

**Reversibility Versus Persistence of GPIIb/IIIa Blocker-induced  
Conformational Change of GPIIb/IIIa ( $\alpha_{IIb}\beta_3$ , CD41/CD61)**

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CD 41	Cluster of differentiation number for the GPIIb subunit
CD 61	Cluster of differentiation number for the GPIIIa subunit
CHO-cells	Chinese hamster ovary cells
FITC	Fluorescein-iso-thio-cyanate
GPIIb/IIIa	Glycoprotein IIb/IIIa
LIBS	Ligand induced binding site
mAb	Monoclonal antibody
PRP	Platelet rich plasma
RGD	Aminoacid sequence on Fibrinogen recognized by GPIIb/IIIa

### Abstract

Clinically used GPIIb/IIIa blockers are ligand-mimetics and thereby their binding can induce conformational changes of the platelet integrin GPIIb/IIIa. Since the reversibility of these conformational changes may be an important determinant of potential adverse effects of GPIIb/IIIa blockers, we produced a new monoclonal antibody (anti-LIBS-mAb) and, by using its binding properties, we investigated the conformational changes of GPIIb/IIIa during the binding and especially the dissociation of GPIIb/IIIa blockers. Production of mAb clones was performed using purified GPIIb/IIIa in a high affinity conformation and using activated platelets. Clone anti-LIBS-145-mAb was chosen, since it allowed the sensitive probing of eptifibatide-induced conformational changes of GPIIb/IIIa. On resting and activated platelets and on GPIIb/IIIa-expressing CHO cells, anti-LIBS-145-mAb binding returned to background binding after dissociation of eptifibatide, indicating a complete reversibility of the eptifibatide-induced conformational change. Furthermore, with mixing of eptifibatide-preincubated and non-incubated cells, a fast reversibility could be demonstrated. However, when fibrinogen was present in a physiological concentration, the GPIIb/IIIa blocker-induced conformation was partially retained after the dissociation of eptifibatide and to the same extent binding of fibrinogen and the activation-specific mAb Pac-1 was induced. In conclusion, a fast reversibility of the conformational change of GPIIb/IIIa after dissociation of GPIIb/IIIa blockers could be demonstrated as an intrinsic property of the GPIIb/IIIa receptor. This mechanism prevents general platelet aggregation after dissociation of ligand-mimetic GPIIb/IIIa blockers. Nevertheless, in the presence of fibrinogen this reversibility is not complete, which may explain some of the side effects of GPIIb/IIIa blockers, especially of the oral GPIIb/IIIa blockers.

Platelet activation results in a conformational change of the membrane spanning platelet integrin GPIIb/IIIa ( $\alpha_{IIb}\beta_3$ , CD41/CD61) enabling the binding of the plasma protein fibrinogen. This binding is primarily reversible, but itself enhances platelet activation by outside-in signaling causing receptor clustering, platelet secretion and finally irreversible fibrinogen binding and platelet aggregation (Shattil et al., 1998). Similar conformational changes of GPIIb/IIIa are induced by several GPIIb/IIIa blockers, since these agents bind at or nearby the fibrinogen binding pocket of GPIIb/IIIa and thereby act as ligand-mimetics (Gawaz et al., 1998; Jennings et al., 2000; Dickfeld et al., 2001). While the conformational change of GPIIb/IIIa after the binding of ligands or ligand-mimetics has been investigated (Kamata and Takada, 2001; Hynes, 2002; Takagi et al., 2002), the conformational status of GPIIb/IIIa after dissociation of ligands or ligand-mimetics has not been the focus of investigations yet.

GPIIb/IIIa blockers have demonstrated limitations as intravenous drugs and have caused an increase in mortality in their use as oral drugs (Cox et al., 2000; Holmes et al., 2000; Chew et al., 2001; Quinn et al., 2002; Quinn et al., 2003; Topol et al., 2003). For several reasons the reversibility of the GPIIb/IIIa blocker-induced conformational change after dissociation of the blocker might be essential for the understanding of the potential adverse effects associated with this class of anti-platelet drugs. GPIIb/IIIa blocker-associated thrombocytopenia and platelet-activating effects of GPIIb/IIIa blockers have been directly associated with the conformational changes of GPIIb/IIIa induced by the binding of ligand-mimetic blockers to the receptor (Peter et al., 1998; Madan and Berkowitz, 1999; Cox et al., 2000; Bougie et al., 2002; Bhatt and Topol, 2003; Quinn et al., 2003). Thus, the reversibility or persistence of these conformational changes can be expected to be determinants of the adverse effects of GPIIb/IIIa blockers.

In the present study, we developed a new anti-LIBS (**L**igand-induced **b**inding sites) monoclonal antibody (mAb), which allows a sensitive probing of GPIIb/IIIa blocker-induced conformational receptor changes and we set up experimental procedures using platelets as well as recombinant GPIIb/IIIa to study receptor conformation after dissociation of GPIIb/IIIa blockers. We could demonstrate that a fast reversibility of the GPIIb/IIIa blocker-induced conformational change is an intrinsic property of the receptor. However, in the presence of fibrinogen reversibility is not complete. This finding is an important new aspect in the discussion of GPIIb/IIIa blocker-associated adverse effects.

## Methods

**Blood Preparation and Cells.** Blood was collected by venipuncture with a 21 gauge butterfly needle from healthy volunteers and anticoagulated with citric acid. Platelet rich plasma was obtained by centrifugation at 100g in plastic tubes at room temperature for 15 minutes in a laboratory centrifuge (Haereus Multifuge 3s). Platelets were counted using a Neubauer-chamber and adjusted to 200 000/ $\mu$ l with plasma.

CHO cells expressing either low affinity (non-activated) or high affinity (activated) GPIIb/IIIa were produced as described elsewhere in detail (O'Toole et al., 1994; Peter and Bode, 1996). These cell express approximately 500 000 to 800 000 GPIIb/IIIa receptors per cell (Marcinkiewicz et al., 1996). Cells were maintained in DMEM, 10 % fetal calf serum, 1% MEM non-essential amino acids, 2 mM L-glutamine, geneticin 700  $\mu$ mg/ml, 100 units/ml penicillin, and 100 g/ml streptomycin (all from Gibco).

**Generation of the Anti-LIBS-145-mAb.** GPIIb/IIIa complex that was purified by affinity chromatography on immobilized KYGRGSD and eluted with GRGDSP was used as the immunogen. Immunization and hybridoma production were performed as described elsewhere (Kotani et al., 2000). The primary screen was performed using stimulated platelets as described previously (Tanoue, 1993). The secondary screen was with immobilized GPIIb/IIIa saturated with GRGDSP on micro titer plates. Positive clones were confirmed by flow cytometric analysis with gel-filtered platelets. Among positive clones, there were a few clones that demonstrated a substantially increased reactivity against platelets in the presence of the GRGDSP peptide. One of these clones, 145, was further analyzed. This mAb was purified on a Protein-G-Sepharose col-

umn (Pharmacia) and labeled with FITC (Pierce). GRGDSP-peptide was purchased from Biomol, eptifibatide from Essex Pharma, tirofiban from MSD and abciximab from Lilly.

**Detection of LIBS-145-Expression in Flow Cytometry.** Platelet rich plasma was diluted in modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO<sub>3</sub>, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1mg/ml bovine serum albumin (BSA), 1mg/ml dextrose; pH 7.4) 1 to 50 (resulting in a platelet density of 4.000/ $\mu$ l) and incubated with ADP [20  $\mu$ M] or GRGDSP [0.6  $\mu$ g/ml], tirofiban [0.5  $\mu$ g/ml], eptifibatide [10 $\mu$ g/ml] or abciximab [10 $\mu$ g/ml] at room temperature for 30 minutes. The GPIIb/IIIa blockers were used in saturating concentrations as determined by titration. These concentrations were adjusted to the concentrations that are therapeutically used (Scarborough et al., 1999; Seshiah et al., 2002). If not otherwise stated, the binding of anti-LIBS-145-mAb (after incubation for 20 minutes) was detected by a secondary FITC-labeled polyclonal anti-mouse antibody (Dianova, incubated for 20 minutes in the dark). If the direct FITC-labeled anti-LIBS-145-mAb was used, incubation time was also 20 minutes in the dark. Finally, platelets were examined on FACSCalibur<sup>®</sup> with CellQuest<sup>®</sup> software (both Becton-Dickinson).

In analogy, GPIIb/IIIa-expressing CHO cells were detached with trypsin-EDTA (Gibco) and washed in modified Tyrode's buffer. 300 000 cells per 50  $\mu$ l in modified Tyrode's buffer were stained and analyzed as described above.

**Quantification of antibody binding sites and GPIIb/IIIa occupancy.** The absolute number of ligand-induced binding sites (LIBS) per platelets and the number of occupied GPIIb/IIIa receptors were measured using the GPIIb/IIIa occupancy kit (Biocytex). The protocol

was performed corresponding to the manufacturers recommendations. For the measurement of LIBS, anti-LIBS-145-mAb was used instead of Mab1 or Mab2.

