Biochimica et Biophysica Acta 1840 (2014) 3423–3433

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

# The sweeter aspects of platelet activation: A lectin-based assay reveals agonist-specific glycosylation patterns

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ARTICLE INFO

*Article history:*
Received 14 May 2014
Received in revised form 17 August 2014
Accepted 21 August 2014
Available online 28 August 2014

*Keywords:*
Platelets
Lectins
Platelet activation
Haemostasis
Glycobiology

ABSTRACT

*Background:* The diversity of platelet functions implies multiple activation states arising in response to different stimuli. Distinguishing between these states has been challenging.
*Methods:* We used fluorescently labelled carbohydrate binding proteins lectins to investigate agonist-induced changes in platelet surface glycosylation.
*Results:* Each of the seven agonists we used caused a unique set of changes in platelet surface glycosylation, eliciting a unique functional state. Some of these changes could be correlated with the expression of granule-specific markers CD62P and CD63, but lectins proved much more sensitive to differences between agonists than antibodies against those markers. This sensitivity appears to arise from the relation between the surface glycosylation changes and the signalling pathways through which various agonists act. In this context it is interesting that the effects of calcium ionophore were significantly different from those of other agonists. We also found that that P-selectin (CD62P) contains haptens for lectins VFA and PTII, because these lectins compete with the anti-CD62P antibody binding and vice a versa.
*Conclusions:* We report for the first time that changes in platelet surface glycosylation are agonist-specific and can be distinguished using lectin-binding assays. Lectin fingerprinting represents a new research and diagnostic tool for studying platelet activation.
*General significance:* The observation of agonist-specific platelet surface glycosylation changes is interesting in the context of the diversity of platelet function, because surface glycans mediate contact interactions between platelets and other cells and serve as binding sites for some of the agonists (galectins).


## 1. Introduction

Platelets are known for their critical role in haemostasis and thrombosis [1–4]. Thrombotic complications such as heart attacks and strokes are major causes of death in the developed world [4–7]. Recent findings further implicate platelets in numerous other physiological and pathological processes [8–14]. These include wound healing [9], angiogenesis and de novo blood vessel synthesis [10], tumour growth and cancer metastasis [11,12], as well as adaptive and innate immune responses [13,14]. Their role in atherosclerosis is intimately related to their role in inflammation [6]. Advances in the understanding of platelet regulation already have a tremendous impact on the treatment of cardiovascular disorders that heavily relies on antiplatelet therapy [4,15,16]. They have the potential to similarly transform the treatment of cancers, infections and their complications (e.g., sepsis) [17,18], recalcitrant wounds, and integration of implants.

Platelet activation can be triggered by numerous agonists: ADP, thrombin, thromboxane A2 (TXA2), tissue factor, collagen, collagen-bound von Willebrand factor, etc. Some are produced by the activated platelets themselves, some form as a part of the clotting cascade, while others become exposed at the injury site [1–3]. Most act through more than one receptor [3,19,20]. They elicit changes in platelet shape and size, translocation of phospholipid phosphatidyl serine (PS), which catalyses clot formation, to their surface, conformational change of the integrin GPIIb/IIIa, which is involved in aggregation and adhesion, and exocytosis of intracellular granules, releasing their content and bringing glycoproteins such as CD62P (P-selectin, α-granule marker) and CD63 (dense granule marker) to the platelet surface. Despite these similarities, differences in platelet aggregation and secretion in response to different agonists have been observed [21,22]. Together with the realization that upon activation, platelets secrete over 200 different molecules, some of which have contradictory functions [1,10,23,24], these observations stimulated the search for functional themes in the

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http://dx.doi.org/10.1016/j.bbagen.2014.08.010
0304-4165/

regulation of platelet activation. Indeed, dense and α-granule secretion is regulated via different signalling pathways [25–28]. However, reports of differential packaging and selective secretion of α-granule content that appeared in the literature [23,24,29] have been challenged [30–32]. There currently is no clear picture of how different platelet functional states arise.

Instead of focusing on the traditional surface or soluble markers of platelet activation, we decided to examine platelet surface glycosylation. Platelets, like all cells, are covered with a coat of carbohydrates, originating mostly from cell surface glycoproteins [33]. The chemical nature of this coat changes as platelets age. These changes serve as a signal for platelet clearance by macrophages and hepatocytes [18,34,35]. Changes upon stimulation with thrombin have also been reported [36], as have differences between platelets from healthy individuals and those from uremic or diabetic patients [37,38]. Platelet surface carbohydrates also serve as receptors for galectins—members of the recently discovered class of platelet agonists [39]. There are also reports of platelet activation by plant lectins [40]. In other cells, changes in surface glycosylation have clear functional implications, such as cells turning malignant [41].

We hypothesized that platelet activation would not only result in changes in the surface glycosylation, but that these changes will differ depending on the agonist used to stimulate platelets. To detect these changes, we used fluorescently labelled lectins that specifically bind to certain carbohydrate sequences [42]. To avoid interference from the interactions between glycosylated plasma proteins and lectins, as well as between plasma proteins and platelets, we used purified platelets. In this way, our observations can be directly related to the events that occur in platelets upon activation, such as granule secretion. We therefore picked granule-specific markers CD62P and CD63 to monitor platelet activation. We demonstrate that each agonist leads to a unique lectin-binding "fingerprint". The differences between them correlate with the differences between the signalling pathways used by the agonists. We consider our results to be highly significant for future research into platelet activation as well as for the development of new diagnostic tests.

## 2. Methods

### 2.1. Materials

FITC-conjugated lectins from *Amaranthus caudatus* (ACA), *Bauhinia purpurea alba* (BPL), *Dolicos biflorus* (DBL), *Aleuria aurantia* (AAL), *Datura stramonium* (DSL), *Sambucus nigra* (SNA), *Canavalia ensiformis* (Con A), *Lens culinaris* (LCA), *Maclura pomifera* (MPL), and *Pisum sativum* (PSA) were purchased from Vector Laboratories (Palex Medicals SA, Barcelons, Spain). Lectins from *Psophocarpus tetragonolobus* (PTII), *Vicia faba* (VFA) and *Maackia amurensis* (MAA) were purchased from EY Laboratories (Hycultec GmbH, Germany). Their haptens are listed in Table 1.

Antibodies used in this study (PerCPCy5.5-conjugated anti-CD41a, APC or PE-conjugated anti-CD62P, PE-conjugated anti-CD63, and FITC-conjugated PAC1) were purchased from Becton–Dickinson (Madrid, Spain).

Acid-citrate-dextrose (ACD), Phorbol 12-myristate 13-acetate (PMA), Calcium Ionophore (CaIoP) and Adenosine 5′-diphosphate sodium salt (ADP) were purchased from Sigma (Madrid, Spain). Protease-activated receptor 1 (PAR)-specific agonist peptide (TFLLR-NH2), Protease-activated receptor 4 (PAR4)-specific agonist peptide (AY-NH2) and thromboxane A2 (TXA2)-receptor agonist (U46619) were purchased from Tocris Biosciences (Oxford, United Kingdom). BAPTA was purchased from Invitrogen (Madrid, Spain). All other chemicals were purchased from Sigma (Madrid, Spain).

