Human platelets express and are activated by galectin-8

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INTRODUCTION

Gals (galectins) are proteins with glycan affinity that are emerging as mediators of atherosclerosis. Despite the similarities in structure and sequence, different Gals exert distinct effects on their target cells. We have shown that Gal-1 triggers platelet activation, suggesting a role for Gals in thrombus formation. Since Gal-8 is expressed upon endothelial activation and also contributes to inflammation, to understand further the role of these lectins in haemostasis, we evaluated the effect of Gal-8 on human platelets. Gal-8 bound specific glycans in the platelet membrane and triggered spreading, calcium mobilization and fibrinogen binding. It also promoted aggregation, thromboxane generation, P-selectin expression and granule secretion. GP (glycoprotein)αIIb and Ib-V were identified as putative Gal-8 counter-receptors by MS. Studies performed using platelets from Glanzmann’s thrombasthenia and Bernard–Soulier syndrome patients confirmed that GPIb-α is essential for transducing Gal-8 signalling. Accordingly, Src, PLC2γ (phospholipase C2γ), ERK (extracellular-signal-regulated kinase) and PI3K (phosphoinositide 3-kinase)/Akt downstream molecules were involved in the Gal-8 signalling pathway. Gal-8 fragments containing either the N- or C-terminal carbohydrate-recognition domains showed that activation is exerted through the N-terminus. Western blotting and cytometry showed that platelets not only contain Gal-8, but also expose Gal-8 after thrombin activation. These findings reveal Gal-8 as a potent platelet activator, supporting a role for this lectin in thrombosis and inflammation.

Key words: galectin, glycan, inflammation, integrin, platelet, thrombosis.

Gals (galectins) are a family of carbohydrate-binding proteins that are involved in homoeostasis and pathological events. On the basis of their structure, they are classified into three groups: a prototype such as Gal-1; a chimaera type with Gal-3 as its only representative; and a tandem-repeat type, where Gal-8 is included among other members [1]. Depending on their structure, these proteins are able to act as bivalent or multivalent agents mediating the cross-linking of cellular surface glycoconjugates, thus being involved in biological processes such as apoptosis, differentiation, cytokine secretion, adhesion and lattice formation [1–3]. More recent studies revealed that Gals are also involved in the pathogenesis of cardiovascular diseases, particularly atherosclerosis [4]. Gals influence many processes that are important for plaque growth as well as stability/instability. For example, the Gal-3 gene has been found activated in aortas of hypercholesterolaemic rabbits, in aortas of rats after balloon injury and in cultured SMCs (smooth muscle cells) [5]. Increased levels of Gal-1 and -3 have also been detected in human atherosclerotic lesions [6].

Recent work from our laboratory has shown that Gal-1 triggers platelet activation and promotes platelet–leucocyte mixed aggregates [7]. These observations point out that Gals might be relevant mediators not only of the inflammation/atherosclerotic process, but also of thrombus formation. In this regard, Gal-3-binding protein was found to be up-regulated in a microparticle proteomics analysis of patients with deep venous thrombosis [8].

Gal-8 is composed of two distinct CRDs (carbohydrate-recognition domains) with different sugar specificities that are connected by a linker peptide of variable length that defines the isoforms [9]. Like other Gals, Gal-8 can be released to the extracellular compartment, and a positive correlation has also been demonstrated between Gal-8 and some human cancers [9]. We have shown recently the presence of two Gal-8 isoforms in murine spleen that play distinctive roles as local enhancers of otherwise borderline immune responses and also stimulate the reactivity at inflammatory foci [10]. Interestingly, also in this work, MS studies shown that GP (glycoprotein)αIIb, a major integrin of the platelet surface involved in thrombus growth was one of the molecules that interacted with Gal-8, suggesting a possible role for this Gal in platelet physiology. Despite the similarities in structure and primary sequence, different Gals exert distinct effects on their target cells. Therefore, to gain a deeper insight into the role of these particular lectins in the thrombotic process, in the present study, we examined the impact of Gal-8 on platelet biology.

Abbreviations used: AM, acetoxymethyl ester; CRD, carbohydrate-recognition domain; C-CRD, C-terminal CRD; N-CRD, N-terminal CRD; C-C, two C-terminal CRD chimaera; N-N, two N-terminal CRD chimaera; ERK, extracellular-signal-regulated kinase; Gal, galectin; GP, glycoprotein; GT, Glanzmann’s thrombasthenia; HRP, horseradish peroxidase; PAR1-AP, protease-activated receptor 1-activating peptide; PE, phycoerythrin; PGI2, prostaglandin I2; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PRP, platelet-rich plasma; SMC, smooth muscle cell; TDG, thiodigalactoside; TRITC, tetramethylrhodamine β-isothiocyante; TX, thromboxane; WP, washed platelet; vWF, von Willebrand factor.