**Washout Experiments.** Platelet rich plasma or GPIIb/IIIa-expressing CHO cells were first incubated 30 minutes with high concentrations of the GPIIb/IIIa blockers as described above. Then, cells were washed once in 4 ml Tyrode's buffer, centrifuged 10 minutes at 2000g and then resuspended in 50  $\mu$ l Tyrode's buffer. LIBS-145 expression before and after washing was determined in flow cytometry as described above.

**Washout Experiments with Fibrinogen.** When working with stimulated platelets or when fibrinogen was present in the washing solution we used a modified protocol with smaller volumes and shorter centrifugation to minimize artificial activation. 28 $\mu$ l ACD (20 mM citric acid, 110 mM sodium citrate, 5 mM dextrose) were added to 200  $\mu$ l PRP and platelets were pelleted 30 seconds at 600 rpm in a microcentrifuge. Platelets were then resuspended in Tyrode's buffer, diluted 1/50 in Tyrode's buffer, and incubated for 30 minutes at room temperature with or without eptifibatide at 5  $\mu$ g/ml, a concentration which allowed the occupancy of most GPIIb/IIIa receptors without excess of eptifibatide. Then, platelets were washed in 2 ml Tyrode's buffer with or without 3 mg/ml fibrinogen and stained with FITC-labeled anti-LIBS-145-mAb, Pac-1 (Becton-Dickinson) or polyclonal chicken anti-fibrinogen antibody (WAK-Chemie).

**Intercellular Transfer of Eptifibatide.** Platelet rich plasma (1/50 in Tyrode's buffer: 4000 platelets/ $\mu$ l) was incubated 30 minutes at room temperature either with or without 2  $\mu$ g/ml eptifibatide. This concentration was chosen to avoid excess of eptifibatide and which corre-

sponds to 50% of the maximal LIBS expression in titration experiments. Then, anti-LIBS-145-mAb was added in saturating concentrations to both vials and incubated for another 30 minutes at room temperature. Cells were then resuspended in 200  $\mu$ l Tyrode's buffer and mean fluorescence of each vial was measured in flow cytometry. Then, cells were mixed and flow cytometry was performed subsequently and thereafter in intervals of one minute. In analogy, mixing experiments were performed with eptifibatide-preincubated platelets [2 $\mu$ g/ml] and GPIIb/IIIa-expressing CHO cells.

**Immunoprecipitation with Human Platelets.** Human platelets were washed once in Tyrode's buffer and then incubated without or with eptifibatide [10 $\mu$ g/ml]. Then, one fraction of the eptifibatide-preincubated platelets was washed again in Tyrode's buffer. Subsequently, all platelets were lysed by incubation for 1 hour in non-denaturing lysis buffer (1% CHAPS, 25mM HEPES, pH 7.5, 2mM NaF, 2mM phenylmethanesulfonyl fluorid, 10 $\mu$ g/ml aprotinin, 10 $\mu$ g/ml leupeptin) as described (Longhurst et al., 1999). After that, debris was pelleted by 10 minutes centrifugation at 10 000g and lysates were precleared by overnight incubation with protein G-Sepharose. Immunoprecipitation was performed by addition of anti-LIBS-145-mAb [20 $\mu$ g/ml] and then by absorption to protein G sepharose with agitation for 2 hours at 4°C. The sepharose beads were washed five times in lysis buffer and then, captured immune complexes were eluted and denatured with reducing Laemmli-buffer for 5 minutes at 94°C. SDS-PAGE was performed on a 5-20% gradient gel and then the proteins were transferred to Immobilon P membrane (Millipore) by Western blotting. After the membrane had been blocked by an overnight incubation in PBS containing 1% BSA and 0.05% Tween-20 the GPIIb/IIIa subunits were hybridized using a murine monoclonal anti-CD41 (anti-GPIIb, anti- $\alpha_{IIb}$ ) (Clone SZ-22, Immunotech) or a polyclonal

goat anti-CD61 (anti-GPIIIa, anti- $\beta_3$ ) antibody (Santa Cruz). Both were stained using secondary horseradish-peroxidase-coupled polyclonal anti-mouse (Immunotech) or goat (Santa Cruz) antibodies and detected with a chemiluminiscent peroxidase substrate (super signal west pico, Pierce). For the eptifibatide-preincubated platelets, eptifibatide was added to all immunoprecipitation steps.

## Results

Monoclonal anti-LIBS antibodies were produced with a selection procedure using RGD peptides (see Methods for details) enriching for mAbs that are able to detect the conformational change on the GPIIb/IIIa receptor induced by GPIIb/IIIa blockers. One of the monoclonal antibody clones (anti-LIBS-145-mAb) revealed strong binding to the GPIIb/IIIa receptor either occupied by RGD peptides or by fibrinogen without inhibiting fibrinogen or ligand-mimetic binding to the receptor. The FITC-labeled anti-LIBS-145-mAb demonstrated a distinct binding pattern on human platelets (Fig. 1) that were incubated with the three clinically used GPIIb/IIIa blockers at concentrations which are based on therapeutically applied plasma levels and which are sufficient to achieve saturated binding to GPIIb/IIIa. Maximal anti-LIBS-145-mAb binding was strongest with the GPIIb/IIIa blocker eptifibatide and lowest with abciximab. Based on these distinct binding properties of anti-LIBS-145-mAb, eptifibatide was used for further experiments.

First, the conformational change of GPIIb/IIIa induced by eptifibatide was characterized. Incubation of platelets with eptifibatide induced a strong shift in fluorescence intensity of FITC-labeled anti-LIBS-145-mAb binding, as seen in the flow cytometric histogram depicted in Fig. 2C (mean fluorescence from 9.1 to 65.1). ADP stimulation and subsequent fibrinogen binding (which was not inhibited by anti-LIBS-145-mAb as it is characteristic for anti-LIBS mAbs, data not shown) also induced anti-LIBS-145-mAb binding to GPIIb/IIIa (Fig. 2B, mean fluorescence from 9.1 to 45), which is notably lower than the anti-LIBS-145-mAb binding induced by eptifibatide (Fig. 2C). Anti-LIBS-145-mAb binding was strongest with the combination of ADP stimulation and eptifibatide incubation (Fig. 2D, mean fluorescence from 9.1 to 106.8). These

binding characteristics demonstrate the advantageous properties of anti-LIBS-145-mAb for the monitoring of the eptifibatide-induced conformational change of GPIIb/IIIa.

To investigate the binding of anti-LIBS-145-mAb and its reversibility independent of the complex platelet-signaling machinery, we also used GPIIb/IIIa-transfected CHO cells. As a model for the activated conformation, CHO cells expressing a GFFKR-deleted cytoplasmic domain of the  $\alpha_{IIb}$ -subunit were used. As depicted in Fig. 3A the anti-LIBS-145-mAb does not bind to CHO cells expressing the non-activated GPIIb/IIIa in the absence of ligands. In contrast, there is a considerable binding on GFFKR-deleted GPIIb/IIIa even if no fibrinogen is present (Fig. 3B). A comparable binding can be detected when non-activated GPIIb/IIIa is incubated with eptifibatide (Fig. 3D). The combination of GFFKR-deleted GPIIb/IIIa and incubation with eptifibatide resulted in the strongest binding of anti-LIBS-145-mAb (Fig. 3E). As a negative control, non-transfected CHO cells without or with incubation with eptifibatide did not demonstrate specific anti-LIBS-145-mAb binding (Fig. 3C + F). Thus, conformational changes of GPIIb/IIIa expressed on CHO cells and their detection by anti-LIBS-mAb are comparable to the changes seen with platelets. Therefore, anti-LIBS-145-mAb applied together with the recombinant expression of GPIIb/IIIa provides the unique opportunity to study conformational changes of GPIIb/IIIa as intrinsic receptor properties.

To address the question whether the conformational change induced by GPIIb/IIIa blockers is reversible after dissociation of the blocker, we washed platelets, which have been preincubated with eptifibatide, in Tyrode's buffer and monitored receptor number, occupancy, and the conformational state simultaneously using three antibodies (Fig. 4A). Mab1 is a complex-

specific antibody that binds to an epitope on GPIIb/IIIa that does not change with the binding of small molecular weight GPIIb/IIIa blockers such as eptifibatide (Quinn et al., 1999). This antibody recognizes 60 000 to 70 000 binding sites on platelets and as an important control this number does not change with incubation or washout of eptifibatide (Fig. 4A). Mab2 binding to GPIIb/IIIa has been described to be inhibited by small molecular weight GPIIb/IIIa blockers and thus allows the monitoring of receptor occupancy (Quinn et al., 1999). Indeed, incubation of platelets with eptifibatide reduced the binding sites from  $(72\,095 \pm 7805)$  to  $(30\,192 \pm 1700)$ . After the washout of eptifibatide, the number of binding sites returned to baseline  $(77\,974 \pm 8686)$ , providing proof of a complete dissociation of eptifibatide from GPIIb/IIIa in the experimental set up used (Fig. 4A). The binding of the anti-LIBS-145 antibody to GPIIb/IIIa before incubation with eptifibatide revealed only  $(5907 \pm 88)$  binding sites, whereas after incubation with eptifibatide anti-LIBS-145-mAb recognizes the majority of receptors  $(49\,827 \pm 259)$ , demonstrating the potential of this mAb to detect occupancy (Fig. 4A). The major finding in this experiment is the full return to the low number of binding sites  $(5657 \pm 427)$  after dissociation of eptifibatide, indicating a complete reversibility of the eptifibatide-induced conformational change of GPIIb/IIIa in this experimental set up.

