**Thrombin induces GPIb-IX-mediated fibrin binding to α Ib β 3
in a reconstituted CHO cell model**

D. Pabón, A. Jayo, J. Xie, P. Lastres, and C. González-Manchón

Department of Molecular and Cellular Pathophysiology, Centro de Investigaciones
Biológicas (CSIC), Ramiro de Maeztu 9, 28040-Madrid, SPAIN

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Address for correspondence:

Consuelo González-Manchón

Department of Molecular and Cellular Pathophysiology

Centro de Investigaciones Biológicas (CSIC)

Ramiro de Maeztu, 9

28040-Madrid, SPAIN

FAX N°: 34-91-5360432

E-mail: cgmanchon@cib.csic.es

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Summary

Background: The interaction of thrombin with platelet glycoprotein (GP) Ib-IX-V has been recently suggested to induce fibrin-dependent platelet aggregation associated to signaling events. The approaches used to avoid the PAR thrombin receptors in platelets have provided controversial conclusions regarding the precise mechanism and molecules involved in the response. *Objectives:* In the present study, we have developed a cellular model to investigate the functional consequences following the binding of thrombin to GPIb-IX. *Methods:* We used Chinese hamster ovary (CHO) cells stably expressing human α IIB β 3 and/or GPIb-IX complexes (CHO- α IIB β 3-IbIX cells) to analyze the effect of thrombin on the binding of polymerizing fibrin by using FITC-fibrinogen as precursor. *Results:* Thrombin induces, in a dose-dependent manner, the binding of polymerizing fibrin to CHO- α IIB β 3-IbIX cells. This response is not observed in cells expressing only one of the receptors, and it can be blocked by monoclonal antibodies against α IIB β 3 and GPIb α . We show that the reaction is not due to simple cell trapping by the fibrin clot, and provide data supporting a role of a signaling pathway in which the 14-3-3 ζ adaptor and calcium-calmodulin-dependent events are involved. *Conclusions:* The present data support a significant role of GPIb-IX and α IIB β 3 receptors in an alternative fibrin-mediated pathway of platelet activation induced by thrombin.

Key words: GPIbIX, thrombin, α IIB β 3, fibrin, CHO cells

Introduction

The platelet glycoprotein (GP) Ib-IX-V complex plays an essential role in primary haemostasis, regulating the early adhesion of platelets to exposed subendothelium through the binding to von Willebrand factor (vWF). The complex comprises four transmembrane subunits: GPIb α covalently linked to GPIb β , GPIX and GPV, in a final stoichiometry of 2:2:2:1. GPIb α functions also as a high-affinity thrombin-binding site facilitating platelet activation at low concentrations of the agonist [1]. Although it has long been recognized that this interaction is required for optimal platelet activation [2], the precise biological significance and the mechanism of GPIb α in thrombin-mediated platelet activation remain unclear. This has been attributed to the presence of the protease-activated receptors (PAR)-1 and 4 that are also involved in the activation of human platelets and are activated by low and high concentrations of thrombin, respectively [3]. In addition, a recent study indicates that GPIb α and ADP act in synergy to amplify PAR-1 coupled responses [4].

Recently, several lines of evidences have provided new insights into the activation signals triggered by the interaction of thrombin with GPIb α . The recently reported crystal structure of platelet GPIb α bound to thrombin demonstrates the simultaneous interaction of GPIb α with thrombin exosites I and II, enabling the contact of GPIb α with two distinct thrombin molecules [5,6]. It has been suggested that this interaction would lead to receptor clustering, an event that commonly triggers activation signals.

Furthermore, strategies have been designed in order to study the functional consequences of the thrombin-GPIb α interaction without interference of the much stronger PAR-mediated pathway. By using platelets with desensitized PAR receptors, several authors have reported that thrombin induces GPIb α -dependent aggregation that requires polymerizing fibrin and is associated to signaling events [7,8]. In these reports, data regarding the identity of the platelet receptor involved in the fibrin-dependent aggregation are controversial.

Ramakrishnan et al. [9] reported a signaling pathway triggered by the binding of catalytically inactive thrombin to GPIb α in GPV depleted platelets. This pathway was dependent on ADP secretion and results in activation of α IIb β 3 and consequently in aggregation. In agreement with these observations, immobilized proteolytically inactive thrombin has been shown to induce GPIb α -dependent platelet activation in which ADP

plays a major role [10]. In this study, activation of the $\alpha\text{IIb}\beta 3$ integrin was also shown to be a consequence of the GPIb α -thrombin interaction. In these models, in contrast to the results obtained from platelets with desensitized PAR receptors, a fibrin receptor would not be required since inactivated thrombin, unable to form fibrin, was used.

More recently, GPIb-dependent interaction of platelets with immobilized thrombin has been demonstrated under flow conditions [11]. The reported findings suggest that the thrombin-GPIb α interaction, by inducing receptor crosslinking, may trigger different responses to facilitate platelet activation by low doses of thrombin. However, it cannot be excluded that some of the observed discrepancies came from technical aspects such incomplete PAR receptor or thrombin inactivation and/or granule secretion of fibrinogen and ADP content. Furthermore, it has been also claimed that the reported fibrin-mediated aggregation is merely due to platelet trapping which is not dependent on platelet activation or GPIb [12].