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MATERIALS AND METHODS

Reagents

Human α-thrombin was purchased from Enzyme Research Laboratories, aprotase, acetylsalicylic acid (aspirin), luciferin-luciferase, TGDD (thiodigalactoside), ATP and TRITC (tetramethylrhodamine β-isothiocyanate)–phallolidin were from Sigma. NHS-biotin (N'-hydroxysuccinimido-biotin) was from Pierce. Eptifibatide was kindly provided by Dr C. Fondevila (Clinica Bazterrica, Buenos Aires, Argentina). FITC-conjugated streptavidin, Alexa Fluor® 488–fibrinogen and trypsin were purchased from Invitrogen. Collagen was from Nycomed Pharma. The inhibitors LY294002, U0126, PP1, PP2, U73122 and wortmannin were purchased from Enzo Life Sciences. Purified rabbit polyclonal anti-Gal-8 antibody was obtained as described previously [3] and from Abcam. Rabbit anti-phospho-PLCγ2 (phospholipase Cγ2) was purchased from Abcam. Lactose and sucrose were obtained from Calbiochem. The cytotox/cytoperm kit, FITC-conjugated anti-CD62P, anti-CD41, anti-CD42b, anti-PAC-1, FITC-conjugated irrelevant IgG1, and IgM, and anti-rabbit IgG and PE (phycoerythrin)-conjugated anti-CD61 were obtained from BD Biosciences. Mouse anti-phospho-ERK (extracellular-signal-regulated kinase) 1/2 (Tyr204), rabbit anti-ERK and anti-phospho-Akt (Ser473) antibodies were obtained from Santa Cruz Biotechnology. Goat HRP (horseradish peroxidase)-conjugated rabbit anti-vWF antibodies were obtained from Dako. Purified rabbit polyclonal anti-Gal-8 antibody was obtained as described previously [3] and from Abcam. Rabbit anti-phospho-PLCγ2 (phospholipase Cγ2) was purchased from Abcam. Lactose and sucrose were obtained from Calbiochem. The cytotox/cytoperm kit, FITC-conjugated anti-CD62P, anti-CD41, anti-CD42b, anti-PAC-1, FITC-conjugated irrelevant IgG1, and IgM, and anti-rabbit IgG and PE (phycoerythrin)-conjugated anti-CD61 were obtained from BD Biosciences. Mouse anti-phospho-ERK (extracellular-signal-regulated kinase) 1/2 (Tyr204), rabbit anti-ERK and anti-phospho-Akt (Ser473) antibodies were obtained from Santa Cruz Biotechnology. Goat HRP (horseradish peroxidase)-conjugated rabbit anti-vWF antibodies were obtained from Dako.

Expression of recombinant Gal-8 isoforms

Clones encoding the human Gal-8L (long) and Gal-8M (medium) isoforms (GenBank® accession numbers NM_006499 and NM_201543) were synthesized (GeneScript) and subcloned into pTrcHisB (Invitrogen). Murine recombinant N-terminal CRD (N-CRD), C-terminal CRD (C-CRD), and chimaeras containing two N-terminal CRDs (N-N) or C-terminal CRDs (C-C) were generated by following different PCR strategies using a mouse Gal-8L-encoding plasmid [10] as a template. For the N-CRD cloning, the coding sequence of the CRD was amplified using the primer set Fwd 5′-AAAGCTAGCATGGTCCTTTAATACCTA-3′/Rev 5′-AAAGATCTGACGTGCCAGC-GTAATC-3′ (NheI and BglII sites are underlined). For C-CRD, the encoding sequence of the CRD was obtained with the primer set Fwd 5′-AAAGCTAGCAAGCTCCAGGCTGAAGTC-3′/Rev 5′-AAAGATCTGGGATACCCATTTCTC-3′ (NheI and BglII sites are underlined). Both amplicons were cloned into the pTrcHisB expression plasmid (Invitrogen). For N-N construction, two CDR amplicons were obtained using the primer sets Fwd 5′-AAAGCTATGGTCCTTTTAATACCTA-3′/Rev 5′-AACTGACGTGGCTTTCTGGATATT-3′ (NheI and PstI sites are underlined) and Fwd 5′-AACTGACGTCGACTGTCTGAGGCCTTTAATACCCATTTCTC-3′/Rev 5′-AAAGCTATGGTCCTTTTAATACCTA-3′ (NheI and PstI sites are underlined). Amplicons were then sequentially cloned into pTrcHisB. A similar scheme was followed for C-C, where two amplicons of the CRD were generated with the primer sets Fwd 5′-AAAGCTATGGTCCTTTTAATACCTA-3′/Rev 5′-AACTGACGTGGCTTTTAATACCTA-3′ (NheI and PstI sites are underlined) and Fwd 5′-AACTGACGTCGACTGTCTGAGGCCTTTAATACCCATTTCTC-3′/Rev 5′-AAAGCTATGGTCCTTTTAATACCTA-3′ (NheI and PstI sites are underlined) and sequentially cloned in pTrcHisB. All constructions were DNA sequenced. The conditions for protein expression in a prokaryotic system and purification were identical with those described previously for mouse Gal-8. The lectin activity of these proteins was tested by haemagglutination assays [10]. The content of LPS (lipopolysaccharide), as checked using a Limulus test, was less than 0.5 ng/mg of the purified protein.

Preparation of human platelets

Blood samples were obtained from healthy donors who had not taken non-steroidal anti-inflammatory drugs in the 10 days before sampling. This study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and received the approval of the institutional ethics committee and written consent from all subjects. Blood was drawn directly into plastic tubes containing ACD (acid/citrate/dextrose) (6:1) or 3.8 % sodium citrate for aggregation in PRP (platelet-rich plasma). PRP from normal donors or from a GT (Glanzmann’s thrombasthenia) patient was obtained by centrifugation of the blood samples at 180 g for 10 min, and the cell number was adjusted to 3 × 10⁸ cells/ml with platelet-poor plasma. PRP from BS (Bernard–Soulier) syndrome patients was obtained by gravity sedimentation at room temperature (25 °C) by placing the tube at 45° inclination. BS and GT PRP counts were 2 × 10⁹ and 2.1 × 10⁹ cells/ml respectively.