Although the extent of binding of anti-LIBS-145-mAb to GPIIb/IIIa on platelets after incubation with tirofiban and abciximab was smaller compared to eptifibatide (see Fig. 1), measurements of anti-LIBS-145-mAb binding could be used to demonstrate that the conformational changes induced by these GPIIb/IIIa blockers were also reversible after dissociation of the blocker (data not shown).

Evaluating the intrinsic GPIIb/IIIa function without the platelet signaling machinery, CHO cells expressing GPIIb/IIIa were incubated with eptifibatide and the LIBS-145 epitope was probed before and after washout of the GPIIb/IIIa blocker in Tyrode's buffer (Fig. 4B). Also for the recombinant GPIIb/IIIa receptor, a full reversibility of this conformational change could be demonstrated.

Immunoprecipitation was used as an additional approach to demonstrate the binding specificity of anti-LIBS-145-mAb for the ligand-occupied form of GPIIb/IIIa. After incubation with eptifibatide, or incubation with eptifibatide and subsequent washout, or without treatment, platelets were lysed and immunoprecipitation was performed with anti-LIBS-145-mAb. Precipitates were separated by SDS-PAGE, and Western blotting was done either with an anti-CD41 (anti-GPIIb, Fig. 5A) or an anti-CD61 (anti-GPIIIa, Fig. 5B) mAb. The molecular sizes for the blotted proteins are 125 kDa for GPIIb and 97 kDa for GPIIIa. Precipitation with anti-LIBS-145-mAb resulted only in strong blotting signals representing either GPIIb (Fig. 5A) or GPIIIa (Fig. 5B) when the GPIIb/IIIa receptor was occupied by eptifibatide. The signal was clearly lost when eptifibatide was washed out from the GPIIb/IIIa receptor. Thus, these data are in agreement with the presented flow cytometric data and confirm by an additional independent method that anti-LIBS-145-mAb specifically reports on the conformation of GPIIb/IIIa.

For the monitoring of the time course of this reversibility of GPIIb/IIIa blocker-induced conformational changes, untreated and eptifibatide-preincubated platelets were mixed. Directly after mixing, two populations were present which were approaching each other and merged after approximately 15 minutes to one single peak in between the middle of both initial peaks (Fig. 6).

This fluorescence shift of the two platelet populations reflects a transfer of eptifibatide from the preincubated platelets to the untreated platelets. Since platelets and CHO cells can be distinguished in flow cytometric dot blots by their different size and granularity, mixing experiments were also performed with these two GPIIb/IIIa sources. Thereby, it was possible to directly follow up platelets after dissociation of eptifibatide. As depicted in Fig. 7, when eptifibatide moves (according to the overall balance of dissociation and association on both GPIIb/IIIa sources) from the eptifibatide-preincubated platelets to the non-preincubated GPIIb/IIIa-expressing CHO cells, the binding of anti-LIBS-145-mAb to platelets decreased. This finding indicates a reversibility of the GPIIb/IIIa blocker-induced conformational change after dissociation of eptifibatide. In parallel, the anti-LIBS-145-mAb mean fluorescence of the GPIIb/IIIa-expressing CHO cells increases, reflecting the conformational change induced by the ligand-mimetic agent eptifibatide. Thus, these experiments indicate that the conformational change of GPIIb/IIIa induced by eptifibatide is reversed after dissociation of the GPIIb/IIIa blocker.

Furthermore, we evaluated whether the GPIIb/IIIa conformation induced by the binding of the native ligand, the macromolecular fibrinogen is comparable in its reversibility with the eptifibatide-induced conformation. Platelets were either preincubated with eptifibatide or pretreated with ADP or both in a Tyrode's buffer containing fibrinogen (3 mg/ml). Then, platelets were washed in Tyrode's buffer not containing fibrinogen. Interestingly, in all settings the anti-LIBS-145-mAb binding after washout returned to the background binding (Fig. 8). Thus, GPIIb/IIIa returns to a conformational state, not expressing the LIBS-145 epitope, after dissociation of both, fibrinogen and eptifibatide.

Finally, we addressed the question whether the presence of fibrinogen could influence the reversibility of the GPIIb/IIIa blocker-induced conformational change. Eptifibatide-preincubated platelets were washed either in Tyrode's buffer alone or in Tyrode's buffer containing fibrinogen. As depicted in Fig. 9A, the reversibility of anti-LIBS-145-mAb binding was not complete when fibrinogen was present in the washing solution. To assure, that the detected anti-LIBS-145-mAb binding reflects the activated, ligand binding competent GPIIb/IIIa receptor, the binding of the activation-specific mAb Pac-1 and the binding of fibrinogen itself, as evaluated by an anti-fibrinogen antibody, was investigated. Indeed, fibrinogen and Pac-1 binding, in parallel with the anti-LIBS binding, demonstrate that the GPIIb/IIIa blocker-induced conformational change of GPIIb/IIIa is not fully reversible in the presence of fibrinogen (Fig. 9B). Induction of fibrinogen binding after dissociation of eptifibatide could also be demonstrated at 37°C and with blood anticoagulated by hirudin (data not shown). The latter condition assures a physiological cation situation and excludes a thrombin-mediated effect on platelets. Overall, under experimental conditions that are close to the physiological situation, the conformational change of GPIIb/IIIa induced by eptifibatide is only partially reversible in the presence of fibrinogen.

## Discussion

The main findings of this study are 1) GPIIb/IIIa blocker-induced conformational changes of GPIIb/IIIa demonstrate a fast reversibility after dissociation of the GPIIb/IIIa blocker. 2) This reversibility can be seen on resting and stimulated human platelets and on recombinant GPIIb/IIIa expressed on CHO cells, indicating that the reverse conformational change is an intrinsic property of the integrin GPIIb/IIIa. 3) In the presence of fibrinogen, the eptifibatide-induced conformational change of GPIIb/IIIa is only partially reversible upon dissociation of the GPIIb/IIIa blocker, as detected by the binding of a mAb specific for a ligand-induced binding site (LIBS), the binding of the ligand-mimetic mAb Pac-1, and the binding of the GPIIb/IIIa ligand fibrinogen.

A new monoclonal antibody (anti-LIBS-145-mAb) was produced that is able to detect conformational changes of platelet GPIIb/IIIa, which are induced by binding of ligands or ligand-mimetics including eptifibatide, tirofiban and abciximab. This antibody also detects the “activated” conformational status of GPIIb/IIIa in the absence of ligands if a deleted and thereby activated version of GPIIb/IIIa is expressed in CHO cells. The strong binding of anti-LIBS-145-mAb to the eptifibatide-induced conformation of GPIIb/IIIa was the prerequisite of our study.

For anti-LIBS-antibodies distinct binding patterns with different GPIIb/IIIa blockers have been reported (Jennings et al.; 2000; Dickfeld et al., 2001). These findings suggest that there are distinct ligand-induced conformational states depending on the individual GPIIb/IIIa blocker. Thus, our finding that the binding pattern of anti-LIBS-145-mAb differs between the tested GPIIb/IIIa blockers is in line with the characteristics of other anti-LIBS antibodies.

Several authors provided evidence for conformational changes of the GPIIb/IIIa receptor induced by ligand binding (Frelinger et al., 1988; O'Toole et al., 1990; Du et al., 1991; Peter et al., 1998; Frelinger et al., 2001). However, for the detection of these conformational changes, experiments were typically performed using a fixation procedure prior to dissociation of the ligands/ligand-mimetics and thus the reversibility of these conformational changes could not be investigated. To address reverse conformational changes, we performed mixing and washout experiments without any fixation. The complete reversibility of anti-LIBS-145-mAb binding after washout of the ligand-mimetic eptifibatide indicates that the GPIIb/IIIa receptor is capable of performing a complete reverse conformational change after dissociation of the ligand. This effect was observed on human platelets as well as on the isolated receptor on GPIIb/IIIa transfected CHO cells. The latter being of particular interest, since it has been described that the ligand-induced conformational change on purified, immobilized GPIIb/IIIa, which is caused by the peptido-mimetic Ro43-5054, is irreversible (Kouns et al., 1992). These authors proposed that the intact platelet is necessary for the reverse conformational change. However, based on the presented data with GPIIb/IIIa-expressing CHO cells, it can be concluded that a correctly folded integrin inserted in a cell membrane is necessary but also sufficient for the reverse conformational change, which appears to be an intrinsic property of the receptor. Since in the clinical settings in which GPIIb/IIIa blockers are used platelets may be activated and since it is reported that GPIIb/IIIa blockers may vary in their binding properties e.g. on platelets activated by shear stress (Wang et al., 2002), it is an important finding that reversibility of the conformational change of GPIIb/IIIa could also be demonstrated in activated platelets. Overall, our findings indicate that the reverse conformational change of GPIIb/IIIa is an intrinsic property of the receptor.

