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Stimulation of platelet glycoprotein IIb-IIIa ($\alpha_{IIb}\beta_3$-integrin) functional activity by a monoclonal antibody to the N-terminal region of glycoprotein IIIa

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Abstract Platelet glycoprotein (GP) IIb-IIIa complex ($\alpha_{IIb}\beta_3$-integrin) changes its conformation upon platelet activation that results in binding of RGD-containing ligands and expression of ligand-induced binding site (LIBS) neoepitopes. Anti-GIIb-IIIa monoclonal antibody (monAB) CRC54 bound to ≤10% of GPIIb-IIIa on resting platelets but binding was enhanced by the occupation of GPIIb-IIIa with RGDS peptide and by platelet activation indicating that CRC54 is directed against LIBS epitope. The epitope was located within the first 100 N-terminal residues of GPIIIa and differed from other LIBS epitopes. CRC54 as well as its Fab fragments were able to induce platelet aggregation. CRC54 also stimulated interaction of GPIIb-IIIa with its ligands (fibrinogen and fibronectin) and conformation-dependent antibodies. The results indicated that changes of GPIIb-IIIa conformation, binding of ligands and platelet aggregation could be stimulated via interaction of anti-LIBS antibody with the N-terminal part of GPIIIa.

Key words: Integrin; Glycoprotein IIb-IIIa; Conformation; Fibrinogen; Platelets

1. Introduction

The main platelet integrin, Ca-dependent glycoprotein (GP) IIb-IIIa complex ($\alpha_{IIb}\beta_3$-integrin), is present on the platelet surface in a resting form unable to bind adhesive ligands. However, agonist-induced platelet activation leads to conformational changes in GPIIb-IIIa resulting in the opening of the ligand-binding pocket, binding of fibrinogen and other RGD-containing plasma proteins and platelet aggregation (reviews [1–3]). Ligand binding induces additional conformational changes which cause exposure of ligand-induced binding sites (LIBS) neoepitopes [4–6]. Similar transformations of GPIIb-IIIa are stimulated via occupation of the receptor by RGD peptides [4–6] or peptidomimetics [7] which bind to a resting form of the complex. Alterations in GPIIb-IIIa are also induced by platelet adhesion [8].

Conformational changes in GPIIb-IIIa could be detected using conformation-dependent monoclonal antibodies (monABs). These antibodies are directed against epitopes within the ligand-binding site accessible only after agonist-induced activation of the complex [9,10] or against LIBS epitopes [4–6]. Some anti-LIBS antibodies could stimulate binding activity of GPIIb-IIIa and subsequent platelet aggregation [6,11]. Activating monABs are also able to aggregate cells expressing recombinant GPIIb-IIIa [12] and to stimulate fibrinogen binding to isolated GPIIb-IIIa [11], indicating that GPIIb-IIIa could be activated without involvement of intracellular pathways. LIBS epitopes are located on both GPIIb and GPIIIa components of the complex [4–6,13]. One of them was mapped to the C-terminus of the GPIIb heavy chain [14] and one to the GPIIIa region adjacent to the membrane domain [15]. However, localization of most regions sensitive to the occupation of GPIIb-IIIa is still unknown.

In this study using monAB against the N-terminal part of GPIIIa it was shown that this region contains the LIBS epitope and that interaction of monAB with this epitope induced conformational changes in GPIIb-IIIa, stimulation of ligand binding and platelet aggregation.

2. Materials and methods

2.1. Monoclonal and polyclonal antibodies

MonAB CRC54 is directed against GPIIIa and has been partially described previously [8]. Antibody pl-80 [13] and antibody PMI-1 [4,14] are directed against LIBS epitopes on GPIIb, antibodies LIBS1, LIBS2, and LIBS6 against LIBS epitopes on GPIIIa [5], and antibody pl-55 recognizes the activated conformation of GPIIb-IIIa [10]. Antibodies LIBS1, LIBS2, LIBS6 and PMI-1 were a generous gift of Dr. Mark Ginsberg (Scripps Clinic, La Jolla, CA, USA). Antibody CRC64 is directed against GPIIb-IIIa complex and inhibited platelet aggregation [8,16] and control anti-GPIIb-IIIa antibody VM16a has no effects on platelet aggregation [17]. Polyclonal anti-GPIIb-IIIa antibodies were produced as described elsewhere [18].