### 2.2. Blood collection

Blood collection was organized by the Biobanco Vasco para la Investigación (Basque Biobank for Research, Galdakao, Spain) and performed with informed consent according to the appropriate legal and ethical guidelines. Donors were healthy volunteers not exposed to medication (such as aspirin) or alcohol in the two weeks prior to blood extraction. Blood was collected by venipuncture with a 21 gauge needle into sodium citrate anticoagulant (two 5 ml glass Vacutainer® tubes, Becton Dickenson, Madrid, Spain) and stored at 37 °C. First 2 ml of the extracted blood was discarded to avoid platelet activation by residual thrombin.

### 2.3. Platelet isolation and purification

Platelets were isolated ~12 h after blood collection. During that time, blood was stored at room temperature without agitation. This procedure was imposed upon us by the Basque Biobank for Research and the administration of CIC biomaGUNE. Platelets were purified by centrifugation using the protocol described in our previous publication [26]. They were resuspended in the buffer containing 145 mM NaCl, 5 mM Glucose, 1 mM MgCl2, 10 mM HEPES, 5 mM KCl, and pH 7.4. The level of platelet activation and their response to agonists were tested immediately after purification by examining expression levels of the classical activation markers by flow cytometry. The % of the purified platelets expressing activation markers CD62P, CD63, PS, and GPIIb/IIIa was in the range of ~0–8%. Upon stimulation with TRAP or PMA, the % of platelets expressing these markers increased to 80–90% for CD62P, CD63 and GPIIb/IIIa, while PS expression fluctuated between 5 and 61%. Purified platelets were used immediately.

We compared platelet activation and response to agonists in platelets isolated from blood immediately after collection and in platelets isolated from blood stored at room temperature for 24 h and found them to behave in the same way as far as marker expression and response to agonists were concerned (Figure S1 in the Supplementary data and our previous work, ref. [26]). This observation is consistent with literature

**Table 1**
List of plant lectins with their corresponding haptens used in this study.
*Experiments with these lectins were performed in the presence of 1 mM calcium.

| Lectin (source) | Abbr. | Hapten specificity | Ref. |
|---|---|---|---|
| *Amaranthus caudatus* | ACA* | Galβ1-3GalNAcα-Ser/Thr (T-antigen), Siaα2-3Galβ1-3GalNAcα-Ser/Thr (cryptic T-antigen) | [66,67] |
| *Bauhinia purpurea alba* | BPL | Galβ1-3GalNAc, Galβ1-3/4GlcNAc, not to terminal α-GalNAc | [68,69] |
| *Dolicos biflorus* | DBL* | GalNAcα-Ser/Thr (Tn) and GalNAcα1-3GalNAc | [70–72] |
| *Aleuria aurantia* | AAL* | Fucose (Fucα1-6,2,3,4GlcNAc)-both core and peripheral fucose | [73–75] |
| *Maackia amurensis* | MAA | Siaα2-3Galβ1 | [76–78] |
| *Sambucus nigra* | SNA | Siaα 2-6Galβ1-4Glc(NAc) | [55,73,77,78] |
| *Pisum sativum* | PSA* | Fucα1-6GlcNAc (core fucose), αMan, αGlc, GlcNAcα | [75,79] |
| *Psophocarpus tetragonolobus* | PTII | D-GalNAcα-(1-3)DGal(β1-3/4)Glc, (α-GalNAc, D-GalNAcα-(1-3)DGal), preference for α over β | [80,81] |
| *Vicia faba* | VFA | Manα | [79] |

reports where platelet activation in platelets prepared from freshly drawn and stored blood was compared [43].

### 2.4. Lectin binding studies

Freshly isolated platelets were either treated with one of the agonists (listed in Table 2 along with the concentrations used ), 50 μM BAPTA-AM, or left untreated. They were then incubated with FITC labelled lectins (20 μg/ml, listed in Table 1) for 30 min at 37° C and analysed by FACS. Platelet concentration was kept at ~$50 \times 10^3$ platelets per μl in all samples (final concentration after the addition of the lectin solution). Measurements were repeated at least three times with blood from different donors.

The effect of lectins on CD62P expression by platelets was determined by staining the FITC-lectin treated platelets with APC-anti-CD62P antibody for 30 min at 37 °C, followed by analysis with flow cytometry.

## 3. Results

### 3.1. Lectin fingerprinting of platelet agonists

Lectins are proteins that bind with high affinity to sugar moieties containing specific oligosaccharide structures [42]. They are ubiquitously expressed in plants, animals, and microorganisms and have been commonly used as tools for studying protein glycosylation and for profiling cell surface carbohydrates [42]. In this study, we used nine fluorescently labelled plant lectins to profile changes in platelet surface glycosylation upon treatment with various agonists. The lectins we used, together with their haptens, are listed in Table 1. Agonists included thrombin, PAR1 and PAR4 receptor agonist peptides (TFLLRN and AY-$NH_2$) that mimic thrombin's action, ADP, TXA2, calcium ionophore (CaIoP), and phorbol 12-myristate 13-acetate (PMA). They are listed in Table 2 along with the concentrations that were used in the experiments.

Prior to the lectin binding experiments, platelet activation state and response to agonists were evaluated by flow cytometry as described in Materials and Methods and in our previous publication [26]. Platelets were used in lectin-binding experiments only if their basal activation levels were below 8% while levels of expression upon treatment with agonists were above 80%, where the values refer to the percentage of platelets expressing the activation markers.

The extent of lectin binding to the resting and the agonist-treated platelets was determined by flow cytometry (Fig. 1). Depending on the agonist and the lectin, mean fluorescence intensity (MFI) of the platelets was found to remain unchanged, increase, or decrease, relative to that of the resting platelets (states ii, iii, or iv, respectively, in Fig. 1A). Responses to different agonists could be distinguished: treating platelets with thrombin did not lead to a change in the ACA binding, while treating them with its analogue PAR1 agonist led to an increase in the level of ACA binding, and with calcium ionophore—to a decrease (Fig. 1A, n = 4). The raw flow cytometry data for other lectins is presented in Figure S2 in the Supplementary data. The conditions for the lectin binding experiments were selected by examining the dependence of the MFI on the incubation time (Fig. 2A) and lectin concentration (Fig. 2B).

For each lectin and agonist, measurements such as those shown in Fig. 1A were repeated with blood from three to five different donors. In order to account for the donor-to-donor variability, the MFI of the agonist-treated platelets was divided by that of the resting platelets from the same donor, giving fold-changes in lectin binding. The average values of the fold-changes, obtained from three to five experiments performed with blood from different donors, are plotted in Fig. 1B, along with the standard deviations that represent donor-to-donor variations. Statistically significant differences ($p^* < 0.05$, n = 3–5) in the extent of lectin binding to different agonist-treated platelets relative to resting platelets were observed across the donor pool. (We also show, in Figure S3 in the Supplementary data, that the variation in the extent of lectin binding to platelets isolated from different donors was reassuringly small).