In the present work we have used a cellular model to further investigate whether the binding of thrombin to GPIb-IX triggers an alternative activation pathway. The results suggest that the thrombin-GPIb interaction induces signaling events leading to the binding of polymerizing fibrin to a conformational state of $\alpha\text{IIb}\beta 3$ that is not competent to bind fibrinogen.

Materials and Methods

Antibodies and reagents

Monoclonal antibodies (mAb) specific for $\beta 3$ (713H7) and αIIb (701E5) were raised in our laboratory using as antigen $\alpha \text{IIb}\beta 3$ heterodimer isolated from human platelets [13]. The mAb 11E5D6, produced in the laboratory, is directed against the anti-Fg A α chain of fibrinogen. mAbs P37 and M3, anti- $\beta 3$ and anti- αIIb , respectively, were provided by Dr. José González (Institute Rocasolano, CSIC, Madrid, Spain). mAbs SZ2 (anti-GPIb α) and SZ1 (anti-GPIX) were from Beckman Coulter (Fullerton, CA, USA), and AK2 (anti-GPIb α) was from Serotec (Oxford, UK). Fluorescein isothiocyanate-conjugated (FITC) F(ab')₂ fragment of rabbit anti-mouse immunoglobulin (Ig) was purchased from Dako A/S (Glostrup, Denmark). The horseradish peroxidase (HRP)-conjugated goat anti-mouse was from BioRad Laboratories (Hercules, CA, USA). FITC-PAC-1 was obtained from Becton Dickinson (San José, CA, USA). Anti-phosphotyrosine mAb PY99 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-p44/42 MAPK polyclonal antibody was from Cell Signaling (Danvers, MA).

Thrombin from human plasma (2,500 NIH units/mg protein), W7 (calmodulin antagonist), genistein (tyrosin protein kinase inhibitor), cytochalasin D (actin polymerization inhibitor), and Ser-Phe-Leu-Leu-Arg-Asn (TRAP-6) and Arg-Gly-Asp-Ser (RGDS) peptides were from Sigma-Aldrich (Madrid, Spain). LY294002 (phosphoinositide 3-kinase inhibitor), PD98059 (MAP kinase kinase inhibitor), Bisindolylmaleimide I (BIM-I, protein kinase C inhibitor) and Gly-Pro-Arg-Pro (GPRP) peptide were purchased from Calbiochem (La Jolla, CA). Recombinant hirudin was a gift from Namos GmbH (Bannewitz, Germany), and purified heparin and bemiparin (low molecular weigh heparin, BMP) were provided by Rovi Pharmaceutical Laboratories (Madrid, Spain).

CHO cell lines stably expressing $\alpha \text{IIb}\beta 3$ and/or GPIbIX wild or mutant

The generation of CHO cell line stably expressing $\alpha \text{IIb}\beta 3$ (CHO- $\alpha \text{IIb}\beta 3$ cells) has been previously reported [14]. Cell clones expressing $\alpha \text{IIb}\beta 3$ and GPIbIX receptors (CHO- $\alpha \text{IIb}\beta 3$ -IbIX cells) were established by transfection of CHO- $\alpha \text{IIb}\beta 3$ cells with the cDNAs encoding GPIb α , GPIb β and GPIX cloned into the pcDNA3 expression

plasmid, using the calcium phosphate precipitation procedure. G-418 resistant cells were cloned using a FACS Vantage cell sorter (Becton Dickinson, San José, CA) after labeling the cells with the anti-GPIb α mAb AK2. The sorted clones were further selected for GPIb β and GPIX expression by using the anti-GPIX mAb SZ1, which recognizes the GPIb β -IX complex. Several clones expressing similar levels of recombinant receptors were used for the experiments.

CHO- α IIB β 3-IbIX(605) cells were established by transfecting a truncated form of GPIb α lacking the last five residues of the cytoplasmic carboxyterminal domain.

CHO cells expressing the GPIb-IX complex (CHO-IbIX cells) were kindly provided by Dr. J. A. López (Baylor College of Medicine, Houston, TX).

Analysis of cell surface and total receptor content

Cells were harvested using 0.5 mM EDTA in phosphate-buffered saline (PBS), resuspended at a density of 10^5 cells/100 μ l, and incubated for 30 min at 4°C with the specific mAb. Cells were then washed and incubated in a 1:25 dilution of fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of rabbit anti-mouse Ig for 30 min. Cells were either analyzed in a flow cytometer (EPICS XL, Beckman-Coulter, Miami, FL) or cloned using a cell sorter.

For total receptor cell content estimation, cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.05% Tween 20, supplemented with 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 5 μ l/100 μ l protease inhibitor cocktail (Sigma) and incubated with biotin-NHS, followed by immunoprecipitation with specific mAbs anti- α IIB or anti-GPIb α , separation in 6-16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting with avidin-horseradish peroxidase (BioRad Laboratories).

