

# Thrombin induces the association of cyclic ADP-ribose-synthesizing CD38 with the platelet cytoskeleton

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**Abstract** The effect of platelet stimulation on the subcellular localization of CD38, a membrane glycoprotein that catalyses the synthesis of cyclic ADP-ribose from  $\beta$ -NAD<sup>+</sup> was investigated. Treatment of human platelets with thrombin caused the association of about 40% of the total ADP-ribosyl cyclase activity with the cytoskeleton, through the translocation of the CD38 molecule from the Triton X-100-soluble to the insoluble fraction. The interaction of CD38 with the cytoskeleton was a specific and reversible process, mediated by the binding to the actin-rich filaments and was inhibited by treatment of platelets with cytochalasin D. This event was regulated by integrin  $\alpha_{IIb}\beta_3$  and platelet aggregation as it was prevented by the inhibition of fibrinogen binding and was not observed in platelets from a patient affected by Glanzmann thrombasthenia. These results demonstrate that the subcellular localization of CD38 can be influenced by platelet stimulation with physiological agonists, and that membrane CD38 can interact with intracellular proteins.

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**Key words:** ADP-ribosyl cyclase; CD38; Cytoskeleton; Platelet; Integrin  $\alpha_{IIb}\beta_3$

## 1. Introduction

CD38 is a type II transmembrane glycoprotein of 46 kDa expressed in both hematopoietic and nonhematopoietic cells [1]. Several studies have revealed a variety of functional properties of CD38, suggesting that this molecule may act as a bifunctional ectoenzyme, an adhesion molecule or a cell-activating receptor. The extracellular domain of CD38 possesses both ADP-ribosyl cyclase activity, able to synthesize cyclic ADP-ribose (cADPR) from  $\beta$ -NAD<sup>+</sup>, and cADPR hydrolase activity, able to hydrolyse cADPR to ADP-ribose (ADPR) [2]. cADPR is an intracellular second messenger that promotes the release of Ca<sup>2+</sup> from specific stores that are insensitive to inositol 3,4,5-trisphosphate [3]. As CD38 is responsible for the transient formation of cADPR, it has been suggested that it could be involved in the regulation of Ca<sup>2+</sup> homeostasis.

CD38 may also function as an adhesion molecule, according to its ability to bind to hyaluronate and to support the interaction of lymphocytes with endothelial cells [4,5]. Recently, a 120 kDa membrane glycoprotein able to interact

with CD38 has been identified in a variety of cells, including endothelial cells, platelets, and monocytes [6].

Several reports showed that ligation of CD38 on the cell surface by selected monoclonal antibodies elicited cellular responses including proliferation, production of cytokines, and protection from apoptosis [7–9]. These findings suggest that CD38 is a cell-activating receptor. Some features of the signal transduction pathways activated by ligation of CD38 have been elucidated and involve the rapid tyrosine phosphorylation of several intracellular signaling proteins including phospholipase C $\gamma$ , the phosphatidylinositol 3-kinase, the proto-oncogene Cbl, and the tyrosine kinases Syk and Btk [10–12].

We have recently demonstrated that enzymatically active CD38 is expressed on the plasma membrane of human platelets [13]. These cells are extremely reactive and can be rapidly activated by a variety of physiological agonists involved in haemostatic and inflammatory processes [14]. One of the earliest events following platelet stimulation is the reorganization of the actin-based cytoskeleton [15]. This structure plays a central role in the regulation of platelet morphology and cell adhesion [16]. Moreover, the cytoskeleton represents an important network connecting several molecules involved in platelet activation. For instance, some tyrosine kinases, like pp60<sup>src</sup>, pp72<sup>syk</sup> and pp125<sup>fak</sup> [17–19], lipid metabolizing enzymes, like phosphatidylinositol 3-kinase and phospholipase C [20], phosphotyrosine phosphatases, like PTP1B [21], low molecular weight GTP-binding proteins, like rap1b and rap2b [22,23], and membrane glycoproteins, like integrin  $\alpha_{IIb}\beta_3$  and PECAM-1 [24,25] interact with the actin filaments during platelet aggregation.