These data are more conveniently represented as a heat-map, where statistically significant increases or decreases in lectin binding are represented with different colours (Fig. 3A). Examining Fig. 3A reveals that each agonist affects lectin binding in a unique fashion, resulting in a "fingerprint". It is noteworthy that lectins are far more sensitive to differences between agonists than the classical activation markers CD62P and CD63 (Fig. 3B).

Further insight can be gained by examining how the changes in lectin binding relate to CD62P and CD63 expression levels. In particular, there is a correlation between changes in the binding of PSA and ACA and the level of expression of CD62P (Fig. 3C; plots for the other lectins are shown in Figure S4 in the Supplementary data), for all agonists except CaIoP. A similar correlation could be discerned in the case of MAA (Figure S4 in the Supplementary data). CaIoP exerted significantly different effects than physiological agonists (Fig. 3C). PMA, on the other hand, exerted effects that were rather similar to those of the physiological agonists. No correlation between changes in the binding of other lectins (AAL, BPL, DBL, SNA, VFA, and PTII) and the level of CD62P expression could be discerned.

With respect to CD63, there was an abrupt change in the level of its expression between ADP-treated platelets and platelets treated with all other agonist except CaIoP (Fig. 3D), and a corresponding change in the binding of DBL and BPL (Fig. 3A). Treatment with CaIoP once again led to discordant behaviour.

It is important to keep in mind that the heatmap shown in Fig. 3A incorporates information about how reproducible the measurements with a particular agonist and lectin were across the donor pool. This is because the colouring of the heatmap depends on the statistical significance of the fold-changes, which, in turn, depends on the variability. For example, there is no statistically significant change in the binding of PTII to PMA-treated platelets relative to the resting platelets (Fig. 3A). Examination of the relevant plot in Figure S4A in Supplementary data shows that this is due to the poor reproducibility of the measurements done with this lectin with PMA-treated platelets.

**Table 2**
List of agonists used in this study and their concentrations.
Agonists used and their concentrations, selected based on the literature studies indicated. Higher-than-usual ADP concentration was probably due to blood storage prior to the experiments (see Materials and Methods). Concentrations of other agonists are well within the accepted ranges. *Experiments with these agonists were performed in the presence of 1 mM extracellular calcium.

| Agonist | Concentration | Ref. |
|---|---|---|
| PAR1 peptide | 100 μM | [82] |
| PAR4 peptide | 150 μM | [22] |
| Thrombin | 10 U/ml | [83,84] |
| ADP* | 100 μM | [50,85] |
| TXA2 | 10 μM | [83] |
| PMA | 10 μM | [83] |
| CaIoP* | 5 μM | [58,59] |

### 3.2. Platelet activation by lectins and competition between lectin and antibody binding

Some of the plant lectins can activate platelets [39,40]. We therefore examined the expression of a common platelet activation marker, CD62P, upon treatment of platelets with the lectins. The results of these experiments are shown in Fig. 4. Platelets treated with the PAR1 agonist were used as a positive control, as they always express CD62P. Platelets treated with BAPTA-AM, a membrane-permeable calcium chelator that prevents platelet activation upon agonist stimulation, were used as a negative control, because they do not express CD62P even upon agonist stimulation. Fig. 4 shows that treating platelets with DBL

