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PLATELET FACTOR XIII IS ACTIVATED BY CALPAIN

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Summary: The action of calpain (EC 3.4.22.17; Ca^{2+}-dependent cysteine proteinase) on platelet factor XIII has been studied. Calpain I activated platelet factor XIII up to 76 % of the maximum level observed with thrombin. Activation was accompanied by the limited proteolysis of the a subunit of platelet factor XIII to produce a 76 kDa fragment which was comparable to the proteolytic product by thrombin. Activation of platelet factor XIII by calpain was inhibited by EDTA, leupeptin, and endogenous calpain-specific inhibitor calpastatin. These findings suggest that calpain is responsible for the intracellular activation of platelet factor XIII.

Factor XIII is a transglutaminase (EC 2.3.2.13; R-glutaminyl peptide: amine γ-glutamyltansferase) and exists in inactive zymogen form. Blood platelets and plasma contain the zymogens of subunit composition of a_2 and a_2b_2, respectively (1). About 50 % of the total blood factor XIII is present in platelets (2). The activation mechanism and function of platelet factor XIII remain obscure, while those of plasma factor XIII are better understood; the latter is activated after limited proteolysis of a subunit by thrombin and cross-links fibrin in the final step of the blood coagulation cascade. It is reported that platelet factor XIII can be activated by thrombin *in vitro*. However, this seems unlikely to occur *in vivo* because platelet factor XIII is present mainly in the cytosolic

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis(β-amino ethylether)-N,N,N',N'-tetraacetic acid; MDC, monodansylcadaverine; p-APMSF, (p-amidinophenyl)methanesulfonyl fluoride hydrochloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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fraction and is not secreted during the platelet release reaction (3, 4). Since there is substantial evidence that platelet factor XIII is intracellularly activated when platelets are stimulated by agonists (5), some activating enzyme must be present in the platelet cytosol.

Calpain is a family of Ca^{2+}-dependent cysteine proteinases and is ubiquitously distributed in cytosolic fractions of various cells. Two distinct forms of the enzymes are known: calpain I requiring low (micromolar) Ca^{2+} and calpain II requiring high (millimolar) Ca^{2+} for activation (6). Calpain is the major neutral protease in platelets (7), and both calpains I and II are present in platelet cytosol (8). When platelets are stimulated by various agents, the intracellular concentration of Ca^{2+} is elevated to enough level to activate calpain I (9). Actually, there have been some observations that active calpain is present in stimulated platelets (7, 10, 11, 12).

The aim of the present study was to investigate the action of calpain I on platelet factor XIII.

MATERIALS AND METHODS

Purification of calpain I and calpastatin Calpain I and calpastatin were purified from human erythrocytes as described previously (13, 14). The preparations used here had specific activities of 180 and 760 U/mg for calpain I and calpastatin, respectively, when assayed with casein as the substrate under the standard conditions (13, 14).

Purification of platelet factor XIII Factor XIII was purified to homogeneity from human platelets using sucessive chromatographies on DEAE-cellulose, Sephacryl S-300, and Phenyl Sepharose CL-4B (Ando, Y., Yamagata, Y., Kikuchi, T., Kannagi, R., and Murachi, T. in preparation). The protein concentration was determined according to the method of Lowry et al.(15) with bovine serum albumin as the standard.

Activation of platelet factor XIII Platelet factor XIII was incubated with calpain I or bovine plasma thrombin (141 NIH U/mg, Sigma, St.Louis, U.S.A.) as described in the legends of Fig. 1. The reaction was stopped by adding leupeptin (Peptide Institute, Osaka, Japan) for calpain I or p-APMSF (Wako Co, Osaka, Japan) for thrombin.

Assay for transglutaminase activity Transglutaminase activity was assayed by measuring the amount of MDC incorporated into casein (16). The standard reaction mixture (0.5 ml) contained 0.1 M Tris-HCl buffer (pH 7.5), 1 mM EGTA, 6 mM $CaCl_2$, 2 mM DTT, 0.2 % N,N'-dimethylcasein, 0.1 mM MDC (Sigma) and enzyme preparation. The reaction was started by the addition of $CaCl_2$. After incubation at 30°C for 30 min, 0.1 ml of 0.1 M EGTA was added to stop the reaction. An aliquot was subjected to high-

performance liquid chromatography to separate casein-bound MDC from free MDC. The amount of the casein-bound amine was calculated based on the fluorescence intensity (excitation at 335 nm, emission at 510 nm, FP-110 spectrofluorometer, JASCO, Tokyo, Japan). An enzyme unit of transglutaminase was defined as the amount which catalyzed the incorporation of 1 nmol of MDC into casein per minute (Ando, Y., Yamagata, Y., Kikuchi, T., Kannagi, R., and Murachi, T. in preparation).