The basis for the regulation of ligand binding to the adhesion molecules belonging to the protein family of integrins is the propensity to change their conformation in response to cell activation (Hynes, 2002). It is generally thought that cell activation results in a conformational change within the cytoplasmic domains of the integrins and that this intracellular conformational change is transferred to the extracellular domains of the integrins, a process which has been termed inside-out signaling. However, signal transduction seems also to be possible as an outside-in signaling. Binding of ligands or ligand-mimetics can cause a conformational change of the extracellular domains of the integrin and this can be transferred to the intracellular domains, resulting in a cell activation signal (Leisner et al., 1999; Hynes, 2002). Having in mind this general principle of integrin regulation, it becomes clear that the conformational changes induced by GPIIb/IIIa blockers, based on their ligand-mimetic properties, indeed may have consequences in signaling and also that reversibility of these conformational changes is a major issue determining side effects of GPIIb/IIIa blockers (Quinn et al., 2003). In the current study, in vitro experiments with incubation of platelets with saturating concentrations of the clinically used GPIIb/IIIa blockers did not result in P-selectin expression on the platelet surface (data not shown). This indicates, that GPIIb/IIIa blockers alone are not sufficient to cause strong platelet activation. However, induction of intracellular calcium release, which is one of the early consequences of the outside-in signaling, has been demonstrated for experimental GPII/IIIa blockers that expose LIBS-epitopes, but it has not been demonstrated for those that do not expose LIBS epitopes (Honda et al., 1998). Moreover, a GPIIb/IIIa antagonist that did not induce LIBS resulted in greater suppression of TXA<sub>2</sub> formation in a canine model of coronary thrombolysis (Murphy et al., 1998). For oral GPIIb/IIIa blockers, that failed to reveal clinical benefits and even increased

mortality, platelet activation markers have been found to be increased (Cox et al., 2000; Holmes et al., 2000; Chew et al., 2001; Topol et al., 2003). The incomplete reversibility of the GPIIb/IIIa-induced conformational change found in the presence of fibrinogen may indeed retain a certain level of outside-in signaling. Moreover, the persistence of the GPIIb/IIIa blocker-induced conformational change may explain previous findings of fibrinogen binding and the induction of a certain level of platelet aggregation at low concentrations of GPIIb/IIIa blockers (Peter et al., 1998). Indeed, the pharmacokinetic profiles of oral GPIIb/IIIa blockers reveal low plasma levels of these drugs repeatedly. Further studies have to elucidate the level and clinical importance of this first report on incomplete reversibility of GPIIb/IIIa blocker-induced conformational changes and also whether these findings can be generalized to all ligand-mimetic GPIIb/IIIa blockers.

The existence of preformed anti-LIBS antibodies in patients, their binding to GPIIb/IIIa and the resulting platelet sequestration has been considered to be a potential mechanism for GPIIb/IIIa blocker-induced thrombocytopenia (Madan and Berkowitz, 1999; Bougie et al., 2002; Seiffert et al., 2003). An incomplete disappearance of the LIBS epitopes after dissociation of the blocker might result in the prolonged sequestration of platelets which have been in contact with the GPIIb/IIIa blocker.

For the integrin  $\alpha_v\beta_3$  a “conformational memory” has been postulated, describing the persistence of a high affinity receptor conformation after dissociation of RGD peptides (Legler et al., 2001). The authors hypothesize that this “conformational memory” is the underlying mechanism by which low concentrations of RGD peptides induce an agonistic effect resulting in the binding of vitronectin to  $\alpha_v\beta_3$  (Legler et al., 2001). The ligand-mimetic properties of RGD pep-

tides are seen as the basis for the “superactivation” of  $\alpha_v\beta_3$  by these peptides (Legler et al., 2001). A similar finding has been reported for to the GPIIb/IIIa receptor: Low concentrations of GPIIb/IIIa blockers can induce fibrinogen binding (Peter et al., 1998). Thus, a “conformational memory” after dissociation of GPIIb/IIIa blockers may be responsible for an agonistic effect at low concentration of blockers. Since pharmacokinetic profiles of oral GPIIb/IIIa blockers repeatedly reveal low concentrations (Quinn et al., 2003; Topol et al., 2003), agonistic effects may be more prominent than with intravenous bolus and infusion therapy (Chew et al., 2001; Quinn et al., 2002).

In general, the concept of integrin blockade by ligand-mimetic agents should be critically assessed. Integrins are not only transducers of mechanical force between the cell inside and outside and vice versa, but integrins are also signal transducers from the inside to the outside and vice versa (Hynes, 2002). Ligand binding can cause outside-in signaling and thus ligand-mimetic blockers may also cause outside-in signaling as paradoxical or agonistic effects (Shimaoka and Springer, 2003). The monovalent character of GPIIb/IIIa blockers seems to prevent a complete outside-in signal, however fibrinogen bound after dissociation of the GPIIb/IIIa blocker is a multivalent ligand and the outside-in signal may thus be complete. Indeed, the disappointing results with oral GPIIb/IIIa blockers and the growing understanding of inherent problems of ligand-mimetic antagonists have initiated the development of anti-integrin therapeutics that are not ligand-mimetics but are stabilizers of the low affinity conformation of integrins (Weitz-Schmidt et al., 2001; Welzenbach et al., 2002; Shimaoka and Springer, 2003).

In summary, the newly produced anti-LIBS-145-mAb allowed the demonstration of a fast reversibility of the GPIIb/IIIa blocker-induced conformational change of GPIIb/IIIa. This is the first report demonstrating this reversibility as an intrinsic property of the GPII/IIIa receptor on resting and activated platelets and as a recombinant receptor expressed in CHO cells. The propensity of the GPIIb/IIIa receptor to reverse its conformation after the dissociation of the GPIIb/IIIa blocker is the essential precondition that the ligand-mimetic GPIIb/IIIa blockers do not cause generalized platelet aggregation after their dissociation. However, our finding that this reversibility is not complete in the presence of fibrinogen provides potential explanations for yet unexplained adverse effects of ligand-mimetic GPIIb/IIIa blockers, especially of the oral GPIIb/IIIa blockers.

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## Legends

### **Fig. 1:**

Binding of the FITC-labeled anti-LIBS-145-mAb to human platelets preincubated with various GPIIb/IIIa blockers under non-activating conditions. Depicted is the average of mean fluorescence as obtained in flow cytometry with standard deviations of three measurements.

### **Fig. 2:**

LIBS-145 expression on resting and activated platelets (black histograms). Depicted are flow cytometry histograms of resting (A+C, left column) and activated (B+D, right column) platelets. The histograms in the second row (C+D) represent anti-LIBS-145-mAb binding in the presence of eptifibatide [10 $\mu$ g/ml]. The grey histogram demonstrates the unspecific binding as evaluated with an unspecific mAb.

### **Fig. 3:**

LIBS-145 expression on GPIIb/IIIa-expressing CHO cells. The binding of anti-LIBS-145-mAb to CHO cells expressing the non-activated (A+D) or the activated conformation of GPIIb/IIIa (B+E), with 10  $\mu$ g/ml eptifibatide (lower row) or without eptifibatide (upper row) was measured in flow cytometry. As negative control non-transfected CHO cells were utilized (C+F).

### **Fig. 4A:**

Reversibility of eptifibatide-induced conformational change. Quantification of binding sites was performed in flow cytometry, using the GPIIb/IIIa occupancy kit (see Methods). Mab 1 indicates the total number of GPIIb/IIIa receptors, Mab 2 the number of unoccupied receptors, and LIBS-145-mAb the number of receptors expressing the LIBS-145 epitope. Measurements were performed without addition, in the presence of eptifibatide, and after incubation and washout of eptifibatide.

**Fig. 4B:**

Reversibility of LIBS-145 expression on GPIIb/IIIa-expressing CHO cells after washing in Tyrode's buffer as detected by FITC-labeled anti-LIBS-145-mAb. In analogy to Fig.1, CHO cells expressing the non-activated GPIIb/IIIa receptor were preincubated with 10 $\mu$ g/ml eptifibatide and washed in Tyrode's buffer.

**Fig. 5:**

Immunoprecipitation of platelets with anti-LIBS-145-mAb. Platelets without pretreatment (first lane), preincubated with eptifibatide (second lane) or preincubated with eptifibatide and washed in Tyrode's buffer (third lane) were immunoprecipitated with anti-LIBS-14-mAb. The GPIIb/IIIa subunits were detected in **A** with an anti-CD41 (anti-GPIIb, anti- $\alpha_{IIb}$ ) or in **B** with an anti-CD61 (anti-GPIIIa, anti- $\beta_3$ ) antibody.

**Fig. 6:**

LIBS-145 expression after mixing of eptifibatide-preincubated and non-preincubated platelets. Depicted are flow cytometry histograms, which demonstrate the binding of the FITC-labeled anti-LIBS-145-mAb to untreated platelets (A), platelets preincubated with eptifibatide (B) as well as directly (C), 10 minutes (D) and 15 minutes (E) after mixing of the two populations.