For WP (washed platelet) suspensions, PRP was centrifuged in the presence of prostacyclin [PGI₂ (prostaglandin I₂)] (75 nM), and the platelets were then washed in washing buffer (140 mM NaCl, 10 mM NaHCO₃, 2.5 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgCl₂, 22 mM sodium citrate, 0.55 mM glucose and 0.35 % BSA, pH 6.5). WPs were resuspended in Tyrode’s buffer and the platelet number was adjusted to 3 × 10⁸ cells/ml, unless stated otherwise. Ca²⁺ (1 mM) was added before platelet stimulation. Highly purified platelets were obtained by depletion of leucocytes by using a high-efficiency leucoreduction filter (Purecell PL, Pall Biomedical Products), as described previously [11].

Gal-8-binding assay

WPs were incubated for 15 min with biotinylated Gal-8 and then platelets were washed with PBS/2 mM EDTA and incubated with FITC-conjugated streptavidin for 30 min. The binding of Gal-8 to the platelet surface was analysed by flow cytometry (FACS Calibur flow cytometer, BD Biosciences). In selected experiments, Gal-8-binding was measured using platelets whose GPIb was cleaved by incubation during 10 min with trypsin in the presence of aspirin, aspirin and PGI₂. The protease treatment was stopped by the addition of 10 % serum and a cocktail containing protease inhibitors.

Platelet spreading

Glass slides were coated with 0.1 μM LPS and blocked with 2 % BSA for 2 h. Then, WPs (5 × 10⁷ cells/ml) were plated and incubated for 20 min. Adhered platelets were fixed in 4 % (w/v) paraformaldehyde, permeabilized with 0.1 % Triton X-100 and stained with TRITC-phallolidin. After mounting, the spread platelets were visualized under confocal microscopy (Olympus FV-1000).

Platelet aggregation and ATP release

Aggregation and ATP release were measured in a luminescence counter (Chrono-Log). ATP levels were measured at the end of the assay by adding a known amount of ATP standard (2 μM).
Expression of platelet CD41, CD61 and CD42b, and P-selectin, fibrinogen or PAC-1 binding

WPs were stimulated, fixed and stained with an FITC-conjugated anti-CD62P antibody (anti-P-selectin) in PBS/0.1% FBS (fetal bovine serum) solution or an equivalent amount of isotype FITC–IgG, as a negative control. To evaluate αIIbβ3 integrin activation or fibrinogen binding, platelets were stimulated in the presence of FITC–PAC-1 or Alexa Fluor® 488–fibrinogen respectively, and then fixed and analysed by flow cytometry. To evaluate platelet-surface expression of CD41, CD61 and CD42b, GT, BS and control platelets were incubated with an FITC-conjugated anti-CD41 or anti-CD42b antibody or with a PE-conjugated anti-CD61 antibody. After fixation, cells were analysed by flow cytometry.

Intracellular Ca²⁺ mobilization

Intracellular Ca²⁺ concentrations in fluo-3-loaded platelets were assessed using flow cytometry as described previously [12]. In brief, platelets were labelled with 5 μM fluo-3-AM (acetoxyethyl ester) (Sigma) at 37°C for 15 min. After washing, 3 × 10⁶ platelets in 500 μl of Tyrode’s buffer were subjected to flow cytometric analysis. After the determination for approx. 10 s of baseline fluo-3 fluorescence from the platelet population, cell aspiration into the flow cytometer was paused briefly and Gal-8 was added. The acquisition was then resumed, and changes in fluorescence over time were recorded. Results are expressed as the percentage of positive cells and represent the events with FL1 values above the threshold.

Measurement of TX (thromboxane) B₂ release

WPs were incubated for 5 min in a platelet aggregometer stirring at 1000 rev/min with Gal-8M or Gal-8L. The reaction was stopped by the addition of ice-cold PBS containing 2 mM EDTA and 0.5 mM aspirin. The samples were centrifuged at 2300 g for 8 min, and TXB₂ levels in the supernatants were measured using an ELISA kit from Cayman Chemical.

Determination of Gal-8 expression in platelets by FACS

WPs were fixed and permeabilized using a cytofix/cytoperm permeabilization kit. After washing, the cells were first incubated with rabbit polyclonal anti-human Gal-8 antibody or an equivalent amount of rabbit IgG (negative control). Then, the cells were labelled with FITC-conjugated swine anti-rabbit antibody, and intracellular Gal-8 expression was determined by flow cytometry.

Determination of vWF release

vWF release was determined by ELISA. Briefly, WPs were stimulated with Gal-8 for 30 min and centrifuged twice at 2300 g for 5 min in the presence of PGI₂ (75 nM). The supernatants obtained were kept at −80°C until assayed. The results are expressed in ng/ml and were extrapolated from serial dilutions of normal pooled plasma, assuming a 10 μg/ml vWF concentration.