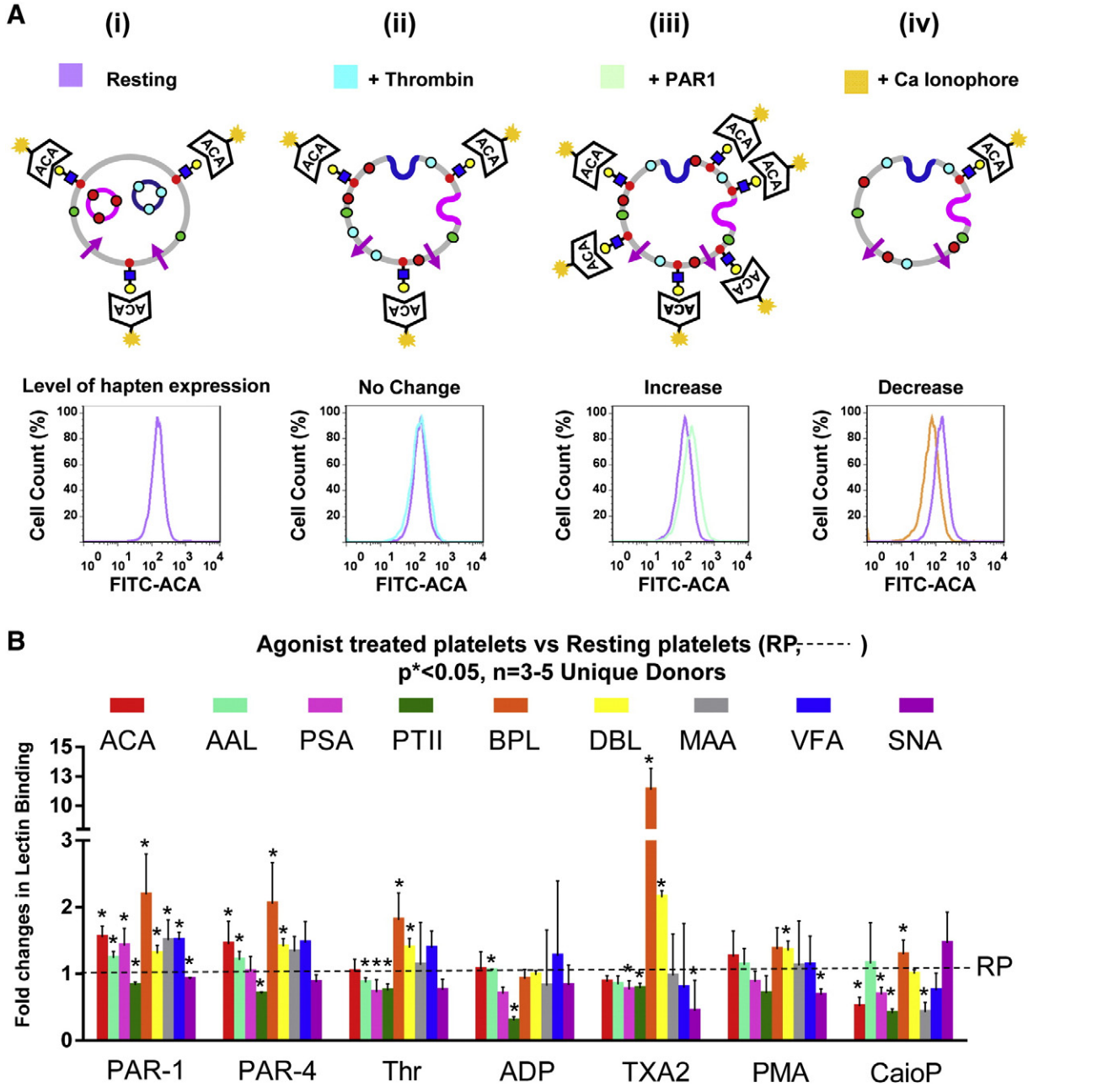



**Fig. 1.** Profiling platelet surface glycosylation changes with lectins. (A) A schematic representation of the flow cytometry experiments. Resting platelets (i) or platelets treated with different agonists (thrombin (ii), PAR1 (iii) and calcium ionophore (iv)) were incubated with FITC-labelled lectins (in this example, ACA). Mean fluorescence intensity (MFI) of the agonist-treated platelets was compared with that of the resting platelets. The plots show representative results of individual experiments. The data for other lectins and agonists is presented in Figure S2 in the Supplementary data. Platelet membrane is shown in grey. Green spheres refer to GPIIb-IIIa integrin in the inactive conformation on the resting platelets. Green ellipses designate the active conformation of this protein. Granules (α, purple, and dense, brown), with their respective markers (glycoproteins CD62P, red, and CD63, turquoise) are shown inside the resting platelet. They undergo exocytosis upon activation, and the two markers appear on the platelet membrane. Purple arrowheads represent phosphatidyl serine (PS)—a phospholipid that catalyses clot formation. It is asymmetrically distributed (facing inwards) in the membranes of resting platelets, but undergoes a re-orientation upon activation with some of the agonists. Orange circles in the platelet membrane represent glycoproteins with the carbohydrate moieties of these glycoproteins projecting outside the membrane. Yellow coloured circles: Galactose (Gal); blue squares: N-acetylglucosamine (GlcNAc). (B) Quantification of the flow cytometry results, such as the ones shown in (A). The results are represented as fold changes of MFI of the agonist-treated platelets over the resting platelets; means of three to five independent experiments ± SD are shown. Each of these independent experiments was performed with blood from a different donor; SD's represent donor-to-donor variation. Note, that although the histograms in (A) are plotted with the fluorescence intensity on the log scale, it was the absolute values of the MFI that were divided to obtain the fold changes. Statistical significance was evaluated using a 1-way ANOVA test with *$p < 0.05$ vs. resting platelets (RP), $n = 3–5$. These results show that the pattern of lectin binding to platelets depends on the agonist used to activate them.

or BPL did not lead to positive staining with the anti-CD62P antibody, while treating platelets with ACA or PSA lectins led to some staining. None of these four lectins interfered with the ability of the antibody to bind to CD62P in platelets treated with the PAR1 agonist, since the levels of antibody binding were similar with and without the lectins. We conclude that DBL and BPL do not activate platelets, while ACA and PSA activated platelets to a limited extent. SNA and AAL lectins interfered with the detection of CD62P expression in the PAR1 agonist-treated platelets (see the green and dashed purple peaks in Fig. 4). Because no binding of anti-CD62P antibody could be detected when PAR1-activated platelets were treated with lectins from this group, it is not possible to ascertain whether these lectins activated platelets or not, but we view this possibility as unlikely because of the results obtained with the lectins from the third