**Fig. 7:**

LIBS-145 expression after mixing of eptifibatide-preincubated platelets with GPIIb/IIIa-expressing CHO cells. The bar graphs depict mean fluorescence of FITC-labeled anti-LIBS-145-mAb binding to preincubated platelets (upper row) and initially unoccupied GPIIb/IIIa-expressing CHO cells (lower row). The movement of eptifibatide between platelets and GPIIb/IIIa-expressing CHO cells is represented by the arrows.

**Fig. 8:**

Reversibility of LIBS-145 expression on resting and ADP-stimulated human platelets after washing in Tyrode's buffer. Platelets were partly stimulated for 30 minutes with 20  $\mu$ M ADP and additionally preincubated with or without eptifibatide. Then, eptifibatide was washed out with modified Tyrode's buffer. Depicted are average values of mean fluorescence of FITC-anti-LIBS-145-mAb as measured in flow cytometry (three experiments).

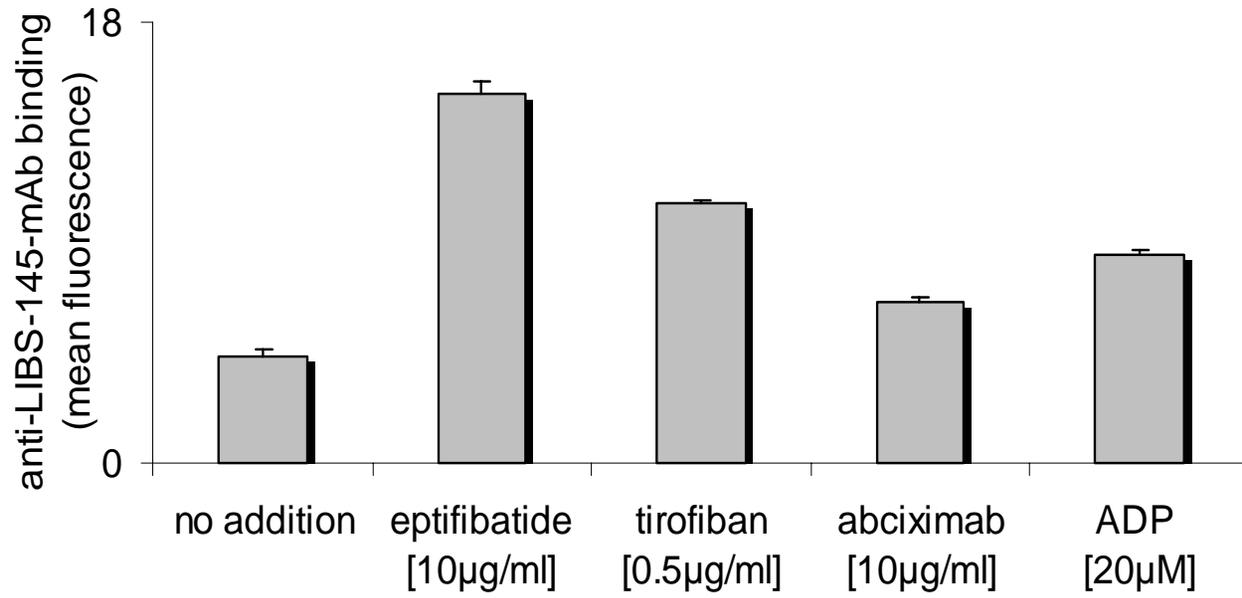
**Fig. 9A:**

Reversibility of LIBS-145 expression on human platelets, preincubated with eptifibatide and after washing in Tyrode's buffer containing fibrinogen. The bar graphs depict average values of mean fluorescence of FITC-anti-LIBS-145-mAb of three experiments.

**Fig. 9B:**

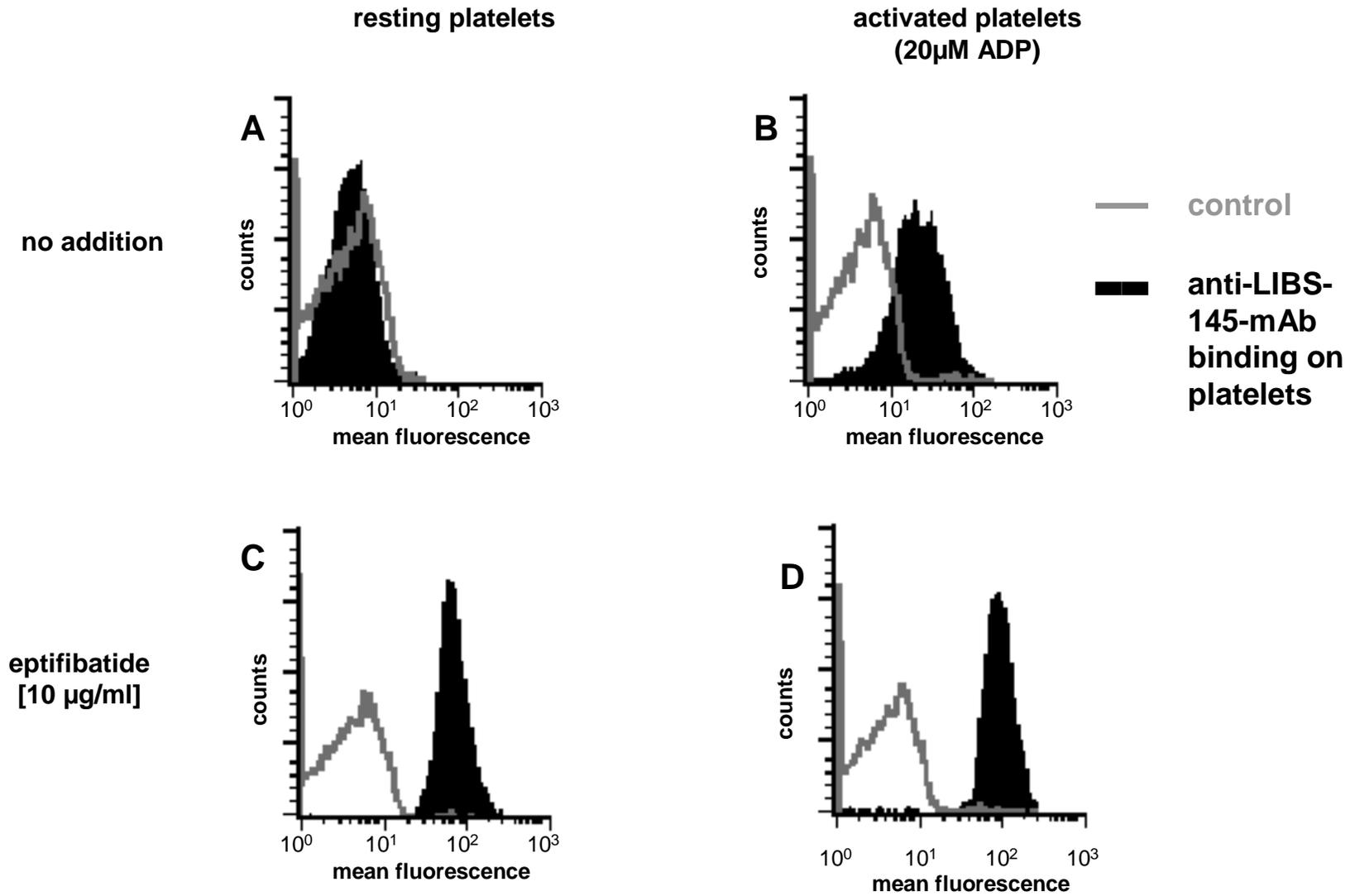
Conformational change induced by eptifibatide and detected by binding of fibrinogen and the ligand-mimetic mAb Pac-1 after dissociation of the GPIIb/IIIa blocker in the presence of fibrinogen. Depicted are average values of mean fluorescence of FITC-anti-fibrinogen Ab or FITC-Pac-1 mAb of three experiments.