Immunoblotting

Highly purified WPs lysates were prepared by solubilizing platelets (10⁶) in loading buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue and 5% 2-mercaptoethanol). Equal amounts of proteins were electrophoresed by SDS/PAGE (12% gels) and electrotransferred on to nitrocellulose membranes (GE Healthcare). After blocking, the membranes were incubated overnight at 4°C with primary antibodies followed by an HRP-conjugated secondary antibody. Protein bands were visualized by using the ECL (enhanced chemiluminescence) reaction. Immunoblotting results were quantified using Gel-Pro Analyzer 3.1 software and the values from blot reprobes were used for monitoring equal protein loads. Western blot assays for Gal-8 detection in WPs lysates were carried out as described previously [10].

Affinity chromatography and MS

Human platelets were lysed with 1 % Triton X-100 in the presence of a protease inhibitor cocktail and passed through a Gal-8-affinity column made by coupling purified human recombinant Gal-8M to N-hydroxysuccinimide-activated HiTrap columns (GE Healthcare). Gal-8 binders were eluted with 150 mM lactose in PBS and concentrated by ultrafiltration. After alkylation with iodoacetamide, samples were resuspended in cracking buffer and resolved by SDS/PAGE (10% gels). After Coomassie Blue staining, discrete bands were cut out, in-gel-digested with trypsin, and subjected to peptide mass fingerprinting by the Mass Spectrometry Service of Institute Pasteur of Montevideo, Montevideo, Uruguay.

Statistical analysis

The results are expressed as means ± S.E.M. Student’s paired t test was used to determine the significance of differences between the groups. P < 0.05 was considered statistically significant.

RESULTS

Soluble Gal-8 binds to the platelet surface and immobilized Gal-8 promotes platelet adhesion and spreading

To investigate whether Gal-8 interacts with glycoconjugates at the platelet surface, we tested the binding ability of Gal-8M and Gal-8L, the prominent isoforms in humans [9,13]. As shown in Figure 1(A), both isoforms bound platelets at a similar rate and in a concentration-dependent manner. Addition of lactose, but not sucrose, strongly reduced Gal-8 binding, highlighting the specific interaction of this lectin with glycan residues on the platelet surface (Figure 1B).

Considering that Gal-8 binds to the platelet surface and that in other cell types it induces cell adhesion [14–16], we examined whether immobilized Gal-8 promoted platelet adhesion and spreading. Whereas platelets did not adhere to BSA-coated slides, they effectively attached, spread and displayed lamellipodia formation on immobilized Gal-8M- or Gal-8L-coated surfaces. Spreading and attachment were significantly reduced by lactose (Figure 1C).

Gal-8 induces activation of αIIbβ3 integrin and calcium mobilization

As a consequence of platelet agonist–receptor interaction, αIIbβ3 integrin suffers conformational changes that lead to the exposure of neoepitopes and allow fibrinogen binding (inside-out signalling) [17]. Flow cytometry studies using anti-PAC-1 antibody that recognizes the activated form of αIIbβ3 and Alexa Fluor® 488-conjugated anti-fibrinogen antibody showed that both phenomena occurred upon platelet activation induced by the Gal-8 isoforms (Figures 2A and 2B). Another feature of inside-out signalling is elevation of cytosolic Ca²⁺ concentrations which is essential for platelet activation. Figure 2(C) shows that, as with other platelet agonists, Gal-8 effectively raised intracellular
platelet Ca\(^{2+}\) levels. Inside-out signalling mediated by Gal-8 was inhibited by lactose (results not shown).

**Platelet aggregation mediated by Gal-8**

Conformational changes of αIIbβ3 integrin allow platelet aggregation to proceed [17]. Figure 3(A) shows that the two splice variants of Gal-8 induced platelet aggregation both in WPs (EC\(_{50}\) 0.19 ± 0.05 μM for Gal-8M and 0.20 ± 0.08 μM for Gal-8L) and in PRP (EC\(_{50}\) 0.9 ± 0.06 and 0.8 ± 0.07 μM for Gal-8M and Gal-8L respectively). Because all of the evaluated platelet responses triggered by both splice variants were almost identical, the rest of the experiments were performed with only the Gal-8M isofrom.

The aggregation response was associated with the ability of Gal-8 to bind to specific glycan molecules on the platelet surface since it was completely suppressed by pre-incubation of platelets with lactose or its related sugar TDG (Figure 3B, I and II), but not by sucrose (Figure 3B, III). Because lectins are known to induce cell agglutination, to determine whether this phenomenon accounted for the Gal-8-induced response, platelet stimulation was performed in the presence of EDTA or eptifibatide, an αIIbβ3 antagonist. Under these conditions, platelet aggregation induced by Gal-8M was almost completely suppressed (Figure 3B, IV). Moreover, Gal-8 was not able to induce aggregation of fixed platelets (Figure 3B, IV), confirming that the observed response was not an agglutination phenomenon and that, similar to classical agonists, Gal-8-mediated platelet aggregation is ultimately dependent on fibrinogen binding to αIIbβ3.