2.2. Preparation of Fab fragments

Fab fragments of monAB CRC54 were prepared by papain digestion. CRC54 was incubated with papain (Serva, Germany) at a ratio of 20/1 (w/w) in 0.2 mM phosphate buffer, 2 mM EDTA, 10 mM cysteine, pH 7.4 for 24 h at 37°C. Reaction was stopped by 20 mM iodoacetic acid and Fab fragments were purified by FPLC.

2.3. Platelets

Platelet-rich plasma (PRP) and washed platelets were prepared from the blood of healthy donors as previously described [19]. Washed platelets were resuspended in Tyrode/HEPES solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 0.1% dextrose, 5 mM HEPES, pH 7.35) with 2 mM $CaCl_2$ and 1 mM $MgCl_2$. In some experiments divalent cations were omitted and EDTA was added at the concentration of 5 mM. For aggregation and adhesion studies Tyrode/HEPES was supplemented with 0.35% BSA and for binding and capture assays with 1% BSA. When the effects of deoxyglucose

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Abbreviations: GP, glycoprotein; LIBS, ligand-induced binding site; PRP, platelet-rich plasma; PBS, phosphate-buffered saline; monAB, monoclonal antibody

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were studied dextrose was omitted from all solutions. In some experiments platelets were labeled with ^{51}Cr as described elsewhere [8].

2.4. Binding assays

Labeling of monABs, Fab fragments and fibrinogen (Calbiochem, USA) with ^{125}I and binding experiments were performed essentially as described elsewhere [8,19]. Washed platelets (10^9/ml) were preincubated without any additions or in the presence of RGDS, RGES (both peptides were generously provided by Dr. G. Bespalova, Cardiology Research Center, Moscow, Russia), CRC54 or EDTA for 30 min at 37°C and with thrombin (Sigma, St. Louis, MO) for 5 min at 22°C. After preincubation ^{125}I-labeled monABs or Fab fragments were added in 50 μl of Tyrode/HEPES/BSA to 50 μl of platelets, incubated for 30 min at 37°C or for 20 min at 22°C (incubation with thrombin) and then bound and unbound radioactivities were separated by centrifugation through 20% sucrose. Specific binding was estimated as total binding minus nonspecific binding (in the presence of at least 20-fold excess of unlabeled antibodies). For evaluation of [^{125}I]fibrinogen binding washed platelets (6.25×10^8/ml) were preincubated for 20 min at 37°C in the absence or presence of monABs. After preincubation 20 μl of 0.15 μM [^{125}I]fibrinogen was added to 80 μl of platelets, incubated for 30 min at 37°C and bound and unbound radioactivities were separated by centrifugation through 20% sucrose.

2.5. GPIIb-IIIa capture assay

Washed platelets at a concentration of 5×10^8/ml were incubated without any additions or in the presence of RGDS, RGES or CRC54 for 20–30 min at 37°C, medium was supplemented with 0.5 mM phenylmethylsulfonylfluoride and platelets were lysed with 1% Triton X-100 for 30 min on ice and undissolved material was spun down at $10\,000\times g$ for 5 min. Plates for capture assay (high-affinity binding 96-multiwells, Nunc, Denmark) were coated with monABs at a concentration of 10 μg/ml in phosphate buffered saline (PBS) for 1 h at 37°C, washed with PBS containing 0.05% Tween (PBS/Tween) and blocked with 1% BSA in PBS for 1 h at 37°C. 100 μl of platelet lysates was added to precoated wells and incubated for 1 h at 37°C. Wells were washed with PBS/Tween and affinity purified rabbit anti-GPIIb-IIIa polyclonal antibodies were added to the wells at a concentration of 1 μg/ml and incubated for 30 min at 37°C. After washing goat anti-rabbit IgG antibodies conjugated with peroxidase (BioRad, Richmond, CA) were added at 1/2000 dilution and incubated for 30 min at 37°C. Plates were washed with PBS/Tween and PBS and color reaction was developed using 1,2-phenylenediamine.