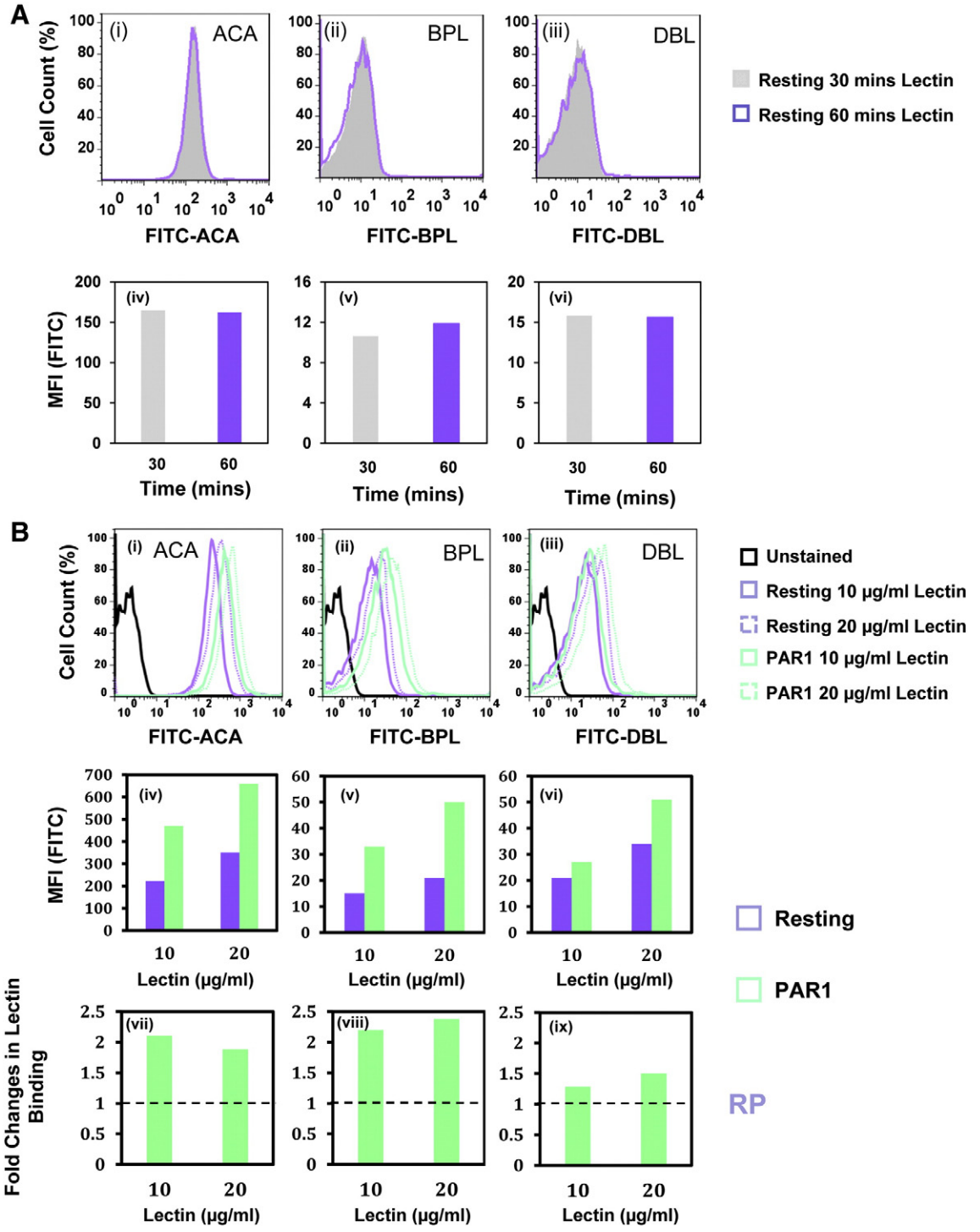
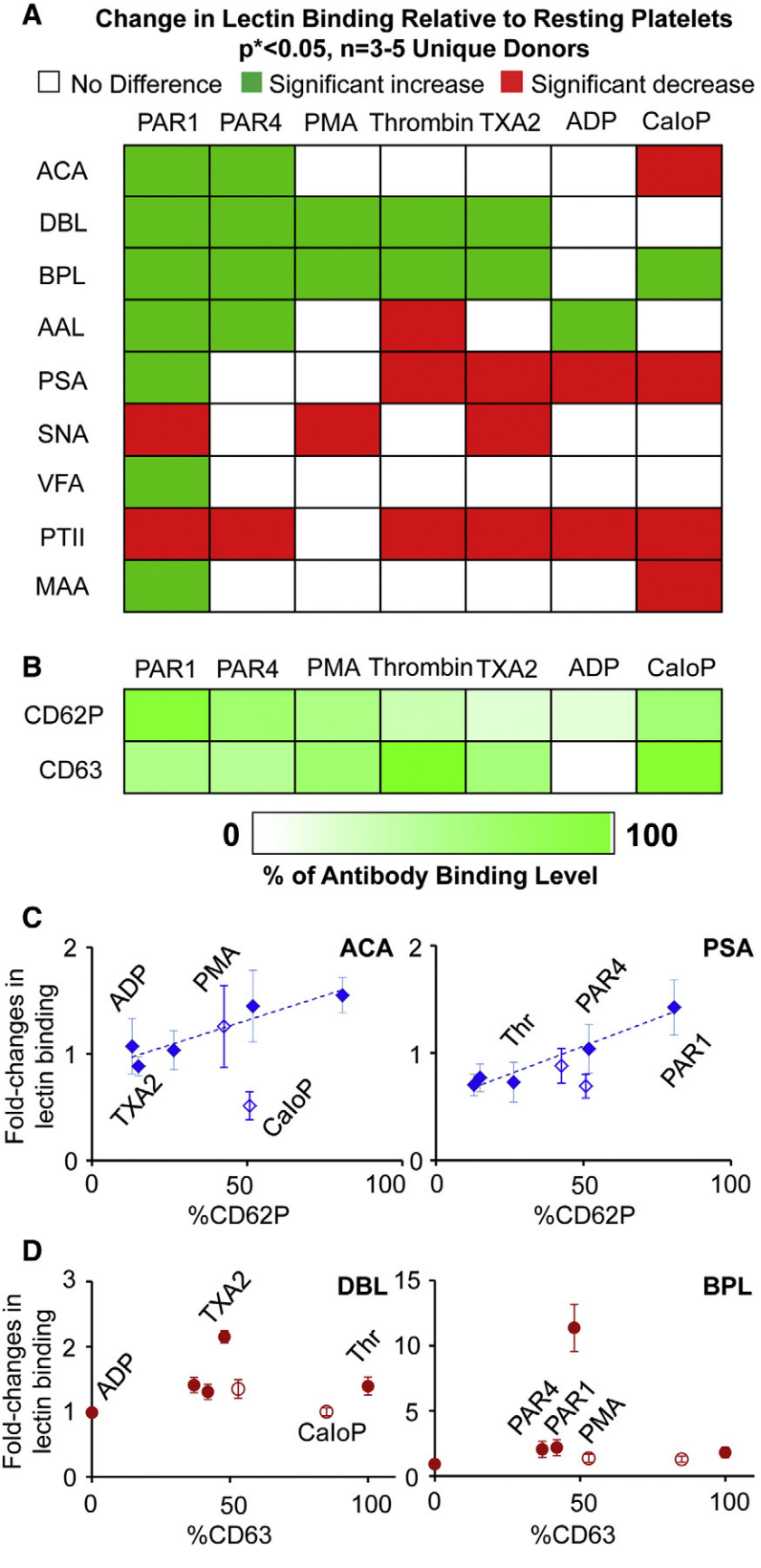



**Fig. 2.** Time and dose response of lectin binding to platelets. (A) Resting platelets were incubated with 20 µg of lectin (ACA, BPL and DBL) for 30 and 60 min and their mean fluorescence intensity (MFI) was determined with flow cytometry. Lectin abbreviation (Table 1) is indicated on each histogram. These results show that binding of lectins to platelets is the same after 30 min (grey) and 60 min (purple). These are representative plots for two independent experiments. (B) Resting platelets and PAR-1 treated platelets were incubated with 10 µg and 20 µg of lectins (ACA, BPL and DBL) for 30 min and their mean fluorescence intensity (MFI) was determined using flow cytometry. Purple colour refers to the resting platelets (RP), while green colour refers to the PAR1 treated platelets. Solid lines—10 µg lectin; dashed—20 µg. The results show that binding levels of lectins to platelets increase with increasing concentration for both resting and PAR1 treated platelets, but the fold-changes (MFI[treated platelets] / MFI[resting platelets]) are independent of the lectin concentration in this concentration range. These are representative plots for 2 independent experiments.

group. This group consisted of VFA, PTII, and MAA, which induced CD62P expression in platelets and interfered with the binding of anti-CD62P antibody to the PAR1-agonist treated platelets. These lectins activate platelets, and in fact, they are not very efficient in distinguishing between agonists (c.f. Fig. 3A).

To better understand the nature of interference between the lectins and the anti-CD62P antibody, we checked whether the reverse was true: whether antibody binding to CD62P affected lectin binding. This was done by comparing lectin binding to platelets previously incubated with the anti-CD62P antibody with that to platelets that were not

incubated with it. The results are shown in Fig. 5. The binding of AAL and SNA was not affected by the anti-CD62P antibody binding (Fig. 5A, B), while the binding of VFA and PTII was. We conclude that CD62P carries glycans containing the haptens for VFA and PTII (Table 1), but not AAL or SNA. Therefore the interference between the binding of AAL, SNA, and the anti-CD62P antibody to the platelets must have a different ori-