**Role of ADP and TXA\(_2\) in platelet activation mediated by Gal-8**

Most classical agonists promote the release of ADP and TXA\(_2\), which, through the interaction with specific surface platelet
Platelet activation mediated by galectin-8

**Figure 2** Soluble Gal-8 promotes inside-out signalling in platelets

(A) αIIbβ3 integrin activation of resting and Gal-8- or thrombin (Thr)-stimulated WPs was determined by flow cytometry, using FITC-conjugated anti-PAC-1 antibody \( (n = 4, * P < 0.05 \) compared with unstimulated (Unst)). (B) Fibrinogen binding of unstimulated and Gal-8- or thrombin-stimulated WPs was determined by flow cytometry using Alexa Fluor® 488-conjugated fibrinogen \( (n = 4, \ * P < 0.05 \) compared with Unst). For comparison, thrombin \( (0.1 \text{ unit/ml}) \) and Gal-8 \( (0.5 \mu \text{M}) \) were the minimal agonists concentration that induced 100% of PAC or fibrinogen binding. (C) Intracellular Ca\(^{2+} \) concentration was determined by flow cytometry using fluo-3-AM \( (n = 3) \). MFI, mean fluorescence intensity.

Platelet secretion mediated by Gal-8

The role of platelets in inflammation and vascular repair is mainly associated with the release of α granule content. [18]. Having demonstrated that Gal-8 effectively triggered secretion of dense granules, we next analysed whether a similar effect was exerted on α granule release. Figures 5(A) and 5(B) shows that both vWF and P-selectin were detected in the supernatants and in the membrane from stimulated platelets respectively, reinforcing the notion of Gal-8 as a pro-inflammatory molecule.

Identification of putative Gal-8 counter-receptors

To screen for possible platelet receptors that interact with Gal-8 in a carbohydrate-dependent manner, a Gal-8-affinity chromatography step followed by MS analysis was performed using platelet lysates. After column elution with lactose and SDS/PAGE resolution, a discrete number of bands was observed. The subunit αIIb from the αIIbβ3 integrin and GPIb and GPV from GPIb–GPIX–GPV complexes respectively, were the only membrane proteins identified that could eventually act as counter-receptors for Gal-8 (Table 1). In addition to these surface proteins,
other relevant molecules that are released after platelet activation such as vWF, coagulation Factor V or multimerin were also identified. In fact, vWF is one of the few ABO glycosylated proteins, and this sugar signature is a known target of Gal-8 [13,19]. In addition, other internal proteins seem to be indirectly co-purified with the Gal-8 counter-receptors as might be the case for actin and filamin, both known to interact with integrins. To characterize further the possible role of αIIbβ3 integrin and GPIb–GPIX–GPV as Gal-8 counter-receptors, functional studies were performed using platelets from a patient with GT, deficient in αIIbβ3 integrin (Figure 6A), from a patient with BS syndrome whose platelets lack GPIb (CD42b) (Figure 6A) or platelets whose GPIb was cleaved by pre-treatment with trypsin. Consistent with MS results, a significant decrease in the MFI (mean fluorescence intensity) was observed at all Gal-8 concentrations employed in GT, BS or trypsin-treated platelets. As expected, owing to the absence of αIIbβ3 integrin, collagen- and Gal-8-induced platelet aggregation were profoundly decreased in GT platelets (Figure 7A, I). However, P-selectin exposure mediated by either collagen or Gal-8 was not affected despite the absence of αIIbβ3 integrin (Figure 7B, I), pointing out that αIIbβ3 integrin is dispensable for Gal-8-induced platelet activation. Notably, platelet aggregation (Figure 7A, II) or the expression of P-selectin mediated by Gal-8 (Figure 7B, II) was almost absent from BS platelets. The mild aggregation response observed at the highest Gal-8 concentration employed in both GT and BS platelets, was not inhibited by EDTA (Figures 7A, I and 7A, II), indicating that it might be an agglutination effect instead.

Together, these results suggest that platelet GPIb, but not αIIb is essential for Gal8-dependent signal transduction and therefore GPIb represents a biologically functional/relevant Gal-8 counter-receptor.

**Signalling pathways involved in Gal-8-mediated platelet activation**

Because GPIb appears to be at least one of the Gal-8 counter-receptors, we next explored, using Western blotting and pharmacological approaches, several downstream signalling molecules known to be involved in GPIb-mediated signalling. Although the model of how GPIb–GPIX–GPV signals in human platelets is currently incomplete, the activation of Src, PI3K (phosphoinositide 3-kinase), PLCγ2 and ERK appear to be involved in GPIb activation mediated by vWF binding [20,21]. In agreement, we found that platelet stimulation by Gal-8 resulted in PLCγ2, ERK1/2 and Akt phosphorylation (Figures 8A–8C).
Platelet activation mediated by galectin-8

541

Figure 4 Role of ADP and TXB2 on Gal-8-induced aggregation

(A) WPs were stimulated with Gal-8 for 5 min under stirring conditions and TXB2 levels in supernatants were determined by ELISA [n = 3, *P < 0.05 compared with unstimulated (Unst)].

(B) ATP release was measured by using a lumi-aggregometer.

(C) Aggregation induced by a range of concentrations of Gal-8 was measured in the presence of apyrase (apy; 2 units/ml) and aspirin (ASA; 0.5 mM), individually and in combination. Results are means ± S.E.M. for three independent experiments. Aggregation is plotted as a percentage of the maximal aggregation achieved (n = 4, *P < 0.05 compared with control).

