Proteolysis of thrombospondin during cathepsin-G-induced platelet aggregation: functional role of the 165-kDa carboxy-terminal fragment

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Abstract The serine-proteinase cathepsin G (CG) is a potent agonist of platelet aggregation inducing the release and surface expression of α-granule adhesive proteins such as fibrinogen (Fg) and thrombospondin-1 (TSP-1). Because Fg and TSP-1 are potential substrates for the enzymatic activity of CG, we investigated the fate of these proteins during CG-induced platelet aggregation using an immunoblot technique. Only a small proportion of secreted Fg was proteolyzed by CG and platelet aggregation was efficiently inhibited by anti-fibrinogen Fab fragments. In contrast, TSP-1 was extensively proteolyzed on aggregated platelets releasing in the milieu a fragment with $M_r \approx 28\,000$, corresponding to the amino-terminal heparin-binding domain (HBD). Several antibodies, directed against the cell-associated carboxy-terminal TSP-1f fragment ($M_r \approx 165\,000$) impaired the formation of stable macroaggregates, indicating that this fragment may contribute to platelet aggregation in the absence of the HBD.

Key words: Platelet aggregation; Thrombospondin; Fibrinogen; Cathepsin G; Proteolysis

1. Introduction

Aggregation of blood platelets following cell stimulation induced by various agonists is a multistep phenomenon which occurs within seconds [1]. A prerequisite is the binding of fibrinogen to its platelet membrane inducible receptor, the integrin $\alpha_{IIb}\beta_3$ [2]. At this stage, the interaction between fibrinogen and $\alpha_{IIb}\beta_3$ remains labile, and platelet aggregation fully reversible. Stabilization and 'locking' of this interaction then occur, leading to the formation of irreversible platelet macroaggregates [2,3]. The surface expression of endogenous adhesive proteins stored in platelet α-granules is thought to play a major role in this latter phenomenon [3]. Based on studies of platelet aggregation induced by the plasma serine-proteinase α-thrombin, the current concept is that secreted platelet thrombospondin (TSP) plays a key role in stabilizing platelet interactions initiated by the binding of fibrinogen to $\alpha_{IIb}\beta_3$ [4].

Platelet TSP, or TSP-1, is the prototype member of an emerging family of cell surface and extracellular matrix adhesive proteins, synthesized and secreted by a number of cell types [4-7]. This large glycoprotein, which has an apparent molecular weight (M_r) of 420 000, is composed of three identical, disulfide-bonded subunits with $M_r \approx 180\,000$ on reduced SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Each subunit has a modular organization typical of multifunctional adhesive proteins, and contains specific domains that support interactions of TSP-1 with cell surface glycoprotein receptors, sulfated proteoglycans and membrane lipids, and various matrix proteins [4-7]. Regarding platelet aggregation, particular attention has been paid to the amino-terminal domain of TSP-1 which contains high-affinity binding site(s) for heparin, thus commonly designated as the heparin-binding domain (HBD) [7]. This domain can be cleaved by various serine-proteinases, producing a fragment with M_r in the range 25 000–35 000 [7,8]. A role for the HBD in platelet-to-platelet interactions is currently supported by the following observations: (i) some antibodies directed at epitopes within the HBD as well as recombinant HBD polypeptides are potent inhibitors of α-thrombin-induced platelet aggregation, and can specifically inhibit the interaction of purified TSP-1 with adsorbed fibrinogen [9-11]; and (ii) the recombinant HBD polypeptides directly bind to adsorbed fibrinogen with high affinity suggesting that the HBD is a major link between fibrinogen and TSP-1 within the macrocomplex made of the two adhesive proteins and their respective membrane receptor(s) on the surface of activated platelets [11].

Cathepsin G (CG) is a secretable leukocyte serine-proteinase which activates platelets in the same way as α-thrombin, including shape change, granular exocytosis and extensive aggregation [12-14]. It has been recently reported that CG proteolyses purified human platelet TSP-1 to a limited extent [15], and CG has also some proteolytic activity on plasma fibrinogen [16]. These observations thus prompted us to investigate whether CG affects the structure of platelet TSP-1 and fibrinogen expressed on the surface of cells activated by this proteinase, and whether modification of these important adhesive proteins affects platelet aggregation.