2.6. Platelet adhesion

24-Well plates (Nunc, Denmark) were coated with fibrinogen or fibronectin at a concentration of 10 μg/ml in PBS for 1 h at 37°C, washed with PBS and blocked with 2% BSA in PBS for 1 h at 37°C. Control wells were treated with BSA only. 200 μl of ^{51}Cr-labeled platelets at indicated concentrations was added into the wells and incubated without any additions or in the presence of monABs for 1 h at 37°C. Unattached platelets were washed off and bound radioactivity was collected with 1 M NaOH.

2.7. Platelet aggregation

Aggregation was studied in PRP (platelet count 2.5×10^8/ml) or in washed platelet suspension (platelet count 3×10^8/ml) by measuring the mean size of platelet aggregates using a highly sensitive method based on the registration of optical density fluctuations in platelet suspension [20]. Measurements were performed in a BIOLA Aggregation Analyzer (BIOLA Ltd., Russia) at 37°C and a stirring rate of 800 rpm.

3. Results and discussion

Anti-GPIIb-IIIa monAB CRC54 has been partially described previously [8] and was shown to be conformation-dependent since it bound weakly to resting platelets but binding was increased after platelet adhesion or dissociation of GPIIb-IIIa complex with EDTA. According to its ability to blot GPIIb-IIIa chymotryptic fragments the CRC54 epitope was mapped within the first 100 N-terminal residues of GPIIIa [8]. In this study we used previously described conformation-dependent monABs to test if CRC54 epitope could overlap or be sterically close to other conformation-dependent epitopes of GPIIb-IIIa. Anti-LIBS antibodies LIBS1, LIBS2 and LIBS6 [5], PMI-1 [4,14], pl-80 [13] and antibody pl-55, which recognizes the activated conformation of GPIIb-IIIa [10], were tested in these experiments and none of them blocked binding of [^{125}I]CRC54 to adherent platelets (Table 1). Some increase of binding in the presence of LIBS1 and LIBS2 might be explained by conformational changes in the complex mediated by the interaction with these antibodies. No competition between CRC54 and other conformation-dependent antibodies demonstrated that their epitopes are located in different regions of GPIIb-IIIa. It was shown earlier that CRC54 is able to blot GPIIIa under both nonreduced and reduced conditions [8]. Unlike CRC54 other conformation-dependent antibodies which have been characterized by immunoblotting under nonreduced conditions were unable to blot reduced GPIIIa [5,6]. All these data taken together indicate the unique specificity of CRC54.

In the previous paper [8] using low concentrations of [^{125}I]CRC54 we were unable to detect significant differences in CRC54 binding to control platelets and platelets treated with thrombin or RGD peptide. In this study we repeated these experiments using high concentrations, up to 0.33 μM (50 μg/ml), of [^{125}I]CRC54. Under these conditions CRC54 binding to resting platelets did not exceed 5000 molecules per platelet which amounted to ~10% of GPIIb-IIIa molecules present on the platelet surface. Binding in the presence of RGDS or thrombin was several times higher than to resting platelets although still lower than to platelets treated with EDTA (Fig. 1A). Thus it has been shown that changes in GPIIb-IIIa detected by CRC54 could be induced by the occupation of GPIIb-IIIa with RGD peptide, thrombin-induced platelet activation or platelet adhesion as was demonstrated earlier [8]. These data indicate that CRC54 is directed against the LIBS epitope. In case of thrombin-induced platelet activation or platelet adhesion exposure of the CRC54 LIBS epitope is, presumably, mediated by the interaction of GPIIb-IIIa with the endogenous fibrinogen which is released from activated platelets.