The phosphorylation of all of these proteins, except for ERK, appears to be relevant for platelet activation induced by Gal-8 since platelet aggregation was significantly impaired in the presence of specific inhibitors of PLC (U73122), PI3K (LY294002 and wortmannin), but not of MAPKK (mitogen-activated protein kinase kinase) (U0126) [22,23] (Figure 8D).

The activity of U0126 was confirmed by the observation that it completely inhibited ERK phosphorylation induced by thrombin or Gal-8 (results not shown). As Src kinases are also involved in GPIb signalling [21] two specific inhibitors (PP1 and PP2) were tested. Pre-incubation of platelets with either of these drugs strongly inhibited Gal-8-induced aggregation (Figure 8D).

Altogether, these data demonstrate that most of the GPIb downstream signalling molecules are activated by Gal-8, giving further support for this GP as a major counter-receptor for Gal-8 in platelets.

N-CRD triggers platelet activation

The absence of platelet agglutination in the presence of Gal-8 (Figure 3B, IV) raises the idea that this lectin may work as a monomer. To gain a deeper insight into the structure–function relationship of Gal-8 on platelet physiology, the effects of fragments containing only N-CRD or C-CRD were tested. Since human and murine Gal-8 structures are highly related [3] and showed almost identical activity on human platelets (Table 2), recombinant N-CRD and C-CRD from murine Gal-8 were used in the following experiments. Notably, only N-CRD was able to trigger platelet activation responses including P-selectin exposure, fibrinogen binding and aggregation (Table 2). Protein chimaeras containing two N-terminal CRDs (N-N) or two C-terminal CRDs (C-C) connected by a linker peptide were also tested. Consistent

Table 1 Galectin-8 protein binders identified by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) from platelets

WPs were lysed and passed through a Gal-8-affinity column. Gal-8 binders were eluted in the presence of lactose and resolved by SDS/PAGE (10% gels). The bands were cut out, and subjected to MALDI–TOF–MS.

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<tr>
<td>von Willebrand factor vWF human</td>
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Figure 6  Binding of Gal-8 to platelets from GT and BS syndrome patients and to trypsin-treated normal platelets

(A) CD41, CD61 and CD42b expression on platelet surface was determined by flow cytometry using FITC or PE-labelled monoclonal antibodies. (B) Gal-8 binding on the platelet surface was determined in PRP by flow cytometry using different biotinylated Gal-8 concentrations (histograms show binding induced by 0.3 μM Gal-8) and FITC-labelled streptavidin. No Gal, no Gal-8 added; C, control (normal donor). MFI, mean fluorescence intensity.

with the results obtained with each separate domain, platelets incubated with the N-N chimaera, but not with the C-C chimaera displayed a strong activation response (Table 2). These results demonstrate that the activation of platelets mediated by Gal-8 is due to the N-CRD, and, moreover, that lectin bivalency is not essential to achieve its activity on platelets.

Human platelets contain Gal-8M and Gal-8L and platelet activation leads to Gal-8 exposure

Gal-8 is a secreted protein that, like other galectins, lacks a signal peptide and uses a non-conventional secretion pathway [24]. Considering that exogenous Gal-8 triggers several platelet effector responses, we investigated whether platelets could also be a source of this lectin. The two principal splice variants of human Gal-8 (M and L isoforms) were detected on resting platelets from healthy donors by Western blot analysis (Figure 9A). These findings were confirmed by detecting Gal-8 in permeabilized platelets by flow cytometry (Figure 9B). These results indicate that platelets, like other vascular cells such as endothelial cells [25], also express Gal-8, which might account for its local effects. Notably, Gal-8 was detected in non-permeabilized platelets only after thrombin stimulation (Figure 9C). However, we were unable to detect the presence of secreted Gal-8 in the supernatants of activated platelets even after washing with lactose (results not shown). These results are in agreement with the previous observations of Hadari et al. [26] where Gal-8 remains attached to the surface of human carcinoma cells after secretion, being released after treatment with trypsin.

To analyse further the role of endogenous Gal-8 on platelet activation, aggregation was triggered by different agonists in the presence of lactose or TDG. Figure 9(D) shows that the aggregation response induced by submaximal collagen, PAR1-AP (protease-activated receptor 1-activating peptide) or ADP concentrations was inhibited by pre-incubation of platelets with both related sugars.

Our findings indicate that platelet Gal-8 is translocated to the outer membrane after activation, allowing us to postulate an amplifying role for this lectin at the site of inflammation/injury.

DISCUSSION

The results reported in the present paper show that two splice variants of Gal-8 bind to the platelet surface and activate different
Platelet activation mediated by galectin-8

Figure 7  Gal-8-mediated activation of platelets from GT (I) or BS syndrome (II) patients

(A) The percentage of platelet aggregation induced by the Gal-8M or collagen (Coll) was determined in PRP. (B) Surface expression of P-selectin induced by Gal-8 or Collagen (Coll) was detected by flow cytometry. Unst, unstimulated. MFI, mean fluorescence intensity.

platelet functional responses, including spreading, activation of integrin αIIbβ3, aggregation and secretion of the content of both dense and α granules. Inhibition of Gal-8 binding and platelet aggregation by lactose or TDG, but not by sucrose, indicated that the interaction of Gal-8 with the platelet surface involves recognition of specific glycans in the platelet membrane.