FITC-labeling of fibrinogen and fibrin polymerization assay

Plasminogen-depleted fibrinogen (Fg) from human plasma (Calbiochem, containing $\geq 95\%$ clottable proteins) was labeled with fluorescein isothiocyanate (FITC). Briefly, Fg was diluted to 2 mg/mL in phosphate buffer solution (PBS) and the pH adjusted to 9.0 with 5% Na₂CO₃. The solution was incubated with 1 mg/mL FITC on celite 10% (Calbiochem) at room temperature (RT) with intermittent vortexing. The celite was separated by centrifugation and unreacted free FITC was cleaned by passing the

reaction mix through a Sephadex G-25 column equilibrated with PBS pH 7.4. The molar ratio of fluorescein to protein varied from 7 to 9, and the concentration of FITC-Fg determined by measuring absorbance at 280 and 495 nm was around 1 mg/mL. 6-8 mg/mL FITC-Fg was obtained by concentrating the labeling reactions with Centriprep columns (Millipore, Bedford, MA) before FITC cleaning. Aliquots of FITC-Fg were stored at -80°C and quickly thawed to RT before used.

For fibrin polymerization assays, Fg or FITC-Fg (200 µg/mL) were incubated with different concentrations of thrombin in a final volume of 50 µL of Tyrode buffer (5 mM Hepes, 2 mM MgCl₂, 0.3 mM NaH₂PO₄, 3 mM KCl, 134 mM NaCl, 12 mM NaHCO₃, pH 7.4) containing 2 mM calcium for 30 minutes at RT. The reactions were stopped by adding a volume of 2x Laemmli buffer (125 mM Tris-Cl, pH 6.8, 8% SDS, 10% 2-mercaptoethanol, 17% glycerol) and 25 µL samples were electrophoresed on 10% SDS-PAGE, transferred to PVDF membranes and stained with amidoblack.

Fibrin and PAC-1 binding assays

CHO cell lines growing in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum were harvested with 0.5 mM EDTA and resuspended in Tyrode buffer containing 2 mM CaCl₂, at a density of 0.25-0.5x10⁶ cells/100 µL and incubated in the absence or presence of different concentrations of thrombin for 5 minutes at RT. FITC-Fg (160-1,600 µg/mL) or FITC-PAC-I (12.5 µg/mL) was then added and, after 20-30 minutes at RT, cells were washed by centrifugation and analyzed by flow cytometry. Before data acquisition, macroscopic fibrin clots were removed from the samples. In order to analyze only single cells, events were gated based on their forward versus side scatter properties, discarding aggregates of cells trapped in microscopic fibrin meshes. When indicated cells were preincubated with different reagents before thrombin addition.

In some experiments, fibrin polymerization was analyzed by transferring 20 µL aliquot of the reaction to a volume of 2x Laemmli buffer. Samples were resolved on 10% SDS-PAGE and transferred to PVDF membranes that were either analyzed by western blot with a mAb anti-A α Fg or stained with amidoblack.

Phosphotyrosine and p44/42 MAPK immunoblot analysis

To determine the pattern of total protein tyrosine phosphorylation, cells were diluted at a density of $0.5\text{-}1 \times 10^6$ cells/50 μL and, then, stimulated with thrombin at RT for different periods of time. After adding a volume of 2x Laemmli buffer to stop thrombin stimulation, a portion of the cell lysate was separated by 6-16% SDS-PAGE under reduced conditions, transferred to PVDF sheets and immunoblotted with the antiphosphotyrosine mAb PY99. After incubation with HRP-conjugated anti-mouse IgG, the bound antibody was visualized using the ECL system. Alternatively, $3\text{-}4 \times 10^6$ cells were lysed in 300 μL of ice-cold Nonidet P-40 buffer (50 mM Tris, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol) supplemented with 1 mM PMSF, 1 mM Na_3VO_4 and 5 μL /100 μL protease inhibitor cocktail, precleared and incubated with 30 μL of agarose-conjugated antiphosphotyrosine mAb 4G10 for four hours at 4°C . The immunoprecipitates were then electrophoresed and blotted with antiphosphotyrosine mAb PY99 as described.

Phosphorylation of p44/42 MAPK subunits by thrombin stimulation of 250,000 cells preincubated or not with 5 $\mu\text{g/mL}$ SZ2 mAb or 40 μM TRAP-1 was determined by western analysis. Cell lysates were electrophoresed and blotted with an anti-phospho-p44/42 MAPK polyclonal antibody.

Results

Establishment of α IIb β 3-IbIX CHO cell lines

CHO cell lines stably expressing α IIb β 3 and GPIbIX complexes were generated by transfecting cDNAs encoding the aminoacid sequence for GPIb α , GPIb β and GPIX into cells stably expressing α IIb and β 3 (CHO- α IIb β 3 cells). Among the stable transfectants, cell lines expressing levels of α IIb β 3 integrin and GPIb α comparable to CHO- α IIb β 3 and CHO-IbIX cells, respectively, were selected. The surface receptor expression was estimated by flow cytometry (Fig. 1) and the total cell content by immunoprecipitation analysis of biotin-labeled total cell lysates (data not shown). GPIbIX dimer expression is higher in CHO-IbIX cells as detected by the anti-GPIX mAb SZ1, which recognizes an epitope requiring the GPIX subunit to be complexed to GPIb β . However, the fluorescence detected by the anti-GPIb α mAb directly correlates with the amount of total complex since GPIb α can not be efficiently expressed at the cell surface in the absence of the other subunits (Fig. 1).