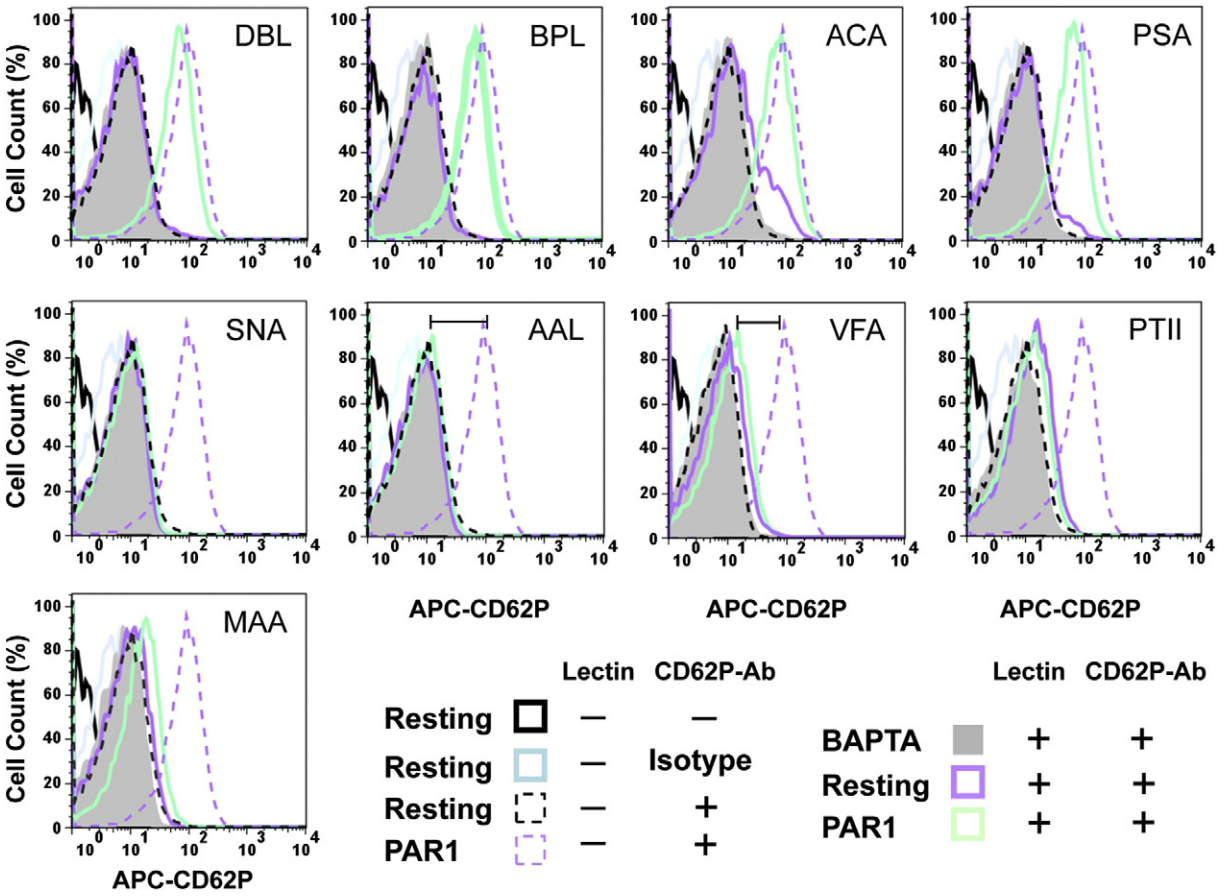



**Fig. 4.** Platelet activation by lectins. Platelets (resting, treated with BAPTA, or with PAR1 agonist) were incubated with one of the nine lectins and then stained with the APC-anti-CD62P antibody as shown in the legend. Surface expression of CD62P marker was evaluated by flow cytometry. Dashed purple and solid green peaks indicate positive controls (PAR1 ± lectin + CD62P). If these two peaks overlap, platelet activation can be judged from the solid purple peak (lectin + CD62P). If these two peaks do not overlap, lectin binding interferes with the binding of the anti-CD62P antibody, and level of platelet activation cannot be ascertained. In the case of VFA, PTII and MAA lectins, there is a further shift in the solid purple and solid green peaks relative to the isotype, indicating both interference between the lectin and the antibody binding and platelet activation by the lectin. These are representative plots of three independent experiments.

gin. These lectins may cause glycoproteins to reorganize in the membrane, hindering the ability of the antibody to bind to CD62P.

## 4. Discussion

The main finding of our work is that changes in platelet surface glycosylation are agonist-specific. To the best of our knowledge, this is the first such observation in platelets, although there are well-documented examples of agonist-specific aggregation and secretion patterns [21,22], and reports of activation-related platelet surface glycosylation changes (e.g., in chilled platelets [34,35,44]).

Stimuli-specific glycosylation changes have been reported in other cells: stimulation of B-cells with different agonists—interleukin (IL)-21 and all-trans retinoic acid (ATRA, a natural metabolite of vitamin A)—leads to different glycosylation patterns of the Fc region of the IgG1 antibodies produced by these cells [45]: IL-21 treatment lead to an increase in their galactosylation and sialylation, while ATRA treatment led to a decrease. Increased galactosylation and sialylation was linked to anti-inflammatory activity of the IgG and vice versa [45]. Similarly, specific patterns of carbohydrates were observed on endothelial cells from different organs, providing "zip-codes" for organ-specific leukocyte traffic [46]. Altogether, these observations in diverse systems suggest that nature uses the substantial combinatorial complexity of carbohydrates to encode subtle differences between otherwise similar cells or molecules. They also provide a starting point for the design of therapeutics targeted to specific platelet activation states.

In the following, we relate surface glycosylation changes to other platelet responses, such as granule exocytosis. In washed, unstirred platelets, ADP does not cause TXA2 production or granule exocytosis, because these effects are aggregation-dependent [47–50]. Indeed, we see in Fig. 3B and D that in contrast to platelets treated with other agonists, including TXA2 [51], ADP treated platelets do not express CD63. The corresponding difference in the binding of DBL and BPL between ADP-treated platelets and platelets treated with all other agonists

**Fig. 3.** Agonist-specific changes in the platelet surface lectin and antibody binding profiles. (A) A three-colour heat map depicting a significant increase (green), a significant decrease (red), or no change (white), in the binding of the respective lectin to platelets treated with the respective agonist as compared with that to the resting platelets. Statistical significance ($p^* < 0.05$, $n = 3–5$) was evaluated using a 1-way ANOVA test run on the lectin binding fold changes (MFI(agonist-treated platelets + lectin) / MFI(resting platelets + lectin)) relative to 1 [MFI(resting platelets + lectin) / MFI(resting platelets + lectin)], obtained from three to five separate experiments for each agonist/lectin pair performed with blood from different donors. (B) The expression of two common platelet activation markers, CD62P and CD63, in response to agonist treatment, evaluated by measuring the level of binding of respective antibodies. Maximum antibody binding for each marker (PAR1 for CD62P and thrombin for CD63) was taken as 100%. Average results of three to five experiments are shown. Marker expression levels depend on the agonist. Agonist concentrations used in these experiments are shown in Table 2. They were picked to induce the maximum response for each agonist. In (A) and (B), the agonists are placed, left to right, in the order of decreasing CD62P expression (from PAR1 to ADP). (C) Fold-changes in lectin binding are plotted as a function of CD62P expression level for ACA and PSA lectins. Lines are linear regression fits with correlation coefficients of 0.94 and 0.97, respectively. (D) Fold-changes in lectin binding are plotted as a function of CD63 for DBL and BPL lectins. Solid symbols in (C) and (D) refer to physiological agonists, while open symbols refer to PMA and CaIoP. Agonists are indicated in the plots. Their order in the two subfigures reflects the level of expression of each marker.