The observation that immobilized Gal-8 functions as an extracellular matrix protein that mediates cell adhesion was reported previously by other groups in other cell types [14–16]. We have now extended these findings, showing for the first time that immobilized Gal-8 promotes platelet adhesion and spreading. Interestingly, the ability to artificially trigger these responses independently of agonist-induced integrin activation, is usually restricted to αIIbβ3 ligands, including fibrinogen, vWF or CD40L [17,27].

The affinity of αIIbβ3 for fibrinogen and other ligands is modulated through conformational changes. Like most traditional platelet agonists, soluble Gal-8 promoted the transition of this integrin from a resting state to a high-affinity state, resulting in the unmasking of neoepitopes in the αIIbβ3 complex, and allowing fibrinogen binding. Furthermore, we also demonstrated that both Gal-8 isoforms were able to induce platelet aggregation as well as TXB2 release. Although the aggregation response triggered by low Gal-8 concentrations was inhibited in the presence of aspirin and/or an ADP scavenger, a full response was restored at higher concentrations, showing that Gal-8 is a strong agonist that can activate platelets independently of TXA2 formation or ADP release.

Stimulation of platelets with Gal-8 also led to the release of both dense and α granule content such as ATP and vWF as well as P-selectin exposure. Platelet secretion is critical for haemostasis and vascular repair [18]. Besides, the ability of platelets to interact with other vascular cells through the expression of P-selectin has been implicated in the progression of inflammatory conditions, the metastatic spread of malignancies and the immune response to bacterial challenge [28,29]. Thus our results suggest that Gal-8 might play a relevant role, not only in the physiopathology of thrombus formation, but also in fuelling the inflammatory response or in metastasis development.

Different cell-surface glycoconjugates, as well as extracellular matrix glycoproteins, appear to be primary receptors for Gals [30]. Among the different receptors, integrins are known to be involved in Gal-mediated biological responses [31,32]. In agreement with these data, MS studies showed that, among the different platelet-surface GPs, subunit αIIb from the αIIbβ3 integrin and GPIb and GPV from GPIb–GPIX–GPV complex, appear to be Gal-8 counter-receptors. The decreased binding of Gal-8 in GT, BS or platelets whose GPIb was cleaved by trypsin confirmed that both GPIb and αIIbβ3 integrin are involved in Gal-8 interaction with the platelet surface. However, the observation that Gal-8-mediated platelet activation was only impaired in BS platelets revealed that GPIb is essential for Gal-8-dependent signal transduction and therefore represents a major functional Gal-8 counter-receptor. Moreover, our findings showing that Src, PI3K/Akt and PLCγ2 (well-known downstream signalling molecules related to GPIb–GPIX–GPV [20,21]) are involved in platelet activation induced
Figure 8 Involvement of Src, PLC\(_{\gamma}2\), ERK and PI3K/Akt in Gal-8-mediated platelet activation

WPs were stimulated with Gal-8 (0.5 \(\mu\)M) at 37°C, and the reaction was stopped by adding loading buffer. PLC\(_{\gamma}2\) (PLC\(_{\gamma}2\)) (A), ERK (B) or Akt (C) phosphorylation was determined in cell lysates. Anti-(total ERK) and -actin antibodies were used to monitor protein loading in all lanes. The images are representative of three independent experiments (*P < 0.05 compared with unstimulated). (D) WPs were pre-treated with vehicle (DMSO), LY294002, wortmannin, U0126, PP1, PP2 or U73122 for 1 min and then aggregation was induced by Gal-8. Aggregation induced by Gal-8 (0.5 \(\mu\)M) in the presence of vehicle was considered as 100% (n = 3, *P < 0.05 compared with control).

by Gal-8, gave additional support to the notion that GPIb is an essential receptor for transducing Gal-8 signalling.

Since all of the platelet activation responses evaluated were exerted by different concentrations of the two splice variants of Gal-8 (M and L) with a similar potency, it could be assumed that the isoforms would function likewise in vivo. Besides, as the isoforms share identical CRDs differing only in the length of the linker region, our results also indicate that platelet activation triggered by Gal-8 does not depend on a specific spacing of the CRDs or does not rely upon lectin bivalency. The importance of the presence of multiple isoforms of human Gal-8 has still not been addressed; however, some authors speculate that they may be tissue-specific, or correspond to certain pathologies such as cancer [9]. Interestingly, N-CRD-containing fragments were able to trigger platelet activation, indicating that lectin bivalency is not essential and that the clustering of surface receptors is not required. This observation was supported further by the fact that Gal-8 failed to induce platelet aggregation in the presence of EDTA or in fixed platelets, precluding agglutination.