SDS-PAGE and immunoblotting Samples were subjected to SDS-PAGE according to the procedure of Laemmli (17), and then transferred to nitrocellulose membrane filter (Schleicher & Schuell, Dassel, Germany) (18). The nitrocellulose paper was first incubated with rabbit anti-human plasma factor XIII-A antibody (Behringwerke, Marburg, Germany) and then with peroxidase-conjugated goat anti-rabbit IgG as the second antibody. The peroxidase staining was developed using o-dianisidine as the substrate (19).

RESULTS

Platelet factor XIII was incubated with calpain I at molar ratios of 20:1 and 10:1 in the presence of 0.2 mM free Ca^{2+} at 30°C for various time intervals (Fig. 1a). The zymogen was rapidly activated by

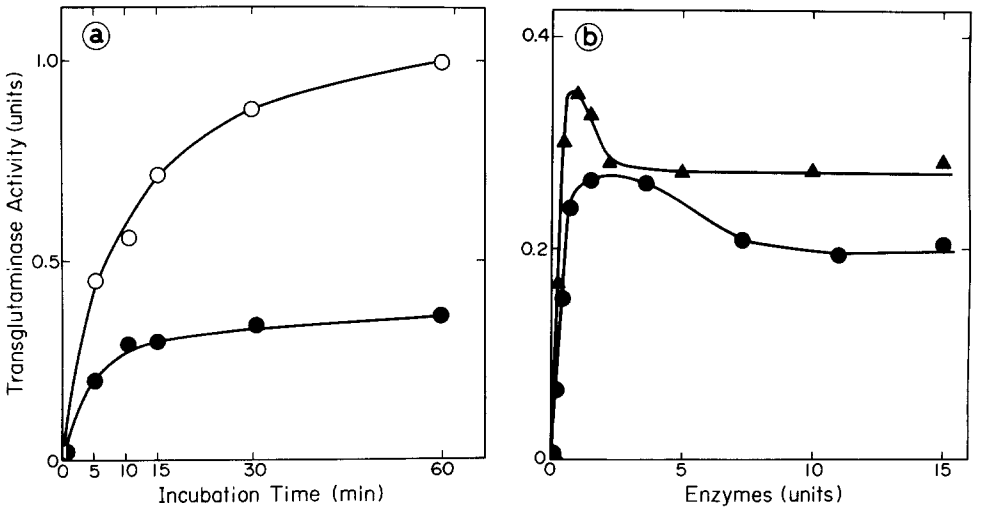

Fig. 1a. Time course of platelet factor XIII activation by calpain I Platelet factor XIII(13 μg) was incubated with calpain I (●:0.1 U, ○: 0.2 U) in 20 mM Tris-HCl buffer(pH 7.5) containing 5 mM cysteine, 1 mM EGTA, and 1.2 mM $CaCl_2$ (final volume of 20 μl) at 30°C. At the indicated time intervals, the reaction was stopped by adding 10 μl of 0.5 mM leupeptin. Transglutaminase activity was assayed as described in METHODS.

Fig. 1b. Activation of platelet factor XIII by calpain I and thrombin For calpain(●), the incubation mixture(50 μl) contained platelet factor XIII (1.2 μg), 0.18-15 U of calpain I, 20 mM Tris-HCl buffer(pH 7.5), 5 mM cysteine, 1 mM EGTA, and 1.2 mM $CaCl_2$. For thrombin(▲), the incubation mixture(50 μl) contained platelet factor XIII(1.2 μg), 0.25-15 NIH U of thrombin, 20 mM Tris-HCl buffer(pH 7.5), 2 mM DTT, and 1 mM EGTA. After incubation at 30°C for 30 min, the reaction was stopped by adding 10 μl of 5 mM leupeptin for calpain or 5 mM p-APMSF for thrombin, then transglutaminase activity was assayed.

TABLE I

Activation of platelet factor XIII by calpain I and effects of inhibitors

Preincubation with [a]	Transglutaminase activity (%)
Thrombin(1.0 NIH_bU [b])	100
Calpain I(1.5 U [b])	76
Calpain I(0.8 U)	68
Calpain I(0.8 U)+ EDTA(2 mM)	<1
Calpain I(0.8 U)+ Leupeptin(50 μM)	1
Calpain I(0.8 U)+ Calpastatin(8 U)	2

[a] The incubation was carried out as described in Fig. 1b.
[b] These doses induced the maximal platelet factor XIII activations under the conditions used.

calpain I, and the activation was leveled off in 30-60 min. The final enhancement of transglutaminase activity by 0.2 U of calpain was about twice of that by 0.1 U. The rather early leveling off can be explained by the fact that calpain easily loses its activity due to autolysis *in vitro* in the presence of Ca^{2+}.