We also compared binding to platelets of CRC54 IgG molecule and CRC54 Fab fragments testing the hypothesis that low binding of CRC54 IgG to resting platelets might be due to the steric inaccessibility of the epitope for the IgG molecule. The level of [^{125}I]CRC54 Fab binding to resting platelets was still low and almost the same as that of [^{125}I]CRC54 IgG (Fig. 1B) although the binding of CRC54 Fab to EDTA-treat-

Table 1
Binding of [^{125}I]CRC54 with adherent platelets in the presence of different conformation-dependent anti-GPIIb-IIIa monABs

Antibody	% of [^{125}I]CRC54 specific binding	*n*
LIBS1	218 ± 21	3
LIBS2	169 ± 26	3
LIBS6	79 ± 8	3
PMI-1	112 ± 47	3
pl-55	120	1
pl-80	134	1

[^{125}I]CRC54 was added to adherent platelets at a concentration of 6.67 nM (1 μg/ml), and unlabeled antibodies were added at ≥100-fold excess. Level of [^{125}I]CRC54 specific binding (inhibited by the excess of unlabeled CRC54 and usually 90–95% of total binding) was taken as 100%. Means ± SEM are given for $n = 3$.

ed platelets was increased to the same extent as the binding of CRC54 IgG, indicating that the Fab fragment has the same binding capacity as the whole IgG. So these data have shown that CRC54 epitope is inaccessible not only to the IgG molecule but to its Fab fragment that is ~3 times smaller than intact antibody.

The effects of CRC54 on platelet aggregation were studied by measuring the mean size of platelet aggregates using a new highly sensitive method based on the evaluation of optical density fluctuations in platelet suspension [20]. CRC54 induced platelet aggregation both in PRP (Fig. 2A) and in washed platelet suspension (Fig. 2B). High concentrations of antibody were added to stimulate detectable aggregation and the intensity of the aggregation response was not saturated even at 1 μM (150 μg/ml) of CRC54 (Fig. 2A). In washed platelet suspension the addition of exogenous fibrinogen was essential for CRC54-induced aggregation (Fig. 2B). Much lower concentrations and a routine turbidometric method were used in the initial study of CRC54 and that is why the aggregation response was not detected in a previous paper [8]. Fab fragments of CRC54 were also able to stimulate platelet aggregation (Fig. 2A,B), providing evidence that CRC54-induced aggregation is not mediated by platelet F_c receptor or possible bivalent binding of monAB. GPIIb-IIIa blocking agents, like RGDS peptide and blocking antibody CRC64, completely inhibited CRC54-induced aggregation (Fig. 2C). However, inhibitors of intracellular metabolic pathways were less effective. Both prostaglandin E_1 and the mixture of deoxyglucose and sodium azide, which are able to block platelet aggregation induced by physiological agonists, only partially inhibited the CRC54-induced aggregation response (Fig. 2D). These data indicate that interaction of CRC54 with GPIIb-IIIa is itself sufficient for the maintaining of platelet aggregation, which is in accordance with the data obtained with antibodies against other regions of the complex and purified or recombinant GPIIb-IIIa expressed in model cells [6,11,12]. However, partial inhibition of CRC54-induced aggregation with prostaglandin E_1 and deoxyglucose/sodium azide suggested stimulation of intracellular pathways that might be mediated by antibody-induced primary aggregation.

Platelet aggregation induced by physiological agonists is mediated by the interaction of fibrinogen with GPIIb-IIIa [1–3]. CRC54, like these agonists, was able to stimulate [^{125}I]fibrinogen binding to platelets and this increase was inhibited by the anti-GPIIb-IIIa blocking antibody, CRC64, showing binding specificity towards GPIIb-IIIa. At a concentration of labeled fibrinogen of 0.03 μM the binding level in the absence of any additions was 490 molecules per platelet, in the presence of 0.5 μM CRC54 it was 1900 molecules per platelet and in the presence of 0.5 μM CRC54 and 0.133 μM CRC64 it was 600 molecules per platelet ($n = 2$).

Table 2
Effects of CRC54 on the binding of anti-GPIIb-IIIa antibodies to platelets

Addition	^{125}I-antibody bound, molecules per platelet		
	CRC64	pl-80	pl-55
None	24960	420	540
CRC54	24000	2840	15120
RGDS	29960	3840	240

Specific binding of ^{125}I-labeled CRC64 (conformation-independent antibody), pl-80 (anti-LIBS antibody) or pl-55 (activation-dependent antibody) added to washed platelets at a concentration of 33 nM (5 μg/ml) was evaluated without any additions, in the presence of 0.5 μM CRC54 or 1 mM RGDS. Means of two experiments are given.