Although Gal-8 is a secreted protein, it is mainly found in the cytosol of different cell types [33]. Western blot and flow cytometric studies revealed that human platelets express the two splice variants of Gal-8. Moreover, whereas Gal-8 was absent on the surface of resting platelets, like P-selectin, it was exposed on the membrane of thrombin-stimulated platelets. In addition, the observation that lactose and TDG inhibited the aggregation induced by classical agonists indicates that platelet-derived Gal-8 could be a mediator of platelet activation. Therefore, in the vascular system, platelets are another source of Gal-8 that would be accessible upon platelet activation not only to eventually
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Table 2 Platelet activation mediated by chimaeric and truncated forms of Gal-8

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fibrinogen binding (% of positive cells)</th>
<th>P-selectin exposure (% of positive cells)</th>
<th>Aggregation (% of light transmission)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>3 ± 1</td>
<td>5 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>Human Gal-8 (0.5 μM)</td>
<td>92 ± 5*</td>
<td>81 ± 8*</td>
<td>90 ± 6*</td>
</tr>
<tr>
<td>Murine Gal-8 (1 μM)</td>
<td>91 ± 3*</td>
<td>68 ± 2*</td>
<td>89 ± 3*</td>
</tr>
<tr>
<td>N-CRD (2 μM)</td>
<td>97 ± 2*</td>
<td>59 ± 1*</td>
<td>61 ± 4*</td>
</tr>
<tr>
<td>C-CRD (2 μM)</td>
<td>2 ± 1</td>
<td>7 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>N-N (2 μM)</td>
<td>96 ± 4*</td>
<td>65 ± 2*</td>
<td>66 ± 5*</td>
</tr>
<tr>
<td>C-C (2 μM)</td>
<td>5 ± 4</td>
<td>5 ± 4</td>
<td>0</td>
</tr>
</tbody>
</table>

Promote further thrombus growth, but also to activate endothelial cells and/or leucocytes. In fact, it has been demonstrated that Gal-8 promotes adhesion and superoxide production in neutrophils [34], and we have shown recently that Gal-8 induces lymphocyte proliferation in the presence or absence of antigen [10]. In addition, high amounts of this Gal were found in the inflamed synovia [35].

Regarding the biological implication of this novel role of Gal-8 as a platelet agonist, it is reasonable to propose that Gal-8 might be relevant in the physiopathological haemostatic response after vessel injury. In fact, the exposure of Gal-8 in the subendothelium or on activated endothelial cells [25] would trigger platelet adhesion, spreading and thrombus formation; the latter event being reinforced by the Gal-8 exposed on platelet membranes after activation. In addition, the effects of Gal-8 on platelets and inflammatory cells could also contribute to the fate of the atherosclerotic plaque. Although the expression of Gal-8 in human atherosclerotic lesions has not yet been explored, the expression of Gal-1 and Gal-3 has been shown previously [5,6]. Among the mechanisms proposed to explain the effects of Gal-1 in the atherogenic process are the ability of this lectin to induce SMC proliferation through a lipoprotein(a)-dependent mechanism and to trigger T-cell apoptosis [36]. Regarding Gal-3, a role for this protein has been recognized in the transformation of macrophages into foam cells as well as in chemotaxis for monocytes and macrophages [37]. Besides, it has been shown that Gal-3 exacerbates vascular inflammation by stimulating macrophages to release superoxide anion [37] and express a range of chemokines and other pro-inflammatory molecules [38]. Our present data, together with our previous observation that Gal-1 triggers platelet activation [7], suggests that Gals might not only be involved in the fate of atherosclerotic plaque, but also could act as potent thrombogenic molecules that are exposed after plaque rupture. Although both Gals are capable of promoting similar platelet functional responses, Gal-8 is ten times more potent

![Figure 9](image-url)

**Figure 9** Gal-8M and Gal-8L are expressed in human platelets

(A) Resting platelets from three different donors (I, II and III) were highly purified and lysed, and the expression of both isoforms of Gal-8 was determined by Western blot analysis (lane 3). Recombinant Gal-8M (lane 1) and Gal-8L (lane 2) isoforms were used as positive controls. The position of the 38 kDa band is indicated. (B) The percentage of cells positive for Gal-8 in permeabilized WPs was determined by flow cytometry. Results are representative of three independent experiments. (C) Non-permeabilized WPs were stimulated with 1 unit/ml thrombin for 20 min and the percentage of positive cells for Gal-8 in permeabilized WPs was determined by flow cytometry. Results are representative of four independent experiments. (D) PRP was pre-incubated with vehicle (PBS, unlabelled traces) and lactose (30 mM) or TDG (30 mM) for 1 min, and then aggregation was induced by submaximal concentrations of PAR1-AP (2 μM), collagen (Coll) (0.5 μg/ml) and ADP (1.25 μM). Traces are representative of three independent experiments.

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than Gal-1. Perhaps the differences in the concentration required to achieve a similar effect could reflect different downstream molecular signals triggered by each lectin and/or the relative abundance of Gal-1, compared with Gal-8, in some cells/tissues [3]. The observed increased levels of Gal-1 and Gal-8 in tumoral endothelial cells as well as in other malignant cells [9,39] could represent a pathogenic mechanism involved in thrombosis and disseminated intravascular coagulation complications, commonly present in cancer patients [40,41]. Furthermore, the formation of mixed-cell aggregates between tumour cells expressing high levels of Gal-8/Gal-1 and platelets might also contribute to tumour progression and metastasis. Nevertheless, further experiments are required to test these hypotheses.

In conclusion, our results reveal Gals as a new family of endogenous platelet agonists capable of triggering activation in either its soluble or immobilized form.

**AUTHOR CONTRIBUTION**

Maria Albertina Romaniuk and Maria Virginia Tribulatti conducted research, collected, analysed and interpreted data, and performed statistical analysis. Valentina Cattaneo and Maria Jose Lapponi conducted research, and collected, analysed and interpreted data; Felisa Concepción Molinas contributed vital patient material, and analysed data. Oscar Campetella and Mirta Schattert designed research, analysed data and wrote the paper.

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