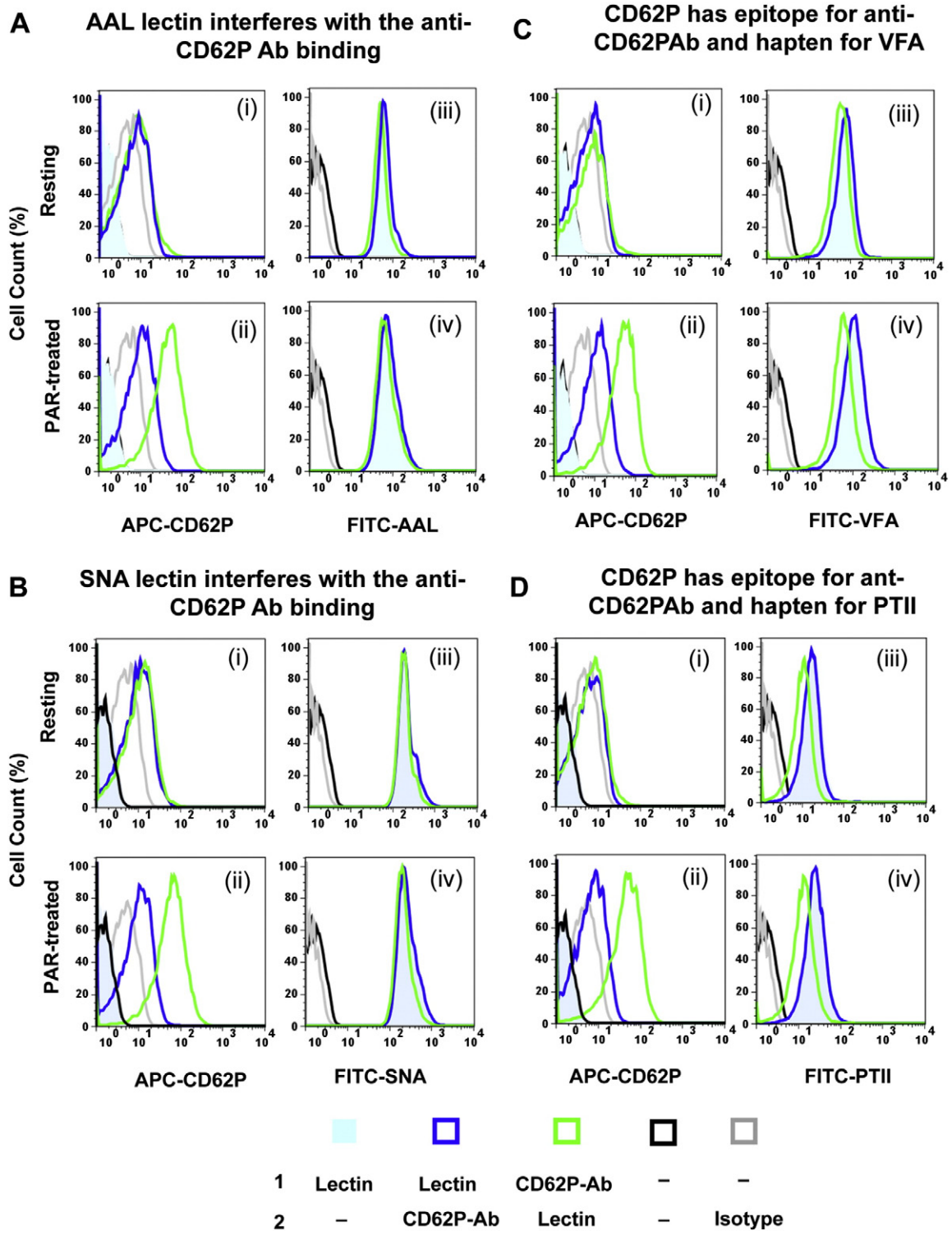



**Fig. 5.** Interference between lectin and antibody binding to CD62P. Resting or PAR1 agonist-treated platelets were treated with FITC-labelled AAL (A), SNA (B), VFA (C), or PTII (D) and the APC-anti-CD62P antibody. Antibody treatment either followed (dark blue) or preceded (green) lectin treatment. Note, how in the case of AAL and SNA, the lectins affect antibody binding (evident from the difference between the solid blue and the solid green curves in panels Aii and Bii), but not vice-a-versa (evident from the overlap between the solid green and solid blue curves in panels Aiv and Biv). In the case of VFA and PTII, the antibody binding also affects the lectin binding. These are representative plots for three independent experiments.

except CaIoP (Fig. 3A) is significant because DBL and BPL haptens contain terminal β-galactose and GalNac residues, respectively (Table 1), which are responsible for the clearance of activated or damaged platelets by the hepatocyte Ashwell-Morell receptors [52].

The observation of CD62P expression in ADP- and TXA2-treated platelets is surprising. Its level is smaller than that in platelets treated with other agonists (Fig. 3B, D). Unfortunately previous studies examining the response of washed, unstirred platelets to ADP focused on ATP release from dense granules [47–50], and therefore they do not shed light on the origin of CD62P expressed in ADP-treated, washed, unstirred platelets. Independently of its origin, CD62P expression in TXA2- and ADP-treated platelets is similar, indicating that there is no difference in α-granule exocytosis between platelets treated with these two agonists. Therefore, differences in glycosylation between

ADP- and TXA2-treated platelets correlate with dense granule exocytosis, which appears to mark platelets for clearance.

Other agonists induce aggregation-independent exocytosis of both sets of granules and therefore significant levels of expression of both CD62P and CD63 (Fig. 3B, C, D). PAR1 agonist induced the highest level of CD62P expression (Fig. 3B) as well as most significant changes in lectin binding (Fig. 3A): an increase in the binding of ACA, AAL, PSA, VFA and MAA and a decrease in the binding of SNA. Since PAR1 treatment leads to ADP release and TXA2 production, the increase in PSA binding to PAR1-treated platelets counteracts its decrease caused by ADP and TXA2. Other examples of such agonist interactions include differences in the binding of AAL between ADP, TXA2, PAR1, and PAR4 treated platelets.

PAR4 agonist led to the second-highest level of CD62P expression (Fig. 3B, C) and correspondingly less pronounced effects on the lectin binding than the PAR1 agonist (Fig. 3A): an increase in the binding of ACA and AAL, but no change in the binding of PSA. Since treating platelets with ADP or TXA2 leads to a decrease in the binding of PSA, this means that PAR4 caused a smaller increase than PAR1. This is confirmed by the plot shown in Fig. 3C. The same explanation applies to the binding of VFA and MAA: where there was an increase in binding upon PAR1 treatment, there is no change upon PAR4 treatment. The binding of SNA shows no difference between PAR4-treated platelets and resting platelets, whereas a decrease in its binding was observed upon treatment with PAR1 agonist, PMA, and TXA2.