Thrombin induces the binding of fibrin to CHO- α IIb β 3-IbIX cells

Fig. 2A shows that thrombin induces, in a dose-dependent manner, an increase in surface fluorescence in CHO- α IIb β 3-IbIX cells incubated in the presence of FITC-fibrinogen (Fg). The maximal effect was normally achieved with concentration ranging 10 to 40 mU/mL; however, in some instances, lower concentrations were also effective. Higher thrombin concentrations increase the rate of clot formation and reduce the fluorescence signal reaching basal levels at a concentration close to 1 U/mL. No significant differences were obtained whether the assays were carried out at 37°C or RT. This effect of thrombin was much less apparent in cells expressing only the α IIb β 3 integrin or the GPIbIX complex, suggesting that both receptors are needed (Fig. 2B). As shown in Fig. 2C, increasing FITC-Fg concentration in a range of 160-1,600 μ g/mL did not significantly modify the effect of 40 mU/mL thrombin on cell binding.

To confirm that both receptors are involved in the binding response induced by thrombin cells were preincubated with monoclonal antibodies directed against α IIb β 3 or GPIbIX. The anti- β 3 mAb P37, that recognizes an epitope located in the 150-216 segment of the 23-kDa N-terminal fragment of β 3, has been shown to inhibit platelet aggregation [14]. As shown in Fig. 3, P37 inhibits in a dose-dependent manner the

binding effect. In contrast, M3, an anti- α IIB mAb without effect on platelet aggregation [15], did not block the effect of thrombin (results not shown). Furthermore, the anti-GPIb α mAb SZ2, that recognizes the sulfated-tyrosine containing sequence involved in the thrombin binding [16], was also found to block the binding effect induced by thrombin (Fig. 3). Fibrin polymerization, assessed by clot formation and detection of soluble fibrin by SDS-PAGE, was not altered by the presence of any of these antibodies (data not shown).

In PAR-desensitized platelets, thrombin-triggered signaling does not induce the conformational changes of α IIB β 3 needed to bind fibrinogen [8]. We explored this possibility by using the mAb PAC-1, which recognizes an epitope of α IIB β 3 integrin in activated platelets. As shown in Fig. 4A, thrombin stimulation does not affect the binding of FITC-PAC-1 to CHO- α IIB β 3-IbIX cells, suggesting that α IIB β 3 is not present in a conformation able to bind fibrinogen.

In our experimental conditions, a fibrin clot becomes macroscopically detectable at thrombin ranging between 16-40 mU/mL, the same concentrations that produced maximal binding effects. Fibrin has been suggested to be involved in platelet aggregation induced by the GPIb-thrombin interaction [7,8]. As shown in Fig. 4B, in the presence of the GPRP peptide, that inhibits fibrin polymerization, the binding effect of low concentrations of thrombin was completely impeded. Furthermore, altering the order of addition of the cells produces a significant change in the extent of the response. When fibrinogen is added to cells that have been first incubated with thrombin, a maximal effect is obtained. However, a significantly reduced binding is observed when the cells are added after a lag period in which the thrombin generation of fibrin is in progress. GPRP inhibited the effect of thrombin in both conditions. Overall, the results suggest that the observed cell binding is due to fibrin- α IIB β 3 interaction.

In order to discard that the effect of thrombin was due to abnormal clottability of fibrinogen as a consequence of its conjugation with FITC, we analyzed the pattern of fibrinopeptide release and fibrin polymerization of FITC-labelled fibrinogen upon incubation with thrombin by SDS-PAGE analysis followed by amidoblack staining (Fig. 4C). Similarly to unlabelled fibrinogen, under reducing conditions, FITC-labelled fibrinogen contained the expected bands representing the A α , B β , and γ chains. A similar pattern of fibrinopeptide (FP) A and B release was also observed after addition of increasing doses of thrombin. As expected if the commercial plasma fibrinogen

preparation was contaminated with plasma FXIII, in the presence of calcium and upon thrombin addition, small amounts of γ chain dimers were seen in both unlabelled and FITC-labelled fibrinogens. In Fig. 4D, binding of polymerizing fibrin to individuals CHO- α Ib β 3-IbIX cells was visualized by fluorescence microscopy analysis.

Since our commercial preparation of fibrinogen contains FXIII, we considered the possibility that cross-linked fibrin(ogen) was a major determinant in the final fibrin species bound to the cell. A similar response was obtained when the cell binding assays were performed in the absence of calcium (data not shown). However, it cannot be ruled out that increased fibrin crosslinking mediated by platelet FXIII was relevant *in vivo*.

To further characterize the thrombin-GPIbIX interaction we analyzed the ability of hirudin and heparin to inhibit the effect of thrombin on the binding of fibrin to CHO- α Ib β 3-IbIX cells. In our experimental conditions, cell binding induced by 16 mU/mL thrombin was completely blocked by 40 mU/mL of the direct thrombin inhibitor hirudin (Fig. 5). By analyzing fibrin formation by SDS-PAGE we found that this effect of hirudin is directly related to its capacity to inhibit the conversion of fibrinogen to fibrin. In contrast, high and low molecular weight heparins were able to block the effect of thrombin (Fig. 5) in conditions in which clot fibrin formation was not impeded (data not shown).