In this work we show that thrombin, the most potent platelet agonist, induces the interaction of enzymatically active CD38 with the intracellular cytoskeleton, and that this event is promoted by the binding to the actin filaments and is regulated by the integrin  $\alpha_{IIb}\beta_3$ .

## 2. Materials and methods

### 2.1. Materials

Nicotinamide guanine dinucleotide (NGD<sup>+</sup>), cyclic GDP-ribose, cytochalasin D, U46619, leupeptin, RGDS peptide, and  $\alpha$ -thrombin, were purchased from Sigma. The thrombin receptor-activating peptide (TRAP) was from Calbiochem. Microcon 3 filters were from Amicon. Triton X-100 was from ICN. Sepharose 2B-CL and protein A-Sepharose were from Pharmacia Biotech. The monoclonal antibody IB4 against CD38 was described elsewhere [7]. All other reagents were of analytical grade.

### 2.2. Platelet preparation and cytoskeleton extraction

Human platelets were isolated by gel-filtration on a Sepharose 2B-CL column, in HEPES buffer (10 mM HEPES, 137 mM NaCl,

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**Abbreviations:** NGD<sup>+</sup>, nicotinamide guanine dinucleotide; cGDP, cyclic GDP-ribose; cADPR, cyclic ADP-ribose; ADPR, ADP-ribose

2.9 mM KCl, 12 mM  $\text{NaHCO}_3$ , pH 7.4) as previously described [22]. Platelet samples ( $500\ \mu\text{l}$ ,  $1 \times 10^9$  platelets/ml) were prewarmed at  $37^\circ\text{C}$  and then treated with HEPES buffer or 10 nM thrombin, 10  $\mu\text{g}/\text{ml}$  collagen, or 10  $\mu\text{M}$  U46619 for 2 min. In some experiments, incubation with 10  $\mu\text{M}$  cytochalasin D or 1 mM RGDS peptide for 2 min was performed before stimulation of the samples. After cell lysis with 1% Triton X-100, platelet cytoskeleton was isolated as previously described [22], and mechanically resuspended in  $500\ \mu\text{l}$  of HEPES buffer, pH 7.4 to measure the ADP-ribosyl cyclase activity.

### 2.3. Preparation of the polymerized actin-rich fraction

This was performed essentially as described [26], with some modifications. Briefly, cytoskeleton from thrombin-stimulated platelets was solubilized with 1 ml of buffer containing 0.6 M KI, 100 mM PIPES, 100 mM KCl, 10  $\mu\text{g}/\text{ml}$  leupeptin, 1 mM PMSF, 100  $\mu\text{M}$  sodium ortho-vanadate, pH 6.5, for 50 min at  $4^\circ\text{C}$  under gentle shaking, and then centrifuged at  $35000 \times g$  for 30 min at  $4^\circ\text{C}$ . The supernatant (KI-soluble) was collected and dialyzed overnight at  $4^\circ\text{C}$  with 1 l of buffer containing 10 mM PIPES, 1 mM EGTA, 2 mM  $\text{MgCl}_2$ , 1 mM PMSF, 100  $\mu\text{M}$  sodium ortho-vanadate, pH 6.8. The insoluble material, containing the repolymerized actin and actin-binding proteins, was recovered by centrifugation at  $13000 \times g$  for 5 min at  $4^\circ\text{C}$ , washed twice with dialysis buffer, and finally resuspended in HEPES buffer, pH 7.4.