**Fig. 1:** Distinct Binding Pattern of Anti-LIBS-145-mAb to Platelets Incubated with GPIIb/IIIa Blockers



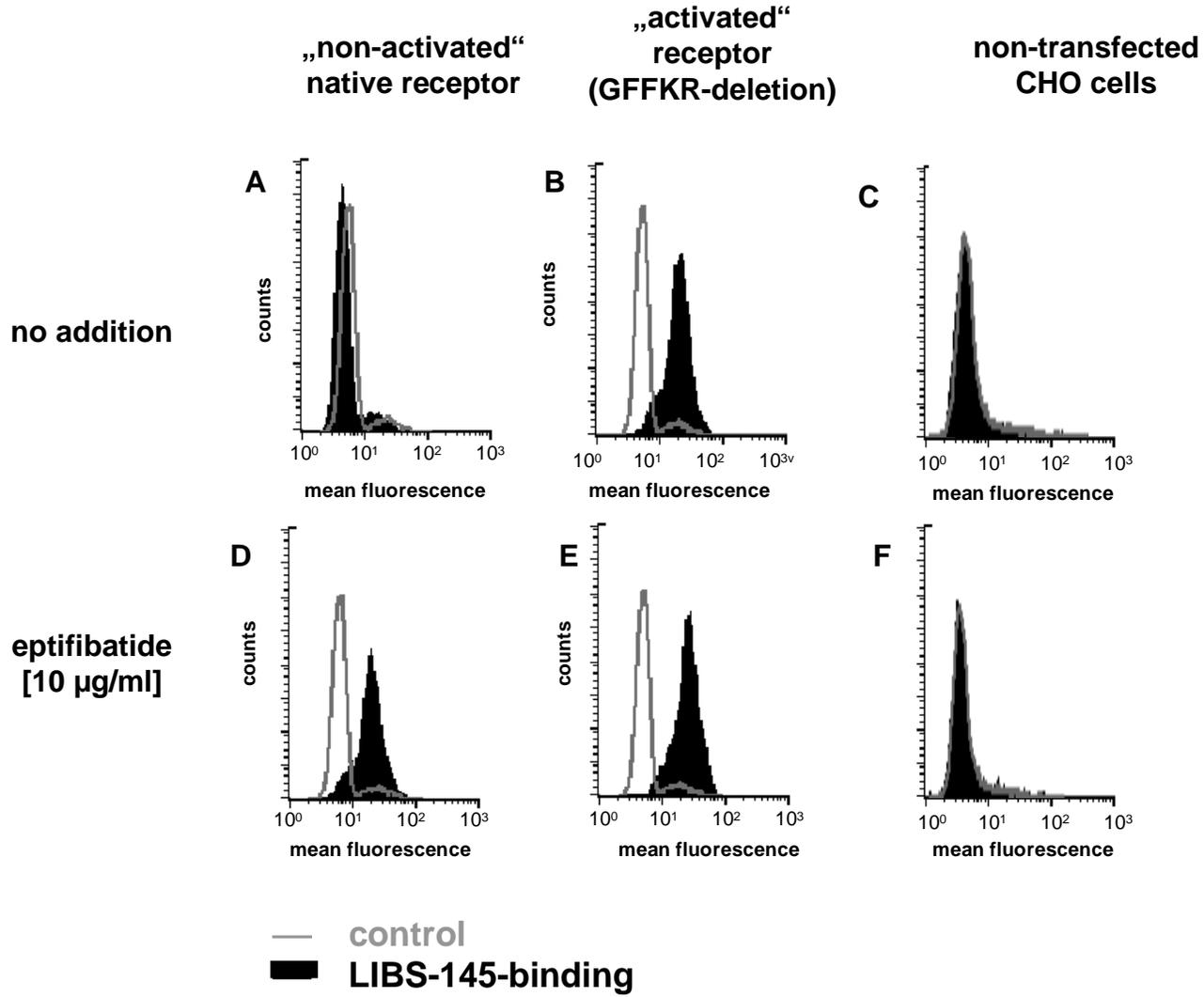
**Fig. 2:**

**LIBS-145 Expression on Platelets**

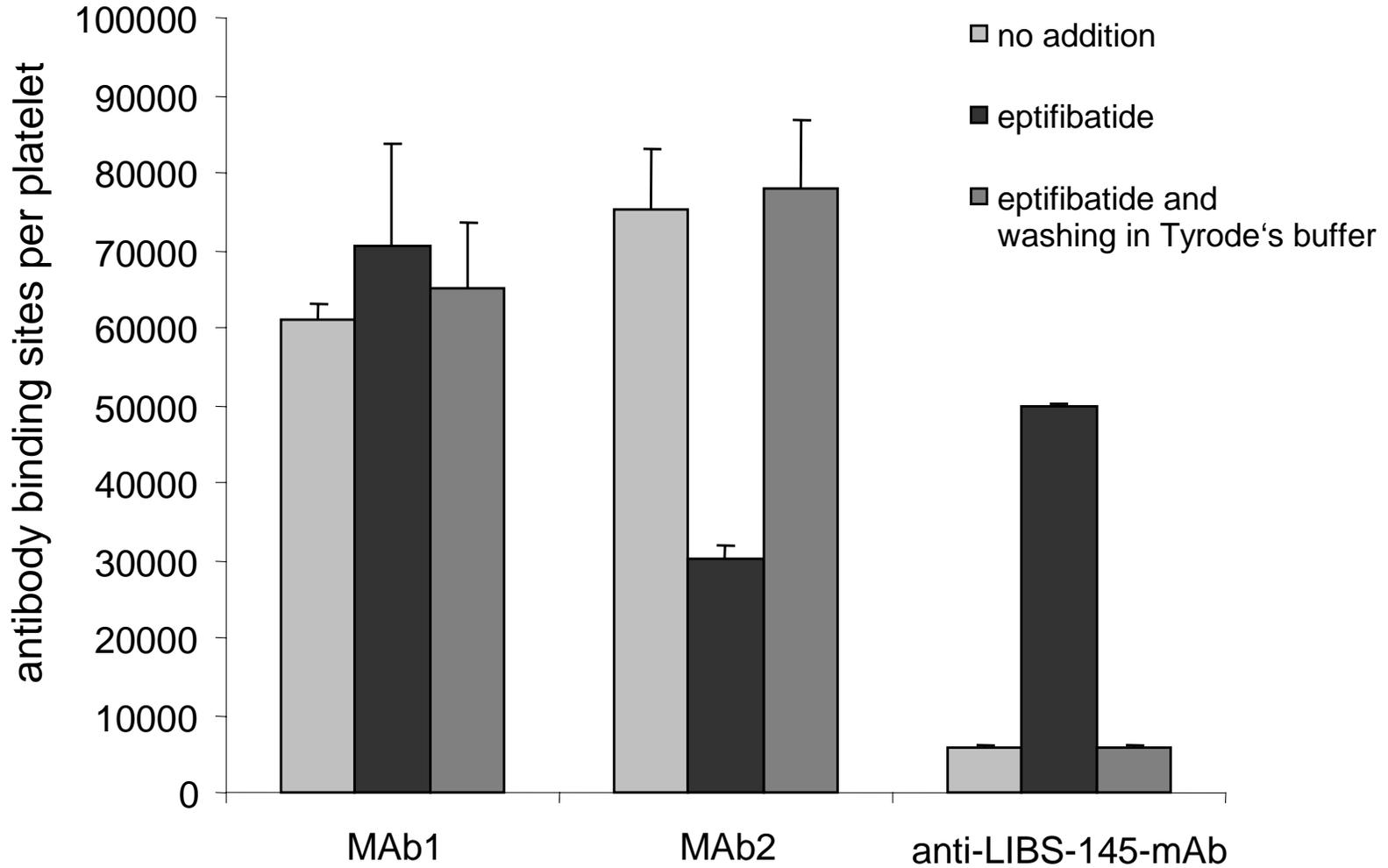


**Fig. 3:**

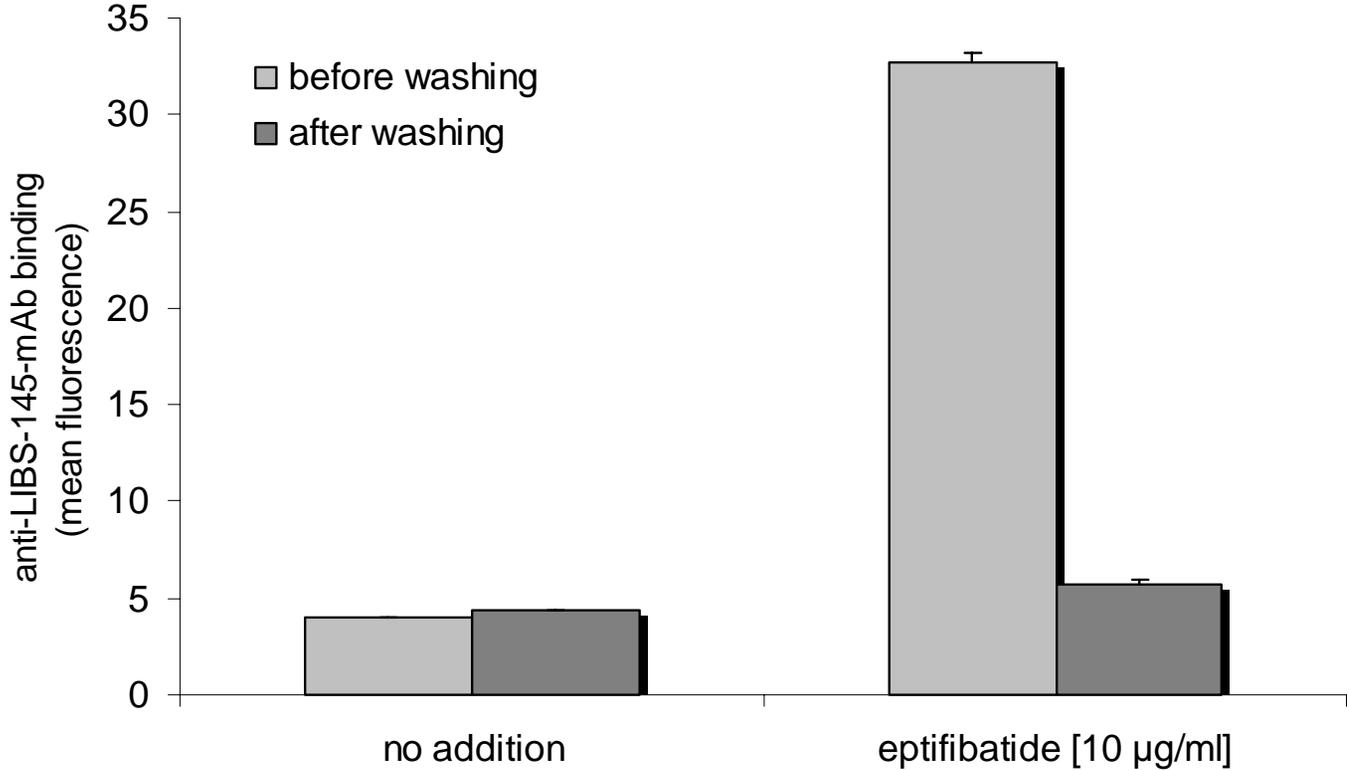
**LIBS-145 Expression on GPIIb/IIIa-expressing CHO Cells**