α Ib β 3 can recognize fibrinogen and fibrin through the RGDS sequence. As shown in Fig. 5, the tetrapeptide RGDS blocked the thrombin-induced effect of binding, supporting the involvement of the transfected α Ib β 3 integrin.

Signaling events associated to the interaction of thrombin with CHO- α Ib β 3-IbIX cells

The intracellular domains of the GPIb subunits contain binding sites for the signal transduction protein 14-3-3 ζ [17]. Since this interaction has been implicated in functional events mediated by signaling through the GPIb-IX complex [18-21], we considered of interest to analyze whether 14-3-3 ζ is involved in the GPIbIX-mediated thrombin response. We generated CHO- α Ib β 3-IbIX(605) cells expressing α Ib β 3 and GPIb-IX containing a 5-residue cytoplasmic tail truncated form of GPIb α . Using a yeast two-hybrid system we determined that, in contrast to the wild type, the mutated GPIb α cytoplasmic domain does not directly interact with 14-3-3 ζ (data not shown). As shown

in Fig. 6, CHO- α IIB β 3-IbIX(605) cells, expressing amounts of α IIB β 3 and GPIbIX similar to control cells, showed a significantly reduced binding response to thrombin.

To gain some more insights into the biochemical mechanism triggered by the thrombin-GPIbIX interaction we analyzed intracellular signaling pathways involved in GPIbIX-mediated responses, by treating cells with specific inhibitors prior thrombin stimulation (Fig. 7A). Although the pattern of total protein tyrosine phosphorylation was not changed by thrombin (results not shown), we found a significant reduction of thrombin-induced binding upon incubation of cells with the tyrosine phosphorylation inhibitor genistein. In addition, the inhibitory effect of the calmodulin antagonist W7 suggests that a calcium/calmodulin dependent pathway is involved in the response to thrombin.

Erk1/2 phosphorylation has been suggested to participate in the signaling events triggered by the thrombin-GPIb α interaction in platelets [8]. As shown in Fig.7B, we detected increase of Erk1/2 phosphorylation upon thrombin stimulation. However, GPIbIX might not be involved since the response was not blocked by the mAb SZ2 and was also detected in cells lacking the GPIbIX complex (data not shown). According to that, the selective inhibitor of MEK-1 PD98059 did not prevent the effect of thrombin on cell binding (Fig. 7A). Moreover, data from Fig. 8A suggest that Erk1/2 phosphorylation induced by thrombin is mediated by endogenous PAR-1 receptors, since the response was significantly decreased in cells desensitized with the PAR-1 agonist hexapeptide TRAP. However, as represented in Fig. 8B, the extent of cell binding was similar in cells untreated or pretreated with TRAP, suggesting that endogenous PAR-1 are not involved in the binding response induced by thrombin.

Discussion

Recently, several studies using different experimental models have provide new insights on the thrombin receptor function of GPIb α . These models were designed in order to differentiate between the GPIb-mediated and PARs-mediated thrombin responses. By using catalytically inactive thrombin [9,10] or by de-sensitizing the PAR receptors [7,8], these studies demonstrate that the interaction of thrombin with the GPIbIX complex can induce platelet aggregation associated to signaling events. However, the results are controversial regarding the precise mechanism and molecules involved in the response. It is possible that technical aspects of platelet experiments may account for some of the reported discrepancies.

In the present work, a cellular model was used to characterize the functional consequences of thrombin-GPIbIX interaction without the interference of the stronger PAR-mediated response and the difficulties inherent to platelet manipulation. Basically, we have analyzed the effect of increasing concentrations of thrombin on binding of FITC-Fg to CHO cells stably expressing the human platelet GPIbIX and/or α IIB β 3 complexes. Using different blocking agents and specific inhibitors we obtained data suggesting an operative pathway triggered by the interaction of thrombin with GPIbIX that leads to the binding of fibrin to α IIB β 3 integrin. The extent of the thrombin response was not significantly affected by the amount of fibrinogen in a wide range of concentration. The involvement of both GPIbIX and α IIB β 3 receptors is supported by the small or undetectable binding effect obtained in cells expressing α IIB β 3 or GPIbIX complexes, respectively. Moreover, an anti-GPIb α mAb that blocks thrombin binding to its high affinity site in GPIb α was found to prevent the effect of thrombin on CHO- α IIB β 3-GPIbIX cells, supporting the role of the thrombin-GPIbIX interaction in this process.

CHO cells expressing α IIB β 3 do not bind soluble fibrinogen but they can be forced to do it if the fibrinogen receptor is activated through ligand stimulation of over-expressed physiological agonist receptors [22,23]. In these cells, enhanced binding of the activation-dependent IgM PAC-1 was also achieved upon agonist stimulation. In contrast, in the present study the undetectable binding of PAC-1 to CHO- α IIB β 3-IbIX cells suggests that thrombin binding to GPIbIX is not sufficient to induce conformational changes that capacitate α IIB β 3 to bind fibrinogen. The following data

support that is polymerizing fibrin what is bound to CHO- α IIb β 3-IbIX cells. First, inhibition of fibrin polymerization by either the GPRP peptide or the thrombin inhibitor hirudin completely blocked the thrombin-induced cell binding. Second, in our experimental conditions, cell binding is detected at thrombin concentrations that produce a slow conversion of fibrinogen to fibrin, allowing the detection of soluble species of fibrin by SDS-PAGE analysis. Thrombin doses higher than 200 mU/mL induce the rapid formation of an insoluble fibrin clot associated to decreased cell binding. Finally, significant differences in cell binding are obtained depending of the order of addition of the reaction components. When cells are added after a lag period in which the thrombin generation of fibrin is in progress the observed effect is markedly reduced. It is also possible that the antithrombin I activity of fibrin contributed to this reduction [24].