### 2.4. Immunoprecipitation

Cytoskeletal samples from thrombin-activated platelets were mechanically resuspended with  $500\ \mu\text{l}$  of HEPES buffer, pH 7.4, containing 1% Triton X-100, 5 mM EGTA, 2 mM PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, using a micropipette. The insoluble material was discarded by centrifugation at  $13000 \times g$  for 10 min, and the soluble fraction was immunoprecipitated with 3  $\mu\text{g}$  of the anti-CD38 monoclonal antibody IB4, or 3  $\mu\text{g}$  of mouse IgG as previously described [13]. Immunoprecipitates were then resuspended in HEPES buffer, pH 7.4, and tested for the ADP-ribosyl cyclase activity.

### 2.5. Enzymatic analysis

The ADP-ribosyl cyclase activity was measured as previously described [13], using  $\text{NGD}^+$  instead of  $\text{NAD}^+$  as a substrate for CD38, because the synthesized product, the cyclic GDP-ribose (cGDPR), which is the guanine nucleotide equivalent to cADPR, is only a poor substrate for the cADPR hydrolase activity of the enzyme, and accumulates in the medium [27,4]. Samples were prewarmed at

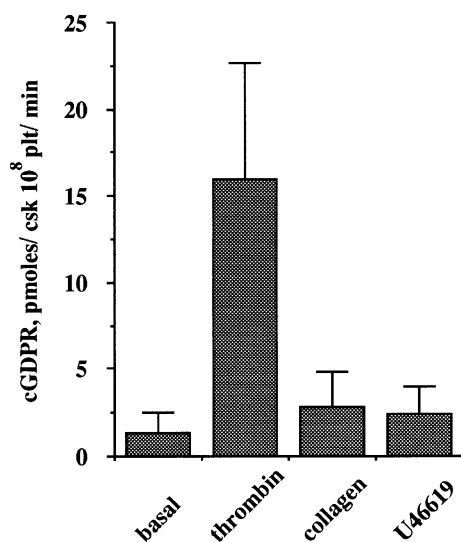


Fig. 1. Association of the ADP-ribosyl cyclase activity with the platelet cytoskeleton. Gel-filtered platelets were stimulated with 10 nM thrombin, 10  $\mu\text{g}/\text{ml}$  collagen, 10  $\mu\text{M}$  U46619, or buffer (basal), and the Triton X-100-insoluble materials were prepared and incubated with 100  $\mu\text{M}$   $\text{NGD}^+$  for 60 min. Results are expressed as pmoles/min of cGDPR produced by the cytoskeleton (csk) isolated from  $10^8$  platelets, and represent the means  $\pm$  S.D. of three to seven separated experiments.

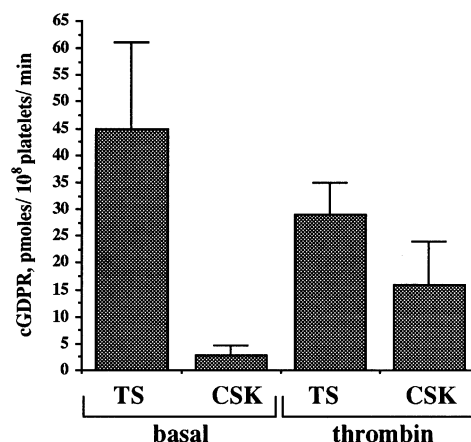


Fig. 2. Analysis of the ADP-ribosyl cyclase activity in platelet subcellular fractions. Gel-filtered platelets were treated with either buffer (basal) or 10 nM thrombin (thrombin) for 2 min at  $37^\circ\text{C}$ , and then lysed with Triton X-100 (1% final concentration). Lysed samples were centrifuged and the Triton X-100-soluble (TS) and insoluble (CSK) fractions were separated and incubated with 100  $\mu\text{M}$   $\text{NGD}^+$  for 60 min. The production of cGDPR by each fraction was determined by HPLC analysis. Results are expressed as pmoles/min of cGDPR produced by the subcellular fraction prepared from  $10^8$  platelets, and represent the means  $\pm$  S.D. of five different experiments.