2. Materials and methods

2.1. Materials and reagents

Human α-thrombin (2000 U/mg protein), hirudin (1000–3000 U/mg protein) from leech, grade V potato apyrase, synthetic prostaglandin E_1 (PGE_1), endotoxin-free fraction V bovine serum albumin (BSA) as a 7.5% (w/v) sterile solution, phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor (SBTI) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). ^{125}I-Protein A (affinity purified, 1600 MBq/mg) was from Amersham International plc (Little Chalfont, UK). All reagents for SDS-PAGE were from Bio-Rad (Richmond, CA, USA). Nitrocellulose membranes (0.45 μm pores) were from Schleicher and Schuell (Dassel, Germany).

CG was isolated from human polymorphonuclear neutrophils according to a previously described procedure. The purified enzyme was devoid of leukocyte elastase and proteinase 3 activities [17].

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Antibodies used in this study were the following: the murine monoclonal antibody MAII, raised against human platelet TSP-1, reacts with an epitope located within the Asn^{1}–Ala^{174} sequence of the amino-terminal HBD [11]. The polyclonal rabbit antibodies designated R3, R6 and R5 are directed against recombinant type 1 (Gly^{385}–Ile^{522}), type 2 (Pro^{559}–Ile^{669}) and type 3 (Asp^{784}–Val^{932}) repeating sequences of human TSP-1, respectively [9]. The immunopurified anti-fibrinogen and control Fab fragments used in functional studies were prepared from rabbit IgG as previously described [18]. For immunoblot analysis, a polyclonal rabbit antibody raised against purified human fibrinogen was obtained from Dako A/S (Copenhagen, Denmark). The polyclonal IgG fraction obtained from a rabbit antiserum raised against mouse IgG (RAM/7S) was purchased from Nordic Immunology (Tilburg, The Netherlands). All antibodies were used as homogeneous IgG (or Fab) fractions.

2.2. Preparation of isolated human platelets

Blood anticoagulated with ACD (NIH formula C) was obtained by venipuncture from healthy adult volunteers. Platelets were then isolated from plasma by differential centrifugations and finally resuspended at 3×10^{8}/ml in Tyrode medium (pH 7.4) containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 3.5 mg/ml BSA, as detailed previously [9].

2.3. Platelet aggregation and secretion

Platelet suspensions (400 μl) were activated at 37°C under stirring (1100 rpm) in a Dual Aggro-meter (ChronoLog Corp., Havertown, PA, USA) with 50–400 nmol/l CG or 0.1 U/ml α-thrombin. When necessary, platelets were preincubated for 1 min with each of the various antibodies to be tested, before addition of CG or α-thrombin. The variation in light transmission (LT) through the platelet suspension was continuously recorded and platelet aggregation expressed as a percentage of the maximal variation in LT [19]. At the given time points, the enzymic activity of CG was blocked by the addition of 1 mmol/l PMSF and 200 μg/ml SBTI [12], whereas α-thrombin was blocked by the addition of 1 U/ml hirudin [20]. Platelets were then separated into two 200-μl aliquots: the first aliquot was centrifuged at $12000\times g$ for 3 min, and both the supernatant and pellet were solubilized in the presence of 2% (w/v) SDS and 5 mmol/l *N*-ethylmaleimide by heating at 100°C for 5 min [17]. Samples were kept at −20°C until SDS-PAGE and immunoblot analysis. The second aliquot was mixed with 40 μl of a solution of 0.1 mol/l EDTA to stop exocytosis and centrifuged at $12000\times g$ for 4 min. The supernatant, and an aliquot of the unsedimented platelet suspension, were kept at −20°C until they were processed for the measurement of secreted β-thromboglobulin (β-TG) [19]. This was done using a commercial ELISA (Diagnostica Stago, Asnières, France) following the manufacturer's instructions. In selected experiments, the aggregated platelets (400 μl) were fixed by adding 50 μl of a 5% (v/v) paraformaldehyde solution in the aggregometer cuvette, then deposited onto 8-well strips and observed using phase-contrast optical microscopy.