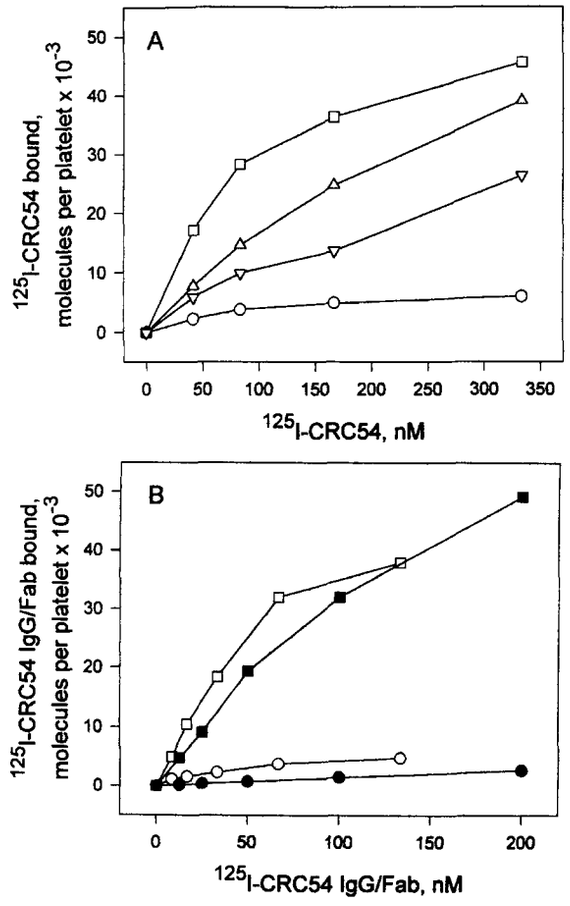

Fig. 1. CRC54 binding to platelets. (A) Specific binding of [^{125}I]CRC54 to washed platelets was measured without any additions (○) or in the presence of 1 mM RGDS (△), 1 U/ml of thrombin (▽) or 5 mM EDTA (□). Mean of two experiments are given. In one experiment platelets were preincubated without any additions and in the presence of 1 mM RGES. Data were combined since no difference was detected. (B) Comparison of CRC54 IgG and CRC54 Fab binding to platelets. Specific binding of [^{125}I]CRC54 IgG (○,□) or [^{125}I]CRC54 Fab (●,■) to washed platelets was measured without any additions (○,●) or in the presence of 5 mM EDTA (□,■).

The effects of CRC54 on platelet interaction with GPIIb-IIIa ligands were also studied by measuring the adhesion of ^{51}Cr-labeled platelets to immobilized fibrinogen and fibronectin. In the presence of CRC54 the level of adhesion to both substrates was increased by 3–4-fold (Fig. 3A,B). Most of the adherent platelets were spread out on the surface and no platelet aggregates were observed by phase-contrast microscopy. The blocking antibody, CRC64, suppressed the adhesion of nonstimulated and CRC54-stimulated platelets to immobilized fibrinogen (Fig. 3A), showing that both basal and CRC54-induced adhesion to fibrinogen was mediated by GPIIb-IIIa. The adhesion of platelets to fibronectin was more complex. CRC64 had no effect on the adhesion of nonstimulated platelets to fibronectin but prevented the CRC54-induced increase of adhesion retaining it at the basal level (Fig. 3B). These results could be explained by the involvement of the specific fibronectin receptor, GPIc-IIa ($\alpha_5\beta_1$-integrin)

[21]. This receptor is present in a functional form on the surface of nonactivated platelets and might therefore mediate basal platelet adhesion to fibronectin which is not inhibited by GPIIb-IIIa blocking antibody. At the same time the increase of platelet adhesion to fibronectin in the presence of CRC54 is mediated by GPIIb-IIIa and thus is inhibited by CRC64.