$37^\circ\text{C}$  for 5 min, and then incubated with 100  $\mu\text{M}$   $\text{NGD}^+$ . Aliquots (100  $\mu\text{l}$ ) were withdrawn immediately after the addition of the nucleotide substrate, and after incubation at  $37^\circ\text{C}$  for 60 min, and centrifuged at  $13000 \times g$  for 5 min. The supernatants were ultrafiltered on Microcon 3 membrane (cut-off 3000 Da), and then analyzed by reverse phase HPLC using a  $25 \times 0.46$  cm Supelcosil LC-18T column as described [13].

## 3. Results

The interaction of the CD38 molecule with the cytoskeleton was investigated by measuring the ADP-ribosyl cyclase activity in the Triton X-100-insoluble material prepared from resting and stimulated platelets. While very little ADP-ribosyl cyclase activity was detected in the cytoskeleton from resting platelets ( $1.4 \pm 1.1$  pmoles/min), a significant amount of cGDPR ( $16.0 \pm 6.7$  pmoles/min) was produced from  $\text{NGD}^+$  by the cytoskeleton obtained from thrombin-stimulated platelets (Fig. 1). By contrast, stimulation of platelets with collagen and the thromboxane  $\text{A}_2$  analogue, U46619, promoted only a slight increase of the ADP-ribosyl cyclase activity recovered in the Triton X-100-insoluble material ( $2.8 \pm 2.0$  and  $2.4 \pm 1.6$  pmoles/min, respectively) (Fig. 1). By analysing the ADP-ribosyl cyclase activity in resting intact platelets incubated with  $\text{NGD}^+$ , we measured the production of  $37.8 \pm 2.9$  pmoles/min of cGDPR. Therefore, we calculated that, in the absence of stimulation, only 3.7% ( $\pm 2.9\%$ , S.D.) of the total ADP-ribosyl cyclase activity was associated with the cytoskeleton. This amount increased to 42.3% ( $\pm 17.6\%$ , S.D.), 7.4% ( $\pm 5.3\%$ , S.D.), and 6.3% ( $\pm 4.2\%$ , S.D.) upon platelet stimulation with thrombin, collagen, and U46619, respectively.

We next focused our investigations on the effect of thrombin, and we analyzed whether the agonist-induced increase of the production of cGDPR by the cytoskeleton was due to the stimulation of the preexisting activity or to the association of more enzyme with the Triton X-100-insoluble material. We compared the ADP-ribosyl cyclase activity in the cytoskeleton

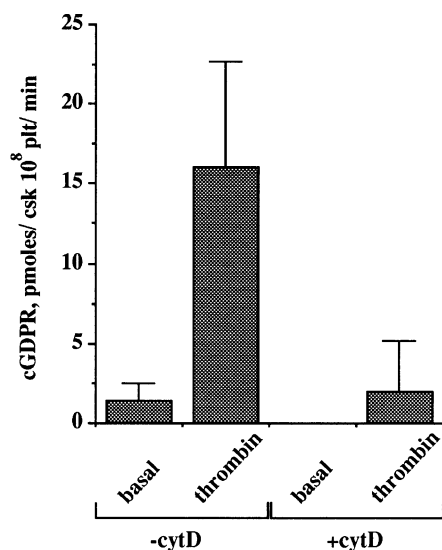


Fig. 3. Effect of cytochalasin D on the association of CD38 with the cytoskeleton. Gel-filtered platelets were incubated at 37°C in the presence or in the absence of 10  $\mu$ M cytochalasin D (cytD) for 2 min, and then treated with buffer (basal), or 10 nM thrombin (thrombin) for 2 min. The cytoskeleton from each sample was prepared and tested for the ADP-ribosyl cyclase activity of CD38 as described in Section 2. Results are the means  $\pm$  S.D. of three to seven different experiments.

and in the Triton X-100-soluble fraction from resting and thrombin-stimulated platelets. As shown in Fig. 2, the increase of cGDP production by the cytoskeleton induced by thrombin was paralleled by a decreased production of this nucleotide by the Triton X-100-soluble material, suggesting a translocation rather than a stimulation of the ADP-ribosyl cyclase activity.