The level of CD62P expression (Fig. 3B, C) and the extent of lectin binding changes (Fig. 3A, B) both suggest that PAR4 agonist is weaker than PAR1 agonist. There is indeed support in the literature for such a conclusion [53]. However, the relationship between PAR1 and PAR4 activation pathways is more complex [54]. The response induced via PAR1 is rapid but transient, while the response induced via PAR4 is delayed but sustained; they are characterized by different kinetics of intracellular calcium changes [54]. The differences in glycosylation we observe may, therefore, reflect different stages of platelet activation: earlier with PAR1 and later with PAR4. Some evidence for this possibility is provided by the effect of thrombin.

We found it curious that the changes in lectin binding induced by thrombin were strikingly different from those induced by PAR1 and PAR4 despite the fact that these two agonists activate the two corresponding thrombin receptors [19,55]. These changes were, however, consistent with the levels of CD63 and CD62P expressed in thrombin-treated platelets (Fig. 3B, C, D). The effects of thrombin that are not mediated by PAR1 or PAR4 receptors [56,57] may offer partial explanation for this puzzling behaviour. Another possibility is that we are observing different stages in a sequence of events triggered by PAR1, PAR4, and thrombin. This is tentatively supported by the observation that persistent stimulation by thrombin is required to sustain PAR4-mediated effects [54].

PMA, for the most part, behaved as a weaker version of PAR1 and PAR4, but stronger than thrombin—an effect that correlates with the level of CD62P expression it induces (Fig. 3C, and Figure S4 in the Supplementary data). Some differences could be noted (e.g., a decrease in the SNA binding, Fig. 3A).

The effects of CaIoP on platelet surface glycosylation were strikingly different from those of all other agonists and were not correlated to the levels of CD62P or CD63 expression. It is the only agonist that led to a decrease in the ACA and MAA binding, and that affected the binding of DBL but not BPL (Fig. 3A). This places CaIoP in a category of its own. This is consistent with its very different mechanism of action: CaIoP does not act through the common receptor pathways but short-circuits them by directly increasing calcium intracellular level. It also affects the platelet membrane structure, although at the concentration we used in our study (5 μM, Table 2), it is not expected to damage platelets [58,59].

In summary, agonist-induced changes in lectin binding could be grouped into four distinct classes. Changes in the first class correlated with the exocytosis of dense granules in response to all agonists except CaIoP. This class includes DBL and BPL lectins, the changes in the binding of which allow ADP to be differentiated from all other agonists. Another set of changes correlated with CD62P expression, and therefore α-granule exocytosis, in platelets treated with all agonists except CaIoP. This set includes ACA, PSA and MAA. The third class of changes did not correlate with the expression of either of the two markers. This set includes PTII, a decrease in the binding of which was observed for all agonists except PMA; changes in the binding of AAL and SNA that varied between agonists; and changes in the binding of VFA that were specific to PAR1. The fourth class of changes was characteristic of treatment with CaIoP.

This classification allows us to formulate hypotheses regarding the mechanisms underlying agonist-induced platelet surface glycosylation changes. Possible mechanisms are schematically shown in Figure S5 in the Supplementary data. The simplest one involves transfer of the granule membrane glycoproteins to the platelet surface. It can be invoked to explain the two classes of glycosylation changes that correlate with granule exocytosis. However, transfer of granule membrane glycoproteins to the platelet surface cannot explain why CaIoP effects are different from those of the other agonists even though the levels of CD62P and CD63 expression evoked by this agonist are similar to those evoked by other agonists. Similarly, specific agonist effects on the binding of several of the lectins cannot be explained through transfer of granule material to the platelet surface. Other mechanisms underlying changes in platelet surface glycosylation must be at play. They include enzymatic modification of platelet surface glycans by glycosyltransferases [60] activated as a part of specific signalling cascades, glycoprotein internalization, and changes in the platelet membrane lateral organization that cause glycoprotein clustering [35] or cluster dissociation. In the end, the only aspect that makes each of the agonists unique is the set of signalling pathways they activate. Therefore, we conclude that lectin binding changes that we observe are specific to the particular set of signalling pathways activated by each of the agonists. This explains both the partial overlap between the changes in platelet surface glycosylation induced by different agonists (because the signalling pathways themselves overlap to a certain extent) and the unique effects of each of the agonists on the binding of some of the lectins. Therefore, lectin binding assays can provide a valuable tool for differentiating between platelet responses that are controlled by different signalling pathways, while agonist-specific platelet functional states may represent the missing mechanistic link between agonist concentration gradients thought to be present at the wound site [30,61] and differences between platelets located in different parts of the clot [62,63].

Finally, a comment needs to be made concerning how the aging of the blood prior to platelet isolation might affect our results. Because of the restrictions imposed on us by the Basque Biobank for Research and the administration of CIC biomaGUNE, we could only receive the blood and begin platelet isolation ~12 h after collection. As we have reported already [26], we found no differences in the basal levels of the classical activation markers, or their levels upon treatment with TRAP or PMA, between platelets prepared from fresh blood and blood that was stored at room temperature without agitation for 24 h (see Figure S1 in the Supplementary data and the Materials and Methods section). This observation might appear surprising in view of numerous reports of platelet activation and degradation upon storage of platelet concentrates [64,65]. However, it is quite consistent with the results of studies where platelets were isolated from fresh blood and from blood 24 h after phlebotomy [43,65]. Therefore we consider it unlikely that our main observation—that changes in platelet surface glycosylation are agonist-specific—is affected by the fact that the blood we used to prepare platelets was 12 h old. However, we cannot exclude the possibility that platelet membranes have undergone some changes during that time, possibly affecting the lectin binding patterns presented in Fig. 3. As a matter of fact, it would be interesting to investigate the ability of lectins to follow storage-related changes in platelet surface glycosylation.

## 5. Addendum

S. Gupta performed all of the experiments, analysed the data, and contributed to study design, interpretation of the results, and writing of the manuscript. I. Reviakine designed the study, contributed to the analysis and interpretation of data, and wrote the manuscript.

## Acknowledgements

We would like to acknowledge funding from the Department of Industry of the Basque Government (program ETORTEK) and from the Spanish Ministry of Science and Innovation (MICINN, grant CTQ2009-11245 to IR); Drs. Jose Manuel Cardenas Diaz De España, Roberto Bilbao Urquiola, Amaia del Villar Álvarez, and Ms. Clara Rodriuez Aierbe (Basque Biobank for Research, Galdakao, Spain); Ms. Mari Carmen Pozo and Dr. Mari Mar Lertxundi (Basque Biobank for Research, San Sebastian, Spain) for organizing the blood collection and testing; Dr. Juan Echevarria and Dr. Niels Reinhardt (CIC biomaGUNE) for helpful discussions about lectins. The idea of looking into platelet glycosylation arose from a discussion with Dr. Reinhardt after a seminar given by one of us (IR).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.08.010.

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