The reported fibrin-dependent aggregation has been attributed to the fact that platelets become trapped during the conversion of fibrinogen to fibrin [12]. A non-specific cell trapping by the fibrin network was observed at the higher concentrations of thrombin used in our experimental conditions. However, by using both flow cytometry and fluorescence microscopy analysis, we demonstrated that binding of polymerizing fibrin to individual cells was specific of the CHO- α IIb β 3-IbIX line.

It has been also pointed out that thrombin may act by facilitating the formation of a fibrin-thrombin-GPIb α ternary complex. Such mechanism could operate *in vivo* serving as the core for further interactions required for clot stabilization [25]. In our experimental model, we did not obtain evidences for the formation of a GPIb-thrombin-fibrin complex, since cells expressing only the GPIbIX complex do not bind fibrin in response to the agonist.

Two studies have previously shown that thrombin-GPIbIX interaction induces fibrin-dependent platelet aggregation. However, they disagree with respect to the identity of the fibrin receptor [7,8]. In our experimental conditions, the small but significant effect of low concentrations of thrombin on CHO- α IIb β 3 cells could be attributed to binding of fibrin to resting α IIb β 3. p37, an anti- β 3 mAb that has been shown to block platelet aggregation by thrombin was found to prevent the effect of thrombin on both CHO- α IIb β 3 and CHO- α IIb β 3-IbIX cells. Together with the inhibition by the RGDS peptide, the results suggest that the binding of polymerizing fibrin is mediated by the α IIb β 3 integrin. Some of the fibrinogen recognition sites for integrins become exposed upon its

conversion into fibrin [26-28]. Also, although widely accepted, it has not been proved that polymerizing fibrin and fibrinogen both bind to $\alpha\text{IIb}\beta 3$ through the same peptide sites during platelet aggregation [29,30]. In the present work, $\alpha\text{IIb}\beta 3$ integrin expressed in CHO cells can bind polymerizing fibrin but no fibrinogen following thrombin-GPIbIX interaction, supporting a unique role for fibrin in mediating an alternative platelet aggregation triggered by the thrombin-GPIb interaction. Further studies may clarify whether fibrin is bound to resting $\alpha\text{IIb}\beta 3$ integrin, as suggested in the studies with platelets [8], or to a partially activated receptor.

Our data provide also evidences of signaling events coupled to the fibrin binding induced by thrombin. The pattern of total protein tyrosine phosphorylation displayed by unstimulated CHO- $\alpha\text{IIb}\beta 3$ -IbIX cells was not altered by thrombin either in the absence or presence of genistein, a tyrosine-specific protein kinase inhibitor. However, a significant reduction of thrombin-induced cell binding was obtained in the presence of the inhibitor, suggesting that tyrosine phosphorylation-dependent signaling events are involved in the response to thrombin. On the other hand, the marked inhibition produced by the calmodulin antagonist W7 may indicate the participation of calcium/calmodulin-dependent kinases. Our observation agrees with data reported by Soslau et al. [7] showing that thrombin-GPIb interaction is accompanied by mobilization of internal calcium in PAR-1-desensitized platelets. However, these results have been argued in a more recent study using platelets desensitized for both PAR-1 and PAR-4 receptors [8].

As reported for other GPIb-dependent responses [31], the Erk-MAPK pathway has been shown to participate in the signal cascade triggered by binding of thrombin to GPIb in platelets [8]. However, our results suggest that phosphorylation of Erk1/2 is not an essential requirement for the GPIb-mediated response to thrombin. Constitutive expression of PAR-1 has been reported in CHO cells [32]. Although in our cellular model thrombin-induced p42/p44 MAPK phosphorylation is largely mediated by endogenous PAR-1 receptors, occupancy of constitutive PAR-1 receptors does not seem to cooperate with GPIbIX to induce CHO- $\alpha\text{IIb}\beta 3$ -IbIX cell binding.

The present results with cells expressing a mutant GPIb α with truncation at residue 605 suggest a role for 14-3-3 ζ protein in regulating the thrombin-induced cell binding to fibrin. This phosphorylation-dependent signaling molecule has been implicated in a number of platelet and megakaryocyte responses mediated by GPIb [18,20]. Although

14-3-3 ζ also recognizes a site in the cytoplasmic domain of GPIIb β , our results agree with previous data suggesting that GPIIb β alone is not sufficient to support high affinity binding of 14-3-3 ζ . The SZ2 mAb recognizes the sulfated-tyrosine containing sequence of GPIIb α involved in the binding of thrombin and has been shown to prevent the effect of thrombin on cells transfected with wild GPIIb α . Binding of SZ2 was similar in both wild and mutant cell types, suggesting that no important structural perturbations have been propagated to the extracellular domain in the GPIIbIX(605) mutant. However, it remains to be determined whether the GPIIbIX-14-3-3 ζ interaction is regulating signaling events or modulating the thrombin binding function of GPIIbIX as described for the GPIIb α -vWF interaction [21].