To demonstrate that the ADP-ribosyl cyclase activity associated with the cytoskeleton from thrombin-stimulated platelets was really due to the CD38 molecule, cytoskeletal samples from thrombin-activated platelets were solubilized and immunoprecipitated with the monoclonal antibody IB4, specific for CD38, or with control mouse immunoglobulins. We found that the immunoprecipitates obtained with the anti-CD38 monoclonal antibody IB4, but not with control mouse immunoglobulins, were able to immunoprecipitate a significant amount of ADP-ribosyl cyclase activity from the cytoskeleton of thrombin-activated platelets, corresponding to a production of  $8.74 \pm 1.48$  pmoles/min of cGDP.

We then analyzed the effect of cytochalasin D, a potent inhibitor of actin polymerization on the thrombin-induced association of CD38 with the cytoskeleton. We had previously shown that treatment of intact platelets with 10  $\mu$ M cytochalasin D for 2 min did not affect platelet aggregation induced by thrombin, but completely prevented the agonist-induced actin polymerization [28]. As shown in Fig. 3, cytochalasin D almost completely inhibited the thrombin-induced association of ADP-ribosyl cyclase activity with the cytoskeleton. To test the possible direct interaction of CD38 with the actin filaments system of the cytoskeleton, a repolymerized actin-rich fraction was prepared from the Triton X-100-insoluble material of thrombin-stimulated platelets as described in Section 2, and tested for the ADP-ribosyl cyclase activity. As shown in Fig. 4, the ADP-ribosyl cyclase activity present in the Triton X-100-insoluble material was also recovered in the

repolymerized actin fraction, indicating that CD38 directly interacts with actin or associates to actin-binding proteins.

It is known that the interaction of several signaling molecules and membrane glycoproteins with the cytoskeleton is regulated by integrin  $\alpha_{IIb}\beta_3$  engagement and platelet aggrega-