2.4. SDS-PAGE and immunoblot analysis

Electrophoretic separation of platelet proteins in SDS-polyacrylamide slab gels was performed according to the procedure of Laemmli as adapted by Nurden et al. [21]. Protein disulfide bonds were reduced by heating the samples at 100°C for 5 min in the presence of 5% (v/v) 2-mercaptoethanol. Separated proteins were electrotransferred onto nitrocellulose membranes and analyzed by immunoblotting as previously described [19]. Primary incubation with the monoclonal antibody MAII was followed by a second incubation with a rabbit anti-mouse IgG antibody (RAM/7S). Bound immunoglobulins were revealed by a final incubation with ^{125}I-Protein A diluted 1/1000 and exposure of nitrocellulose membranes on Kodak X-Omat MA films (Kodak-Pathé, Marne-la-Vallée, France). The extent of proteolysis of one given protein was quantitated by densitometric scanning of autoradiographs, performed using an LKB Ultroscan XL densitometer (LKB-Produkter AB, Bromma, Sweden) equipped with a 633 nm laser beam.

3. Results

3.1. Activation of platelets by cathepsin G

Platelets were activated with increasing concentrations of CG at 37°C under stirring, and aggregation and secretion of α-granules were followed over time. Whether considering the concentration dependency or the time course of CG-induced platelet activation, the rate of increase in aggregate formation strictly paralleled that of α-granule exocytosis as measured by the secretion of β-TG (Fig. 1). Under optimal conditions of activation by CG (i.e. 200–400 nmol/l for 5 min), maximal values for platelet aggregation and α-granule exocytosis were 67.3 ± 4.5 and $92.3\pm3.8\%$ (mean ± S.D., n=12), respectively. α-Thrombin (0.1 U/ml) was used in parallel as it gave similar values of platelet aggregation and secretion, i.e. 70.2 ± 8.2 and $89.5\pm9.4\%$ (n=9), respectively. Control unactivated platelets stirred for 5 min at 37°C showed no detectable aggregation and basal secretion of β-TG was $9.1\pm1.4\%$ (n=5).

3.2. Cleavage of TSP-1 and fibrinogen during CG-induced platelet aggregation

In order to evaluate the putative proteolytic modifications of TSP-1 and fibrinogen upon secretion from α-granules, we separated the aggregated platelets from the extracellular milieu and analyzed these proteins by SDS-PAGE and immunoblotting. A representative time-course analysis of the proteolysis of TSP-1 and fibrinogen during aggregation of platelets with 200 nmol/l CG is illustrated in Fig. 2. Immunoblotting analysis of cell-associated TSP-1 performed under reducing conditions indicated that TSP-1 was rapidly proteolyzed during activation (Fig. 2A, left-hand panel), as the intact TSP-1 monomer with an M_r of 180400 ± 6100 (mean ± S.D., n=5) progressively decreased in intensity, while a cell-associated fragment, noted TSP-1_f, with M_r=164400 ± 5000 (n=5) increased concomitantly. Densitometric analysis of the autoradiograph shown in Fig. 2 indicated that the conversion of TSP-1 into TSP-1_f closely paralleled the rates of α-granule exocytosis and platelet aggregation (indicated at the bottom of the figure), with TSP-1_f corresponding to 12, 40, and 81% of the total TSP-1-reactive material at 1, 3, and 5 min of activation, respectively (Fig. 2A, left-hand panel). On seven different platelet preparations, TSP-1_f represented $85\pm6\%$ (mean ± S.D.) of the cell-associated TSP-1 molecules at 5 min of activation, when exocytosis and aggregation were maximal. The residual intact TSP-1 (≈15% of the initial content)

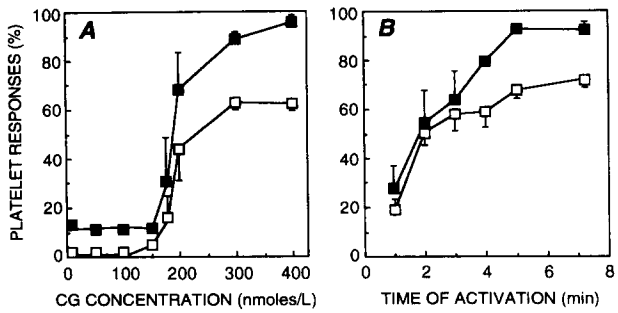

Fig. 1. Concentration dependency and time course of CG-induced platelet exocytosis and aggregation. Platelets (3×10^{8}/ml) were incubated in an aggregometer cuvette at 37°C under stirring for 5 min with increasing concentrations of CG (A), or with 200 nmol/l CG for increasing periods of time (B). At the end of incubation, measurements were performed for the extent of cell aggregation (□), and secretion of β-TG (■), as described in Section 2. Each point is the mean ± S.E.M. of 2–4 experimental values.