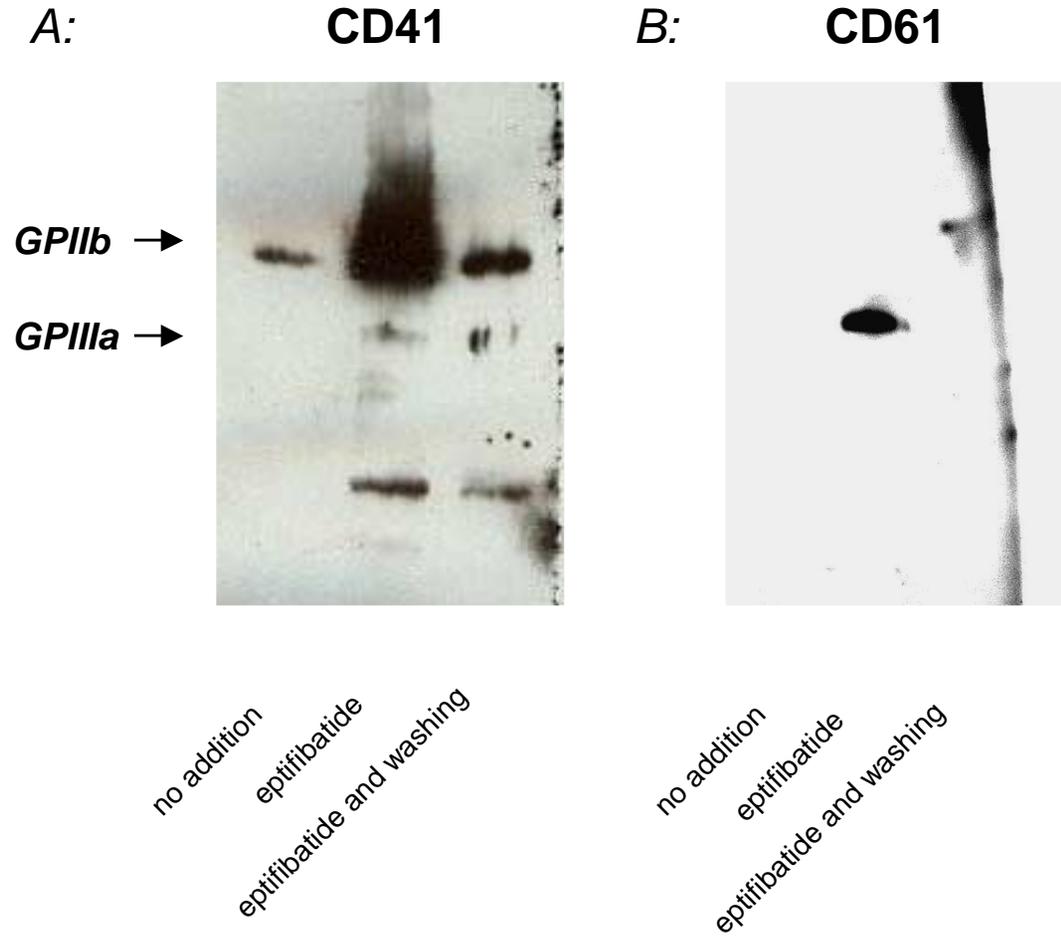
**Fig. 4A:** Reversibility of Eptifibatide-induced Conformational Change of GPIIb/IIIa