Although a number of biochemical, functional and structural data demonstrate the participation of thrombin exosites-I and II, the precise nature of the GPIIb α -thrombin interactions remains controversial [33]. Furthermore, it is unclear whether the negatively charged region of GPIIb α containing sulfated tyrosine residues plays a role in the interaction with both anion-binding exosites of thrombin [5,6]. The involvement of thrombin exosites was addressed by analyzing the ability of hirudin and heparin to inhibit the effect of thrombin on the binding of fibrin to CHO- α IIb β 3-IbIX cells. We can not conclude about the participation of exosite-I since in our experimental conditions the blocking effect of hirudin could be accounted for by the prevention of fibrin polymerization. In contrast, heparin was found to inhibit the effect of thrombin on cell binding in the presence of polymerizing fibrin, supporting that exosite-II is involved in the GPIIb α occupancy by thrombin.

Despite the fact that less than 5% of the approximately 25,000 copies of GPIIb α expressed at the platelet surface has been shown to function as high-affinity thrombin-binding sites, a number of evidences suggest that the platelet GPIIb-IX receptor might play an important role at the start of the haemostatic process, when trace amounts of thrombin are generated [34]. In this context, the thrombin-GPIIbIX interaction-dependent pathway may be the first to operate in the presence of low thrombin concentrations [35]. Based on our experimental observations with a cell model, it is not unreasonable to suggest that the proposed GPIIb-dependent platelet aggregation mediated by fibrin may represent a basal haemostatic balance mechanism operating at thrombin concentrations that keep a rate of fibrin polymerization low enough to permit the binding of polymerizing fibrin to platelets. Thus, the consequences of the thrombin-

GPIbIX interaction may be influenced *in vivo* by factors such as quantitative and/or qualitative changes of thrombin and fibrinogen that could alter fibrin polymerization rate. Additional experiments with selected conditions closer to the physiological situation would provide further insights on the role of GPIbIX in platelet activation by thrombin.

In summary, the present work supports previous reports suggesting a novel role for fibrin in an alternative platelet aggregation pathway secondary to the thrombin-GPIbIX interaction. Our results provide evidence of the binding of polymerizing fibrin to a conformational state of the α IIb β 3 integrin unable to bind soluble fibrinogen. While more studies are required to assess the potential pathophysiological implications of this pathway, our cellular model provides a useful experimental approach to further investigate the responses triggered by the thrombin-GPIbIX interaction.

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Legends to the Figures

Fig. 1. Surface expression of α IIB β 3 and GPIb-IX complexes in stably transfected CHO cells

CHO cell lines stably expressing the α IIB β 3 and/or GPIb-IX complexes were established as described in Methods. Surface receptor expression was determined by flow cytometry using mAbs directed against α IIB (701E5), β 3 (713H7), GPIb α (AK2) and GPIX (SZ1). Numbers in the figure panels represent the values of the mean fluorescence levels in arbitrary units.

Fig. 2. Flow cytometry analysis of cell binding induced by thrombin in the presence of FITC-fibrinogen.

(A) CHO cells stably expressing α IIB β 3 or both α IIB β 3 and GPIbIX complexes were stimulated with increasing concentration of thrombin for 5 minutes and, then, FITC-Fg (160 μ g/mL) was added and incubation continued for 30 minutes either at RT or 37°C. After washing, cell binding was determined by flow cytometry. (B) The bar graph shows the values of mean fluorescence intensity upon addition of 160 μ g/mL FITC-Fg to CHO cell lines stimulated with increasing thrombin concentrations. Data represent the means $\pm$ S.D. of, at least, four experiments. (C) Effect of increasing FITC-Fg concentration on cell binding induced by thrombin (40 mU/mL). The non-specific basal binding of Fg was determined in each condition and the values subtracted from those obtained in the presence of thrombin. Data are representative of three independent determinations.

Fig. 3. Effect of anti- α IIB β 3 and anti-GPIbIX mAbs on cell binding induced by thrombin.

(A) CHO- α IIB β 3-IbIX cells were preincubated in the absence or presence of increasing doses of anti- β 3 (P37) or anti-GPIb α (SZ2) for 30 minutes and, then, stimulated with thrombin for 5 minutes. Cells were then incubated for 30 minutes at RT with 160 μ g/mL FITC-fibrinogen, washed and analyzed by flow cytometry. Histograms show a representative experiment. (B) Bar representation of the mean fluorescence intensity values expressed as percent of the thrombin effect. Data are means $\pm$ S.D. of, at least, three independent determinations.

Fig. 4. The effect of thrombin on cell binding is mediated by polymerizing fibrin.