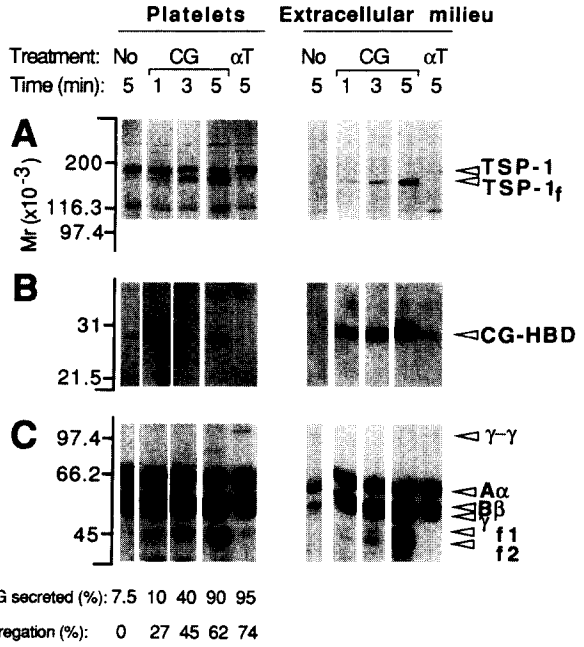

Fig. 2. Immunoblot analysis of the proteolytic activity of CG on secreted TSP-1 and fibrinogen. Platelets were treated with 200 nmol/l CG for 1 to 5 min, or with 0.1 U/ml α-thrombin or with no proteinase for 5 min. After sedimentation of the platelets (see Section 2), proteins from the platelets (10 μg) or from the corresponding volume of milieu were solubilized by SDS in the presence of 5% 2-mercaptoethanol and separated by SDS-PAGE on 7% (A,C) or 10% (B) acrylamide gels. Proteins transferred onto nitrocellulose membranes were probed with the following antibodies: anti-TSP-1 R5 (A), anti-TSP-1 MAII followed by RAM/7S (B), anti-fibrinogen IgG (C), all used at 10 μg/ml. Bound immunoglobulins were revealed by incubation with ^{125}I-Protein A, followed by autoradiography. The percentages of β-TG secretion and cell aggregation measured for this particular experiment are indicated at the bottom.

can be considered mostly as nonsecreted molecules, on the basis that the maximal release of β-TG was ≈90%.

A fraction of the total TSP-1_f generated during activation of platelets by CG was also recovered in the extracellular milieu, particularly at the later time points (Fig. 2A, right-hand panel). Thus, based on densitometric analysis, 48 ± 12.5% of the total TSP-1_f fragment was in the supernatant at 5 min (mean ± S.D., n=6). Conversion of the TSP-1 monomer into TSP-1_f was accompanied by the generation of a smaller fragment with M_r=28 200 ± 1450 (n=3), which was identified as the amino-terminal HBD (CG-HBD) since it was recognized by the monoclonal antibody MAII [7] (Fig. 2B). The most striking feature, however, was that, being generated during the course of platelet exocytosis and aggregation, the CG-HBD was almost entirely released into the extracellular milieu, with only traces (<5%) detected in the cell extracts (Fig. 2B). These data were compared to those obtained when platelets were activated for 5 min with 0.1 U/ml α-thrombin. In this case, the bulk of TSP-1 remained associated with the cell surface, and compared to the control sample, there was no evidence for an α-thrombin-dependent proteolysis (Fig. 2A). Immunoblotting analysis of fibrinogen in the cell extract and extracellular milieu performed under reducing conditions indicated that ≈50% of secreted fibrinogen remained associated with the platelet surface during aggregation induced by CG (Fig. 2C). Proteolysis of the secreted fibrinogen appeared to be much less efficient than that of TSP-1 as it remained largely incomplete by 5 min both on the platelet surface and in the milieu. The major fragments detected were the f_1 fragment (M_r = 46 400 ± 400, n = 3), only detected in the milieu, and the f_2 fragment (M_r = 44 000 ± 2200, n = 4), present with the platelets and in the milieu (Fig. 2C). Of the three fibrinogen subunits, A_α (M_r = 65 600 ± 4300, n = 4), B_β (M_r = 56 100 ± 1700, n = 4), and γ (M_r = 52 500 ± 1450, n = 4), the densitometric signal given by the Aα chain in the CG-treated samples, relative to that of the control non-treated samples, was decreased by ≈30% at 5 min of activation, while those given by the Bβ and γ chains were unchanged. As for CG-aggregated platelets, ≈50% of secreted fibrinogen was retained on α-thrombin-aggregated platelets (Fig. 2C). There was no detectable proteolysis of this adhesive protein during platelet activation by α-thrombin, and only a small percentage of the γ chains was converted into γ-γ dimers (M_r=102 700 ± 1500, n=3), both on the platelet surface and in the milieu.