**Fig. 4B:** Reversibility of LIBS-145 Expression on GPIIb/IIIa-expressing CHO cells.

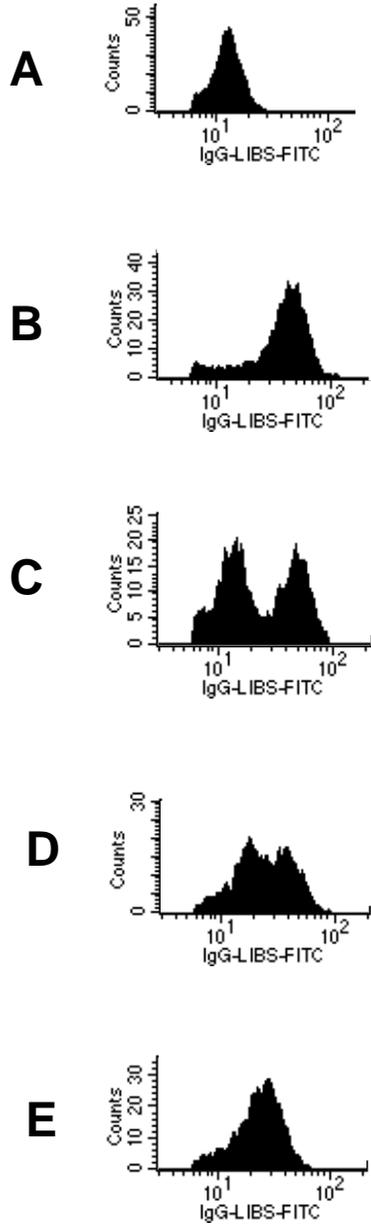


**Fig. 5:** Immunoprecipitation of Platelets with Anti-LIBS-145-mAb

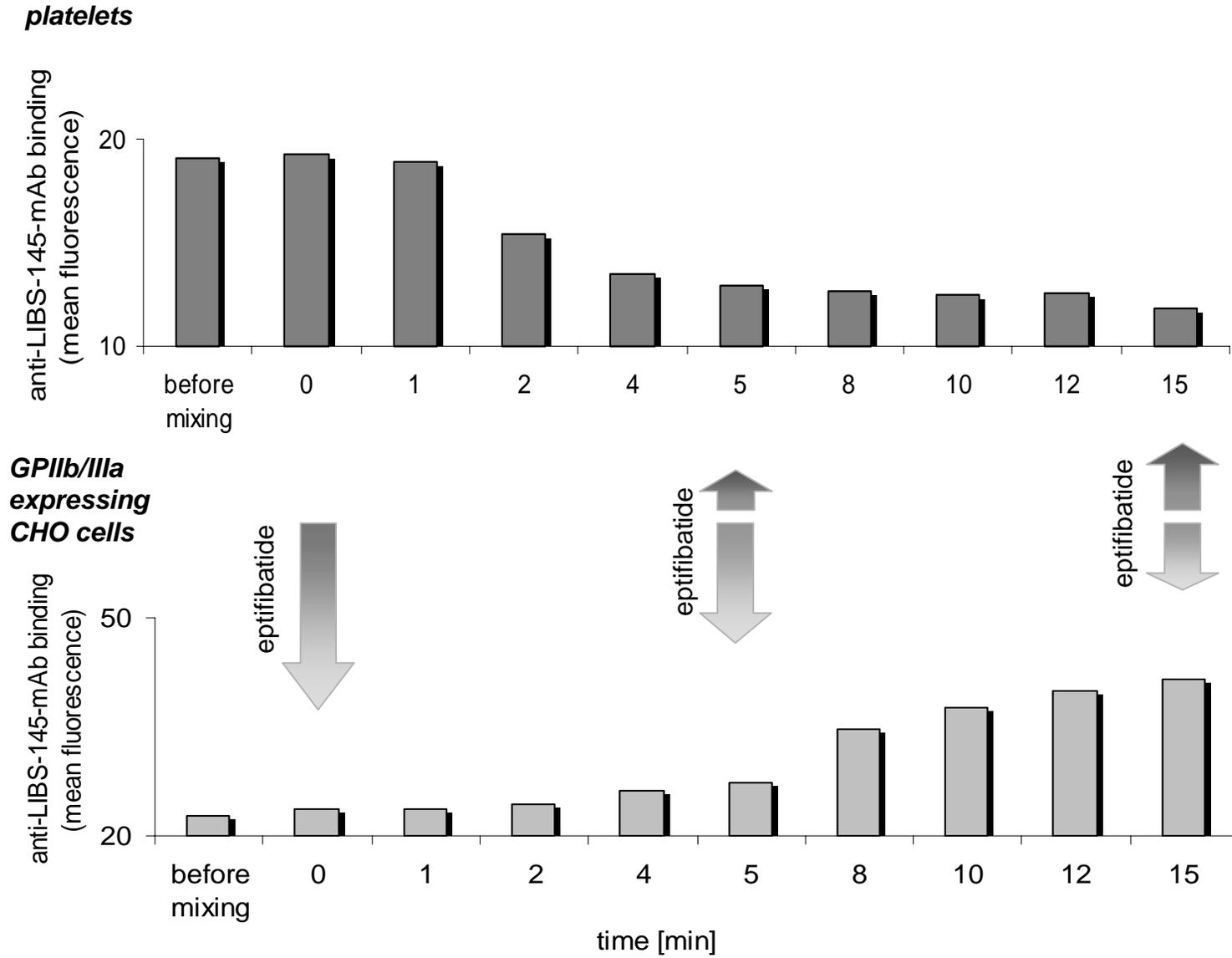


## LIBS-145 Expression after Mixing of Platelets

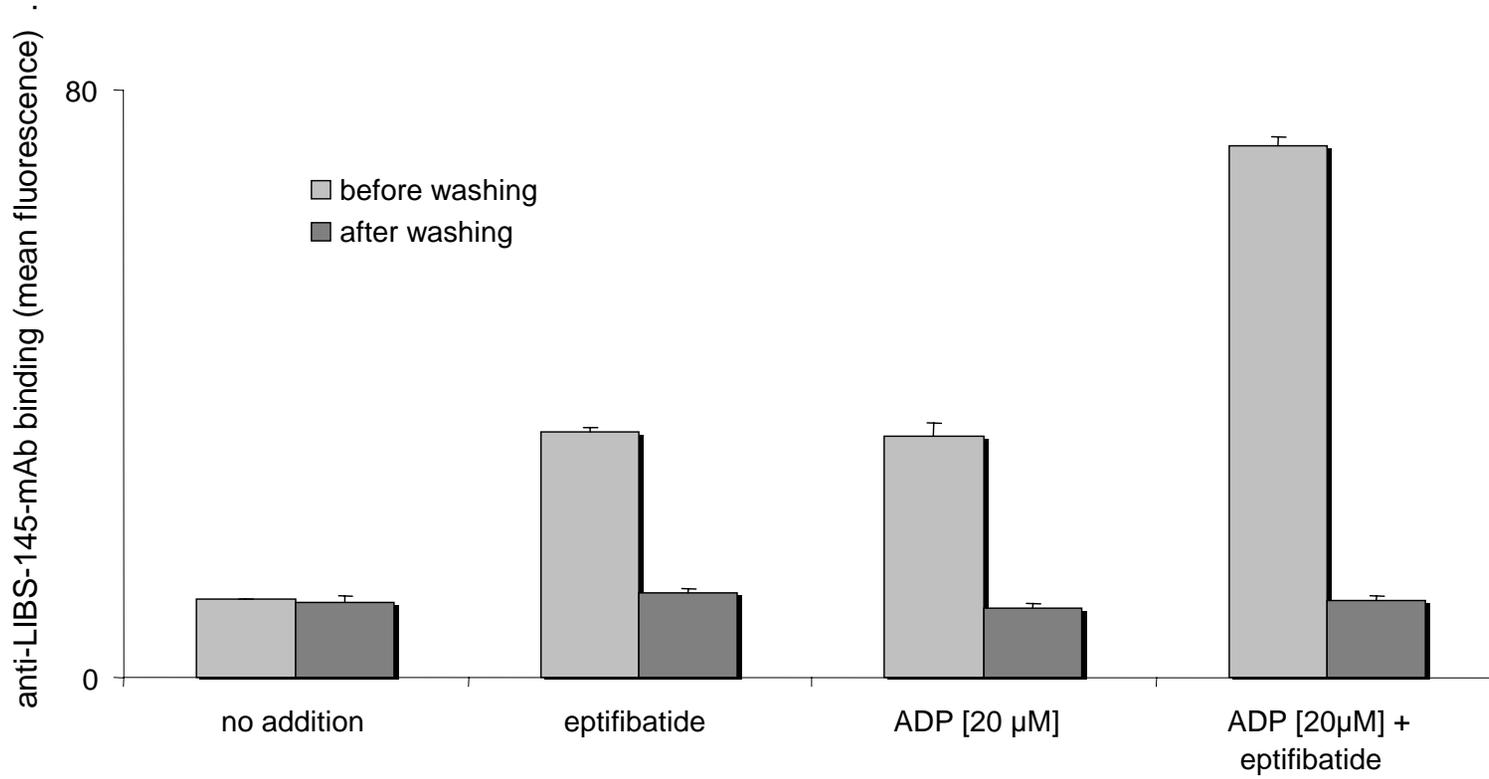
Fig. 6:



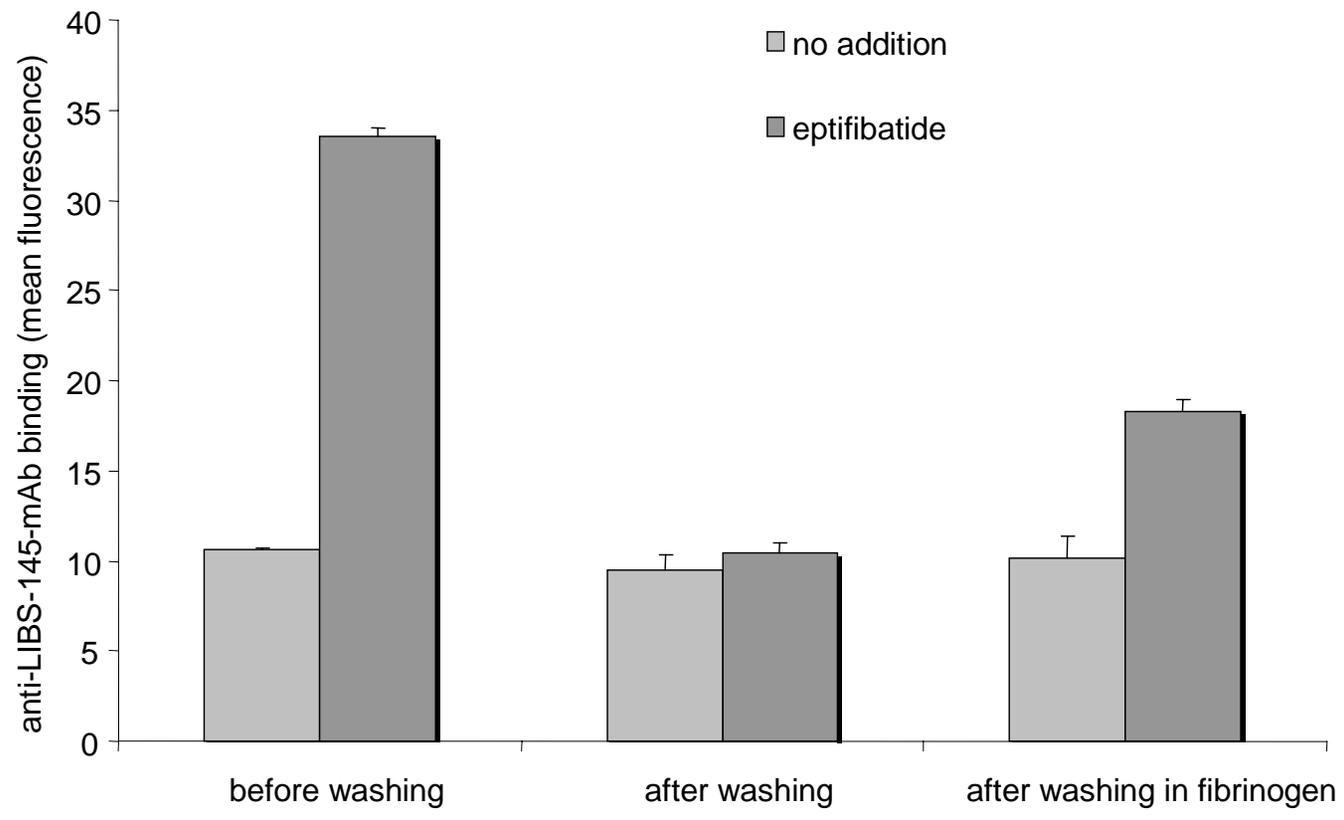
**Fig. 7:** Loss of LIBS-145 Expression after Transfer of Eptifibatide from Preincubated Platelets to GPIIb/IIIa-expressing CHO Cells



**Fig. 8:** Reversibility of LIBS-145 Expression on Resting and ADP-stimulated Human Platelets after Washing in Tyrode's Buffer



**Fig. 9A:** Reversibility of LIBS-145 Expression on Human Platelets after Washing in Tyrode's Buffer Containing Fibrinogen



**Fig. 9B:** Conformational Change Induced by Eptifibatide and Detected by Binding of Fibrinogen and the Activation-specific mAb Pac-1 after Dissociation of the GPIIb/IIIa Blocker