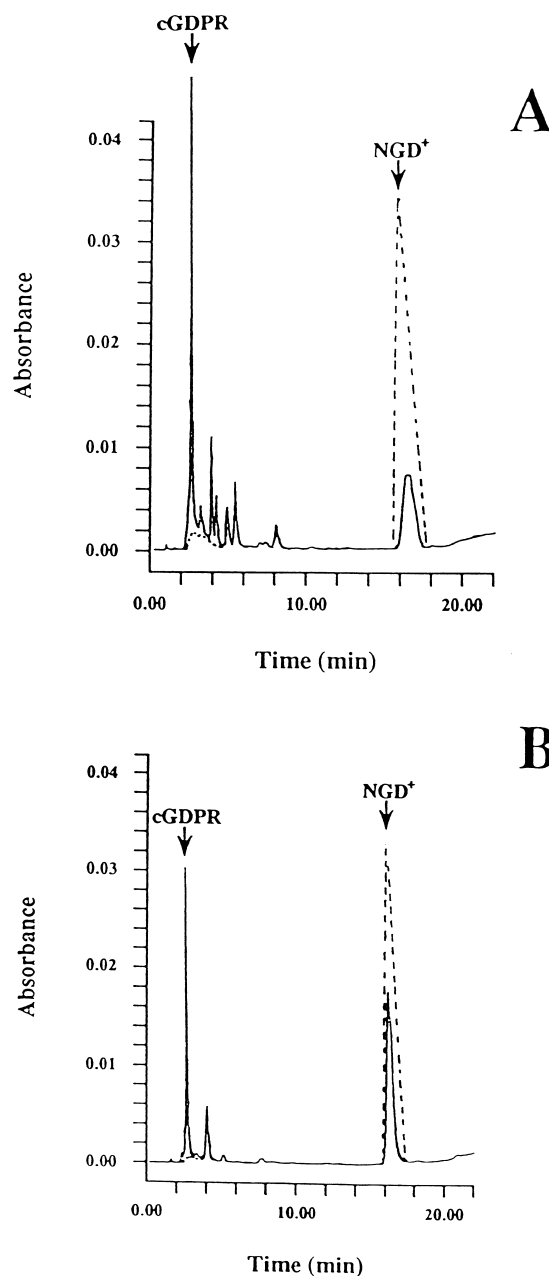


Fig. 4. CD38 interacts with the actin filaments. Triton X-100-insoluble material from thrombin-stimulated platelets (A) and actin-rich filaments (B), prepared by solubilization of the Triton X-100-insoluble material with KI followed by induction of actin repolymerization as described in Section 2, were incubated with 100  $\mu$ M NGD<sup>+</sup>, and the production of cGDP was analyzed by HPLC. Typical chromatographic profiles of the aliquots of the samples processed immediately after the addition of the substrate (broken lines), or after 60 min of incubation (unbroken lines) are reported. The reduced activity of the repolymerized fraction ( $9.2 \pm 2.8$  pmoles cGDP/min) compared to that of the Triton X-100-insoluble material ( $16.0 \pm 6.7$  pmoles cGDP/min) may reflect the fact that some unpolymerized actin persists even after dialysis of the KI-soluble material. Results are representatives of at least three different experiments.

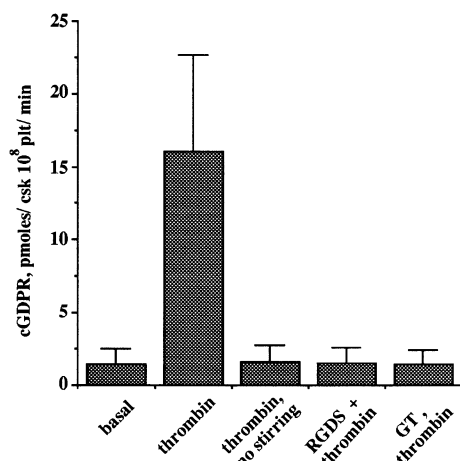


Fig. 5. Role of integrin  $\alpha_{IIb}\beta_3$  and platelet aggregation on the translocation of CD38 to the cytoskeleton. Normal platelets were incubated at 37°C in the absence or in the presence of 1 mM RGDS peptide. Samples were then treated with buffer (basal) or with 10 nM thrombin. In one sample, stirring was omitted. Platelets from a patient affected by Glanzmann thrombasthenia (GT) were stimulated with 10 nM thrombin for 2 min under constant stirring. Cytoskeletons were prepared and the ADP-ribosyl cyclase activity was tested using NGD<sup>+</sup> as substrate. Results with normal platelets are the means  $\pm$  S.D. of three to seven experiments. Analysis of thrombasthenic platelets was performed in duplicate.

tion [17–25]. For this reason, we measured the ADP-ribosyl cyclase activity in the cytoskeleton of platelets stimulated with thrombin in the presence of 1 mM RGDS peptide, which inhibits both fibrinogen binding and platelet aggregation, or in the absence of stirring, a treatment that allows fibrinogen binding but prevents platelet aggregation. Moreover, we analyzed platelets from a patient affected by Glanzmann thrombasthenia, which lack the integrin  $\alpha_{IIb}\beta_3$  and fail to aggregate. As shown in Fig. 5, inhibition of both fibrinogen binding and platelet aggregation prevented the thrombin-induced interaction of CD38 with the cytoskeleton. Fig. 5 also shows that the ADP-ribosyl cyclase activity was not detected in the cytoskeleton of thrombasthenic platelets stimulated with thrombin.