3.3. *Effects of anti-TSP-1 and anti-fibrinogen antibodies on CG-induced platelet aggregation*

Since the TSP-1f fragment was the only one to be retained on CG-aggregated platelets, we evaluated its role in supporting platelet-to-platelet interactions. For this, we tested a panel of polyclonal antibodies directed against the TSP-1f fragment, designated R3, R5, and R6 [9], on CG-induced aggregation of several platelet preparations. Results of a typical experiment are shown in Fig. 3. As compared to a nonimmune rabbit IgG, these antibodies were found to inhibit CG-induced platelet aggregation (Fig. 3A). The main effect was a decrease in the size of the aggregates formed as shown by the reduction in amplitude of the oscillations in the aggregometer tracings. Examination of the aggregates by light microscopy confirmed that large platelet aggregates were not formed in the presence of these antibodies (Fig. 3B). In contrast, the monoclonal antibody MAII that reacts with the HBD of TSP-1 had no effect on CG-induced platelet aggregation.

By comparison, the anti-fibrinogen Fab fragments produced almost total inhibition of platelet aggregate formation (Fig. 3A).

4. Discussion

Formation of platelet macroaggregates is clearly dependent upon exocytosis and surface expression and organization of fibrinogen and TSP-1 [3,4]. Thus, using α-thrombin as the agonist, it was originally shown that TSP-1 stabilizes the binding of fibrinogen to its membrane receptor, the $\alpha_{IIb}\beta_3$ integrin [4]. Recent studies have indicated that the amino-terminal heparin binding domain (HBD) of TSP-1 may interact with membrane-bound fibrinogen [9,11], whereas there is also a role for other domains of TSP-1 in platelet aggregation [9,22,23].

Cathepsin G is another potent platelet agonist [12-14,24] which can induce extensive cell exocytosis and aggregation, even in the absence of exogenously added fibrinogen ([17] and this study). This study is so far the first to focus attention on the role of secreted adhesive proteins, namely fibrinogen and TSP-1, in CG-induced platelet aggregation considering the fact that, once secreted from α-granules, these are potential substrates for the enzymatic activity of CG [15,16]. A major role of secreted fibrinogen in CG-induced aggregation

