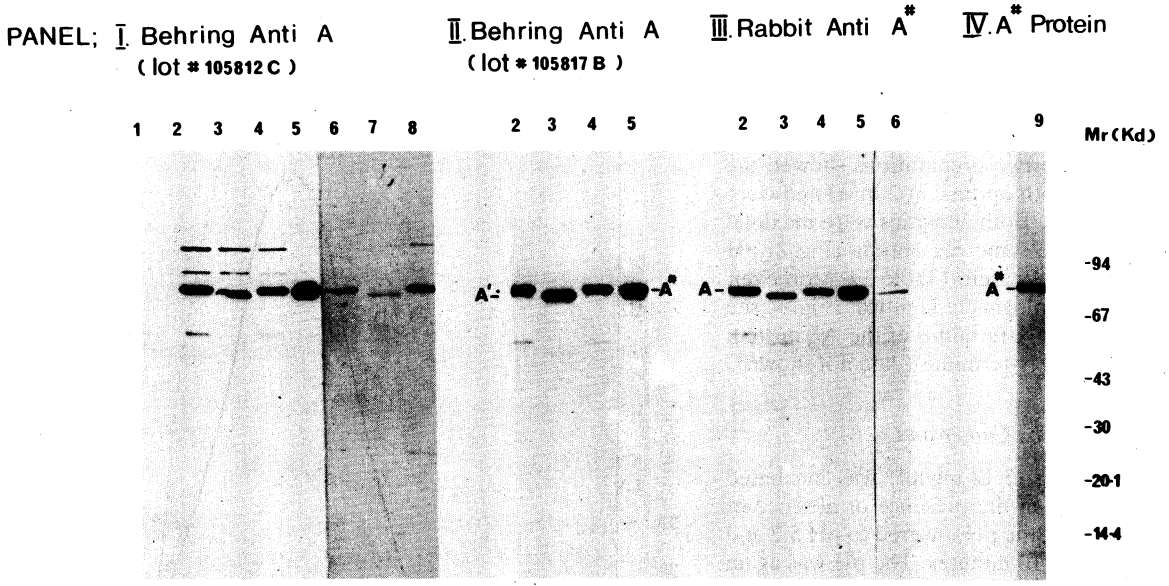

Fig. 3 Immunological cross-reaction between the factor XIII A subunit and A*.
Commercial antisera to factor XIII A, Behring lot. 105812 C-(Panel I), Calbiochem-Behring lot. 105817 B-(Panel II), and rabbit antisera raised against purified A* protein-(Panel III) were tested by immunoblotting as described in materials and methods, against a number of factor XIII preparations:

1. Erythrocytes (0.42×10^9 red cells/ml)
2. Platelet Lysate (0.62×10^9 platelets/ml)
3. Platelet Lysate + thrombin (10 U/ml)
4. Platelet Lysate treated at pH 5.2 for 10 minutes at 37° C
5. A* protein (5 μg)
6. Purified factor XIII zymogen (1.94 μg)
7. Purified factor XIII activated by 10 U/ml thrombin
8. Purified factor XIII activated by 1 U/ml cathepsin C at pH 5.2

For comparison Coomassie stained A* protein (10 μg) is shown in Panel IV lane 9

the addition of thrombin for activity. Such an enzyme has been described previously (9–12) and has led to the suggestion that platelets contain two distinct TGases; a factor XIII A zymogen and a tissue-type TGase (transamidase). However, in this present study, the capacity of protease inhibitors to block the appearance of the transamidase-like activity during platelet lysis suggested otherwise. The inability of Tsukada (11) to observe comparable inhibition using TAME and OM-189 inhibitors, possibly resulted from addition of inhibitors to their platelet suspensions after platelet lysis, rather than before. The heat lability of this thrombin-independent TGase (A*) agrees with the observations of McDonagh and McDonagh (9) but not those of Tsukada (11).

It was possible that thrombin or factor Xa might have been involved in A* formation, since both can activate factor XIII (31, 32), and because TAME and hirudin prevented A* formation. However, for a number of reasons, it is unlikely that they are the enzymes involved. 1. To generate A' from factor XIII zymogen to levels of TGase comparable to the A* activities rapidly formed on platelet lysis would require thrombin levels of approximately 2 U/ml. 2. No thrombin or factor Xa could be detected in these platelet preparations by very sensitive assays. 3. A* was generated in lysates at pH 5, but not at alkaline pH (pH >7.9) at which thrombin and factor Xa are most active (21). 4. The most potent inhibitors of A* formation were dipeptides which are substrates for cathepsin C and carboxypeptidase but not for thrombin. 5. Activation of factor XIII to A* did not cause the same change in molecular weight as observed with thrombin.

The involvement of the calcium-dependent neutral platelet protease (33–35) was also excluded because leupeptin and EDTA, potent inhibitors of this enzyme, failed to inhibit A* enzyme formation.

Human platelets are known to possess cathepsin (36–40) and catheptic carboxypeptidase activities (39), which are optical at acid pH. All the substrates for cathepsins A, B, C and catheptic carboxypeptidase examined inhibited generation of A*, but did so to varying degrees. Thus, it is possible that there is more than one acid protease involved. Alternatively cross-reactivity of the peptide substrates (29) may be responsible. Because antipain and pepstatin, inhibitors of cathepsin A, B and D activities, had no effect on A* generation in platelet lysates, the involvement of these cathepsins (A, B and D) would appear to be minimal. Unfortunately, selective inhibitors for cathepsin C and catheptic carboxypeptidase were not available. A* generation in platelet lysates by a cathepsin C was supported by the finding that a commercial preparation of cathepsin C alone can activate purified platelet factor XIII zymogen. However, possible catheptic carboxypeptidase involvement cannot be excluded.

When platelet factor XIII was purified from platelets lysed at alkaline pH, predominantly zymogenic factor XIII was obtained, as has been previously described (4). However, acidified platelet lysate yielded a fully active TGase which co-purified as for factor XIII, but which had been converted to an active form (A*) in the absence of thrombin. Although these proteins differed in their requirement for thrombin activation, they were identical with respect to their immunologic characteristics, molecular weights, pH activity curves, enzyme kinetics for casein substrate, and their requirements for calcium and reduction. Activation of factor XIII by thrombin, involves cleavage of an arginyl-glycine bond from the N terminus of the A subunit and release of a 4,000 dalton activation peptide (8, 41). In contrast generation of A* activity by acid treatment of platelet lysate or cathepsin C treatment of factor XIII zymogen, did not result in a decrease in molecular weight and purified A* protein migrated identically with the factor XIII A subunit zymogen but not with the thrombin activated form (A'). This indicates that cleavage of a peptide of significant size did not precede the generation of A*. N terminal analysis of the purified A* protein indicated that the N terminus was blocked.

We have also observed activation of macrophage factor XIII A subunits during cell disruption. This activation was also prevented by alkaline pH and inclusion of TAME during cell lysis (42, 43). Macrophages were previously thought to contain non-factor XIII thrombin-independent tissue TGase (44, 45). But, as with

platelets, activation of factor XIII during lysis accounts for the majority of the TGase activity of this cell (42, 43). Human epidermal TGase has also been observed to be activated by an acid protease (cathepsin D) (46).

It is not yet clear whether platelet A^* activity is of biological significance. It could be important in producing factor XIIIA within the degrading platelets of a thrombus, where its formation could stabilize the fibrin/platelet aggregate. Alternatively, because platelets release acid protease on stimulation with thrombin (38), a possible alternative pathway in the activation of plasma factor XIII during clotting might be envisaged.

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