CRC54-induced conformational changes were registered using two other conformation-dependent monABs: anti-LIBS GPIIb antibody pl-80 [13] and activation-dependent antibody pl-55 [10]. Binding of both ^{125}I-labeled antibodies to platelets was increased when platelets were incubated with CRC54, while binding of the conformation-independent antibody CRC64 was unaffected (Table 2). Binding of anti-LIBS antibody pl-80 was also increased when platelet GPIIb-IIIa was occupied by RGDS, but the binding of activation-dependent antibody pl-55 was inhibited (Table 2), in accordance with the data obtained previously [13].

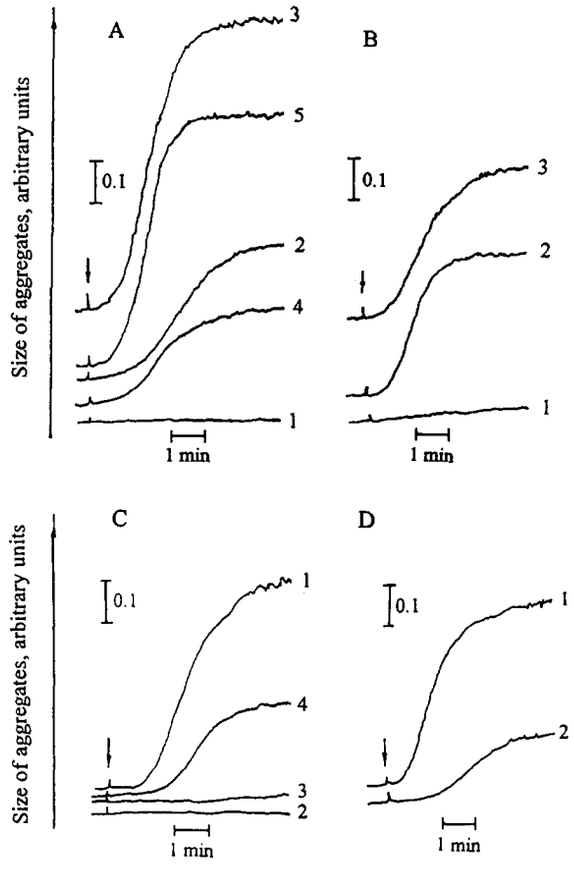

Fig. 2. Platelet aggregation induced by CRC54 IgG and CRC54 Fab fragments. (A) Platelets in PRP were incubated with: 1 μM of control anti-GPIIb-IIIa antibody VM16a (1), 0.5 μM CRC54 IgG (2), 1 μM CRC54 IgG (3), 0.8 μM CRC54 Fab (4) and 1.6 μM CRC54 Fab (5). (B) Washed platelets were incubated: without fibrinogen and with 1 μM CRC54 IgG (1), with 1.65 μM fibrinogen and 1 μM CRC54 IgG (2), and with 1.65 μM fibrinogen and 1.6 μM CRC54 Fab (3). (C) Platelets were preincubated in PRP for 5 min: with no additions (1), with 0.133 μM CRC64 (2); 1 mM RGDS (3) or 1 μg/ml prostaglandin E_1 (4) and then CRC54 was added at the concentration of 0.5 μM. (D) Washed platelet were preincubated for 10 min with 10 mM dextrose (1) or 10 mM deoxyglucose and 0.2% sodium azide (2) and then CRC54 was added at the concentration of 1.0 μM. Additions of CRC54 IgG or CRC54 Fab are indicated by arrows.