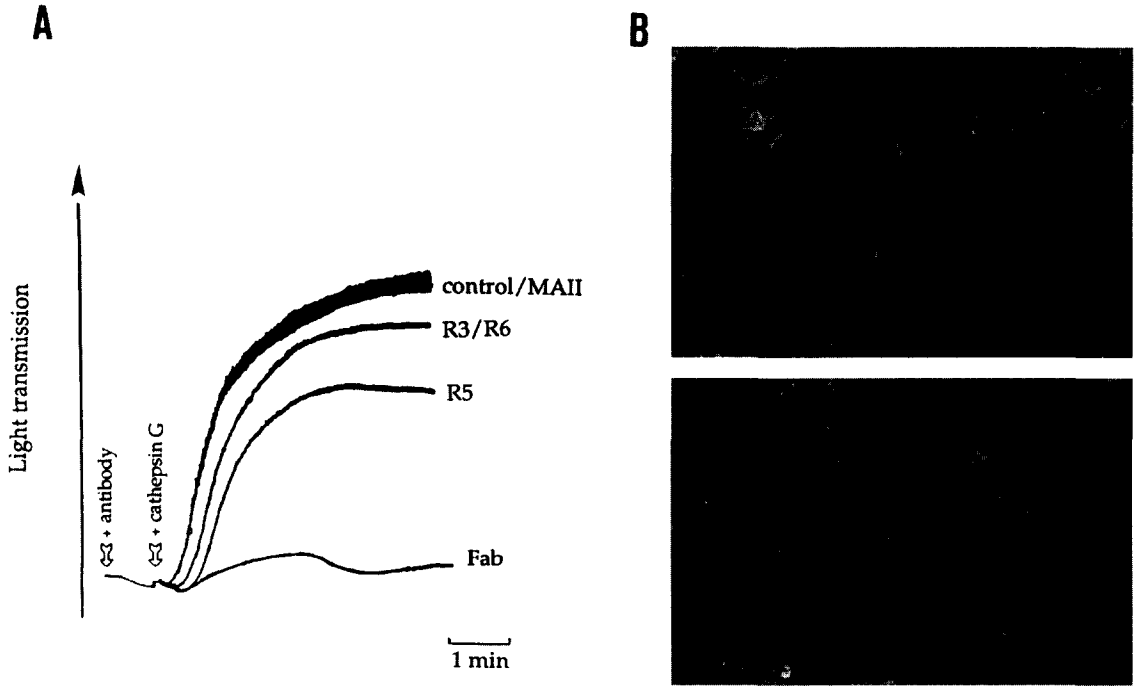

Fig. 3. Effects of anti-TSP-1 and anti-fibrinogen antibodies on CG-induced platelet aggregation. In A, platelets were tested for aggregation induced by 400 nmol/l CG, following a 1 min preincubation with the anti-TSP-1 monoclonal murine IgG MAII (0.2 mg/ml), or with the anti-TSP-1 polyclonal rabbit IgG R3, R6 or R5 (0.4 mg/ml), or with the anti-fibrinogen rabbit IgG Fab fragments (1 mg/ml). Control platelet suspensions received buffer, nonimmune rabbit IgG (0.4 mg/ml) or Fab fragments (1 mg/ml). In B, platelets aggregated for 5 min with 400 nmol/l CG in the absence or presence of R_5 were fixed with 0.5% (v/v) paraformaldehyde and photographed under microscopy observation.

is apparent from our observation that anti-fibrinogen Fab fragments are potent inhibitors of this aggregation. In agreement with these data, previous reports have indicated that CG-activated platelets express the active conformation of the $\alpha_{IIb}\beta_3$ fibrinogen receptor [13,24]. As previously suggested from coagulation assays [16], we have shown that CG has some fibrinogenolytic activity, which is, however, rather limited under the conditions used in our experiments, especially for the membrane-bound fibrinogen (see Fig. 2C). Late during CG-induced aggregation, a limited fraction ($\approx$30%) of the Aα chains of cell-associated fibrinogen is cleaved, most likely within the carboxy-terminal region which is known to be highly susceptible to early proteolytic attack by various serine proteinases [25].

A major observation in our study is that TSP-1 is very efficiently proteolyzed as soon as it is released from the α-granules of CG-activated platelets. Proteolysis generates a fragment with $M_r \approx 28\,000$, and a partially degraded TSP-1 remnant made of polypeptide chains with $M_r \approx 165\,000$ (TSP-1f). This pattern of cleavage, similar to that reported for other serine proteinases acting on purified TSP-1 [7,8], as well as the fact that the shorter fragment contains the epitope for the monoclonal antibody MAII [7], clearly identifies the 28 000-M_r fragment as the amino-terminal heparin-binding domain (CG-HBD). The most striking and interesting feature in this study is that the CG-HBD is readily released from the platelet surface as soon as TSP-1 is proteolyzed in situ by CG, and almost totally absent from the cells at the time macroaggregates have formed and stabilized (see Fig. 2B). Thus, this domain which was previously shown to play a major role in platelet aggregation induced by α-thrombin [9,11] appears not to be essential for platelet aggregation induced by CG, as also indicated by the fact that MAII had no effect on CG-induced platelet aggregation. In addition, we have demonstrated in this study that the TSP-1f carboxy-terminal fragment which remains associated with the platelet surface is functionally active. This is shown by the potency of several antibodies directed against this fragment to limit the size of the aggregates formed. Whether the TSP-1f fragment interacts with fibrinogen or other plasma membrane component(s) on CG-activated platelets remains to be determined.

In conclusion, we have shown here that extensive and stable platelet aggregation can occur with CG despite the fact that a major functional domain of the TSP-1 molecule (i.e. the HBD) is rapidly removed from the cell surface by this proteinase. In a more general aspect, the potential capacity of secretable leukocyte serine proteinases such as elastase [26] and CG (this study) to proteolyze TSP-1 on the surface of cells where it can be expressed, or in the extracellular matrices where it is incorporated [6], could be an important pathway in the regulation of its biological activities in various physiopathological situations.

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