#### 4. Discussion

Human platelets express enzymatically active CD38 on the plasma membrane [13], and can be activated by a variety of extracellular agonists that lead to shape change, secretion, and aggregation [14]. One of the more important effects of platelet activation is the reorganization of the intracellular cytoskeleton, which is not only responsible for the morphological changes of activated platelets, but also represents a functional network connecting several signaling molecules and membrane glycoproteins [15,17–25]. In this work, we showed that a little ADP-ribosyl cyclase activity was associated with the cytoskeleton in resting platelets, and that stimulation with the strong agonists, thrombin, collagen, and the thromboxane A<sub>2</sub> analogue U46619, induced an increase of the ability of the cytoskeleton to produce cGDP from NGD<sup>+</sup>. This effect was particularly evident in platelets stimulated with thrombin, where about 40% of the ADP-ribosyl cyclase activity expressed by resting intact platelets was recovered with the Triton X-100-insoluble material. We also found that the effect of thrombin was totally mediated by the activation of its mem-

brane receptor, as identical results were obtained using the thrombin receptor-activating peptide, TRAP (data not shown). Moreover, immunoprecipitation experiments demonstrated that the ADP-ribosyl cyclase activity in the cytoskeleton was actually due to the CD38 molecule.

In thrombin-stimulated platelets, the increase of the ADP-ribosyl cyclase activity in the cytoskeleton was concomitant with the reduction of the same activity in the Triton X-100-soluble fraction. Moreover, the sum of the ADP-ribosyl cyclase activity in the two fractions prepared from activated cells was identical to that of resting cells, and we did not measure any increase in the specific ADP-ribosyl cyclase activity (as pmoles of cGDP/min/mg of proteins) in the subcellular fractions analyzed (data not shown). Therefore, we conclude that thrombin induced a real redistribution of the CD38 molecules in activated platelets, rather than the stimulation of the activity of the CD38 molecules already associated with the cytoskeleton.

We found that the translocation of CD38 to the cytoskeleton in thrombin-stimulated platelets required actin polymerization, since it was almost completely prevented by cytochalasin D, and was a reversible and specific process mediated by the interaction of the enzyme with the actin filaments or with some actin-binding proteins. This is demonstrated by the evidence that cytoskeletal CD38 was solubilized when actin filaments were selectively depolymerized, and spontaneously re-associated with actin when repolymerization was induced ‘in vitro’. In addition to actin polymerization, the association of CD38 with the cytoskeleton also required fibrinogen binding to the integrin  $\alpha_{IIb}\beta_3$  and platelet aggregation, as it was prevented by the RGDS peptide and by the omission of stirring, and was not observed in platelets from a patient affected by Glanzmann thrombasthenia. In this regard, thus, CD38 behaves like other signaling enzymes, whose association with the cytoskeleton is dependent on integrin engagement [17,20,21].

The association of CD38 with the cytoskeleton in thrombin-stimulated platelets may play multiple roles. One of the most intriguing questions about the cellular function of this molecule rises from the evidence that the product of its enzymatic activity, the cADPR, supposed to act as an intracellular second messenger involved in the regulation of Ca<sup>2+</sup> release, is produced outside the cell. In this context, it is possible that the association with the actin filaments represents a way to mediate the internalization of the enzymatically active CD38. It is also possible that the interaction of CD38 with the cytoskeleton results in a relocation of the enzyme in a suitable membrane environment to promote the influx of the produced cyclic nucleotide into the cell. In addition, it has been shown that ligation of CD38 on cell surface initiates transmembrane signaling processes leading to tyrosine phosphorylation of several proteins. Since in aggregated platelets the cytoskeleton represents a network connecting several signaling molecules, including the tyrosine kinases pp60<sup>src</sup>, pp72<sup>syk</sup> and pp125<sup>FAK</sup> [17–19], the translocation of CD38 molecule described in the present work may represent a way to link this membrane glycoprotein to intracellular effectors.

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