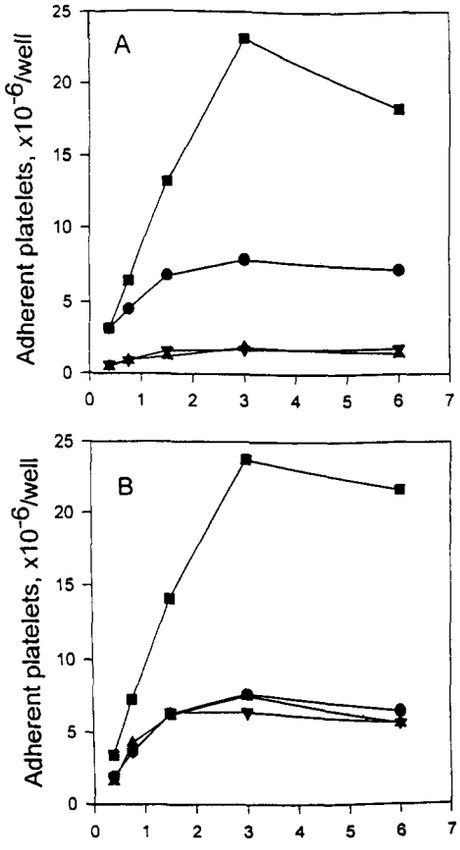

Fig. 3. Effects of monABs CRC54 and CRC64 on the adhesion of ^{51}Cr-labeled platelets to fibrinogen (A) and fibronectin (B). Platelet adhesion was performed without antibodies (●), or in the presence of 0.4 μM CRC54 (■), 0.133 μM CRC64 (▲), or 0.4 μM CRC54 and 0.133 μM CRC64 simultaneously (▼). Level of adhesion in control wells coated with BSA only at platelet concentration of 6×10^8/ml in all samples was approximately the same and amounted to $1.2–1.6\times10^6$/well. Means of two experiments are given.

The results obtained by measuring the binding of labeled antibodies in suspension were confirmed in the capture assay using platelet lysate as a source of GPIIb-IIIa and immobilized pl-80 and pl-55 as capture antibodies. Binding of GPIIb-IIIa from the lysate of CRC54-treated platelets to both antibodies was several times higher than binding from the lysate of control platelets (Fig. 4). In accordance with the data obtained in the binding assay with whole platelets, CRC54-induced binding to pl-80 was comparable to that induced by RGDS peptide and binding to pl-55 in the presence of RGDS was slightly lower than in control samples. Thus these results demonstrate that CRC54 could induce conformational changes in GPIIb-IIIa registered by binding of other conformation-dependent antibodies.

The data presented in this work and in a previous paper describing antibody CRC54 [8], where the mapping of its epitope was performed, have demonstrated that the N-terminal part of GPIIIa contains a LIBS epitope and thus is involved in conformational changes of the GPIIb-IIIa complex. It was shown that interaction of monAB with this epitope could stimulate binding of ligands to GPIIb-IIIa and subsequent platelet aggregation. Using other conformation-dependent antibodies we detected conformational changes of GPIIb-

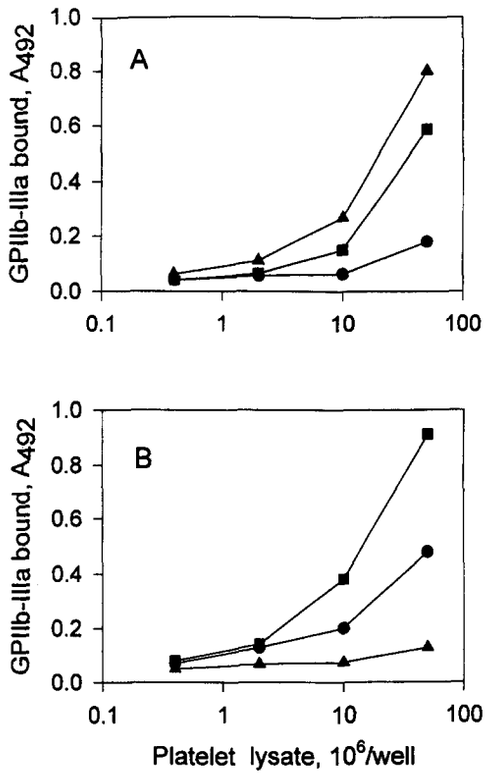

Fig. 4. Binding of GPIIb-IIIa from the platelet lysate to immobilized anti-GPIIb-IIIa conformation-dependent monABs. Washed platelets were preincubated without any additions (●), with 0.5 μM CRC54 (■) or 1 mM RGDS (▲), lysed with Triton X-100 and binding of GPIIb-IIIa from the platelet lysate to anti-GPIIb LIBS antibody, pl-80 (A), or anti-GPIIb-IIIa activation-dependent antibody, pl-55 (B), was evaluated by sandwich ELISA.

IIIa which were induced by activating anti-LIBS antibody and reflected the increased binding ability of the complex.

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