(A) Thrombin does not induce the binding of the activation-dependent mAb PAC-1 to CHO- α Ib β 3-IbIX cells. Cells were stimulated or not by thrombin and, then, incubated with 12.5 μ g/mL FITC-PAC-1 for 30 minutes, washed and analyzed by flow cytometry. The figure is representative of three determinations. (B) GPRP blocks the cell binding induced by thrombin. In the first set of experiments, [Cells + TB + Fg], FITC-Fg was added to cells preincubated with thrombin for 5 minutes, in the absence or presence of 0.5 mM GPRP, an inhibitor of fibrin polymerization. In the second set of experiments, [(TB + Fg) + Cells], cells were added 10 minutes after thrombin and FITC-fibrinogen were mixed in the absence or presence of GPRP. After 30 minutes, the cells were washed and analyzed by flow cytometry. Data are representative of three determinations (C) SDS-PAGE analysis of unlabeled and FITC-labeled fibrinogens. Fg and FITC-Fg (200 μ g/mL) were incubated with increasing concentrations of thrombin during 30 minutes at RT in buffer containing 2 mM calcium. Reactions were stopped and resolved on 10% SDS-PAGE as described in Methods. (D) Fluorescence microscopy image of CHO- α Ib β 3-IbIX cells bound to polymerizing FITC-fibrin. Cells were stimulated with 40 mU/mL thrombin for 5 minutes and, then, incubated with 160 μ g/mL FITC-Fg during 30 minutes, washed and visualized using a Zeiss Axioplan fluorescence microscope equipped with a CCD device. Original magnification, x400.

Fig. 5. Effect of heparins, hirudin and RGDS on thrombin-induced fibrin binding to CHO- α Ib β 3-IbIX cells.

Cells were stimulated with thrombin (16 mU/mL) for 5 minutes in the absence or presence of the indicated concentrations of high or low molecular weight heparins. Hirudin and RGDS peptide were added to cells preincubated with thrombin during 2-5 minutes. Then, FITC-Fg (160 μ g/mL) was added and incubation continued for 30 minutes. The black filled bar represent cells incubated in the absence of FITC-Fg, and the unfilled bar cells non-stimulated by thrombin. Data are means $\pm$ S.D. of, at least, three independent determinations and are expressed as percent of the thrombin effect. Statistical significance: * $P < 0.05$, ** $P < 0.01$.

Fig. 6. Truncation of the cytoplasmic tail of GPIb α blocks the cell binding induced by thrombin.

(A) Flow cytometry analysis of cell binding induced by thrombin. CHO cell transfectants were stimulated with 40 mU/mL thrombin for 5 minutes and, then, FITC-Fg (160 μ g/mL) was added and incubation continued for 30 minutes. After washing, cell binding was determined by flow cytometry. The values of fluorescence are calculated as a product of the percent of gated positive cells and the value of the mean channel of fluorescence intensity, and expressed as percent of the thrombin effect in CHO- α IIB β 3-IbIX cells. Results are means $\pm$ S.D. of three experiments (* $P < 0.01$). (B) Surface expression of GPIbIX and α IIB β 3 complexes in CHO stable transfectants used in the experiments shown above. The expression of GPIb α , GPIX, β 3 and α IIB was analyzed by flow cytometry using specific mAbs, as described in Methods.

Fig. 7. A calcium/calmodulin but not MAPK-dependent pathway is involved in the response of CHO- α IIB β 3-IbIX cells to thrombin.

(A) Phosphorylation of p44/42 MAPK by thrombin is not impeded by the anti-GPIb α mAb SZ2. Cells were preincubated with SZ2 (5 μ g/mL) for 30 minutes and, then, stimulated with 40 mU/mL thrombin for the indicated times. Cell lysates were analyzed on 10% SDS-PAGE as described in Methods. (B) Effect of different inhibitors on thrombin-induced cell binding. Cells were preincubated for 30 minutes at 37°C in the absence or presence of the inhibitors, stimulated with 16 mU/mL thrombin for 5 minutes and, then, FITC-Fg (160 μ g/mL) was added and incubation continued for 30 minutes at RT. The unfilled bar represent cells incubated with FITC-Fg in the absence of thrombin. The values of fluorescence are calculated as products of the percent of gated positive cells and the value of the mean channel of fluorescence intensity, and expressed as percent of the thrombin effect. Results are means $\pm$ S.D. of three experiments. Statistical significance: * $P < 0.05$, ** $P < 0.01$.

Fig. 8. Endogenous PAR receptors do not participate in CHO- α IIB β 3-IbIX cell binding induced by thrombin.

(A) Phosphorylation of p44/42 MAPK by thrombin is blocked by preincubation with the activator hexapeptide TRAP-1. Cells were preincubated in the absence or presence of 40 μ M TRAP-1 for 45 minutes at RT and, then, stimulated with 40 mU/mL thrombin

for 5 minutes. Cell lysates were analyzed on 10% SDS-PAGE as described in Methods.

(B) Flow cytometry analysis of fibrin binding induced by thrombin. Cells were preincubated with TRAP-1 as above and, then, stimulated with thrombin plus/minus TRAP for 5 minutes. FITC-Fg (160 $\mu\text{g/mL}$) was added and incubation continued for 30 minutes. After washing, cell binding was determined by flow cytometry. The values of fluorescence are calculated as a product of the percent of gated positive cells and the value of the mean channel of fluorescence intensity, and expressed as percent of the thrombin effect. The non-specific basal binding of Fg was determined in each condition and the values subtracted from those obtained in the presence of thrombin. Results are means $\pm$ S.D. of three experiments.

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