Fig. 1b shows the activation of platelet factor XIII by various concentrations of calpain I and thrombin. The activations were dependent on the doses between 0-1.5 U for calpain I and 0-1.0 NIH U for thrombin, and higher concentrations of both enzymes slightly depressed the transglutaminase activity. Calpain I activated platelet factor XIII up to 76 % of the maximum level observed with thrombin, and the activation was markedly prevented by well-known inhibitors of calpain such as EDTA, leupeptin, and calpastatin (Table I).

The proteolytic products of platelet factor XIII after incubation with calpain I and thrombin were analysed by immunoblotting using antibody against the a subunit of human factor XIII (Fig. 2). Lane 1 contains untreated platelet factor XIII, lanes 2 and 3 contain materials preincubated with calpain I and thrombin, respectively. After partial activation with calpain I, both a and cleaved a bands are visible. The apparent molecular weight of cleaved a band by calpain is 76 kDa, which is similar to that by thrombin. The increase of transglutaminase activity of factor XIII by calpain occurred in parallel with the appearance of 76 kDa fragment.

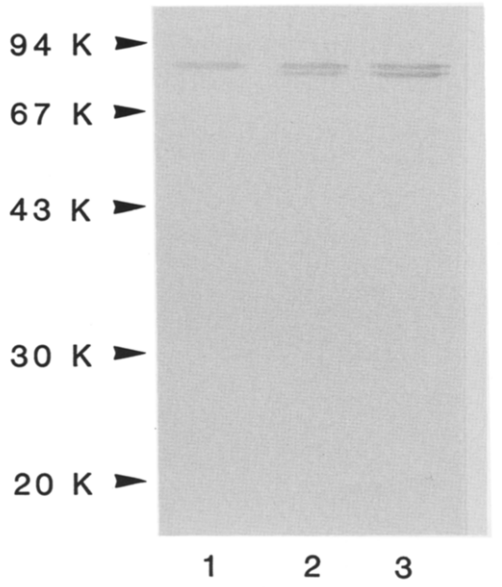

Fig. 2. Immunoblotting analysis of platelet factor XIII after limited digestion by calpain I and thrombin Platelet factor XIII(1.2 μg) was incubated with calpain I(1.0 U) or thrombin(0.5 NIH U) at 30°C for 30 min as described in Fig. 1b. Aliquots were subjected to SDS-PAGE, and then transferred to a nitrocellulose filter which was stained with anti-human factor XIII-A antibody. Samples are: lane 1, untreated; 2, preincubated with calpain I; 3, preincubated with thrombin. K, kDa.

DISCUSSION

In this paper we report that calpain I activates platelet factor XIII with limited proteolysis. This activation was demonstrated using purified preparations of both enzymes, and was absolutely Ca^{2+}-dependent and inhibited by leupeptin and endogenous inhibitor calpastatin. It was thus concluded that the activation was the consequence of the specific action of calpain, not by any other contaminant proteases. The limited proteolytic products by calpain I and by thrombin had similar molecular weight, and the maximal transglutaminase activity induced by calpain was comparable with that by thrombin. It remains to be determined whether calpain cleaves the a subunit of platelet factor XIII at the same site as thrombin or whether the proteolytic product by calpain has the same specific activity of transglutaminase as that by thrombin.

Although we carried out the reaction in relatively high concentrations of free Ca^{2+} (200 μM), calpain I can be half-maximally activated by as low as 2.3 μM Ca^{2+} *in vitro* (13). Similar levels of cytosolic free Ca^{2+} have been observed during platelet stimulation by thrombin (9). In fact, Fox et al.(12) have reported that calpain is activated during thrombin- or collagen-induced platelet aggregation. Some other authors also shown that activation of the endogenous calpain with $CaCl_2$ plus Ca^{2+}-ionophore A 23187 is accompanied by hydrolysis of several platelet proteins (7, 10, 11).

Platelets contain considerable amounts of the zymogen form of factor XIII, the function of which is not well known. The enzyme is mainly present in the cytosolic fraction and remains within the cell after platelet aggregation (3, 4). Recently, Cohen et al.(5) have reported that significant amount of platelet factor XIII is activated when platelets are stimulated by $CaCl_2$ and Ca^{2+}-ionophore A 23187 and that it forms bridges between the cytoskeletal and membrane proteins.

Ca^{2+} plays an important role in stimulus-response coupling in activated platelets. Ca^{2+}-dependent enzymes such as calpain, transglutaminase, protein kinase C, and phospholipases A_2 and C are thought to be involved in such process. There have been some reports suggesting that calpain activates protein kinase C by limited proteolysis (20). Phospholipase C is also suggested to be proteolytically modified by calpain (21). Our results indicate that calpain activates intracellular platelet factor XIII by limited proteolysis. These findings collectively suggest that there is some cascade reaction of Ca^{2+}-dependent enzymes in stimulated platelets which is triggered by the activation of calpain.

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