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Isolation of Galectin-1 from Human Platelets: Its Interaction with Actin

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Abstract Galectins are a family of animal lectins defined by their β-galactoside-binding specificity and a consensus sequence in their carbohydrate-recognition domain. Galectin-1 (Gal-1) is expressed as a non-covalently linked homodimer present in a variety of tissues. Here we describe its isolation from human platelets by a procedure involving ionic exchange chromatography and affinity chromatography on lactose-agarose. Platelet Gal-1 co-purifies with actin, forming an actin-Gal-1 complex which does no dissociate even after treatment with sodium dodecyl sulfate. The presence of both proteins was confirmed by Western blot and by trypsin digestion followed by mass spectrometry identification. By hemagglutination assays we studied the response of recombinant Gal-1/actin, mixed and pre-incubated in different proportions, and then tested against neuraminidase treated rabbit red blood cells. The complex formation was confirmed by confocal microscopy, showing that both proteins co-localised in resting platelets as well as in thrombin-activated ones. These results suggest that endogenous Gal-1 forms an intracellular complex with monomeric actin and that, after platelet activation, Gal-1 could play a role in the polymerization-depolymerization process of actin, which concludes in platelet aggregation.

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Abbreviations

ACN	Acetonitrile
BSA	Bovine serum albumin
CDR	Carbohydrate recognition domain
CM	Confocal microscopy
DEAE	Diethylaminoethyl cellulose
E64	Cysteine proteinase inhibitor
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
MEPBS	Mercaptoethanol phosphate buffered saline
PBS	Phosphate buffered saline
PGE_1	Prostaglandin E1
Plth	Human platelet
PMN	Polymorphonuclear neutrophils
PMSF	Phenyl methanesulfonyl fluoride
PRP	Platelet enriched plasma
rGal	Recombinant galectin
RP-HPLC-MS	Reversed phase-high performance liquid chromatography–mass spectrometry
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tr	Thrombin
TRICT	Tetramethylrhodamine isothiocyanate

1 Introduction

Galectins are a family of soluble animal proteins defined by their β-galactoside-binding specificity and a consensus sequence in their carbohydrate-recognition domain (CRD)

[3]. They are involved in multiple and essential biological functions such as cell adhesion, apoptosis, growth regulation, RNA splicing and tumor metastasis, mostly mediated by their specific recognition of glycoconjugates [9–11, 23, 28, 29, 36]. Fifteen different galectin types have been identified in mammals, and their CRDs show affinity for *N*-acetyllactosamine, a common disaccharide present in many cellular glycoproteins. However, individual galectins can recognize modifications of this ligand, developing multiple specificities [9].

Galectin-1 (Gal-1) is expressed as a non-covalently linked homodimer of 14.5 kDa subunits, with two CRDs per dimer. The lectin requires thiol group for its binding activity and is independent of divalent metals. From the structural point of view the CRD is defined by 134 aminoacids organized into a β-sheets sandwich of a six stranded and a five stranded anti-parallel ones. Although some galectins-1 were purified, characterized and sequenced there is interest to detect new members. So, recently the last molecule reported was from goat heart and was deeply studied employing a number of physicochemical methods [2]. The Gal-1 gene was named LGALS1 and mapped to the q12–q13 region of human chromosome 22 [25]. Gal-1 is present in different human tissues, such as thymus [4] and spleen [1] and is widely distributed from lower to higher vertebrates [22]. Another relevant aspect related to functional roles is its localization, Gal-1 can be present in or outside of the cells depending of their metabolic status [10].

Platelets are anucleated, discoid shaped blood cells, derived from megakaryocytes, which are essential for hemostasis and exert important functions in the modulation of inflammatory and immune processes [21]. Their main function in wound healing is sealing vascular lesions through their interaction with matrix components not normally exposed. When encountering vascular damage, platelets become adhesive to the subendothelium through receptors that bind glycoproteins such as GPIb, which plays a fundamental role in the activation process together with von Willebrand factor and fibrinogen. Adhesion triggers platelet activation, which induces a conformational change in GPIIb/IIIa receptors, which in turn leads to platelet aggregation and thrombus formation [18].

Previous studies on the interaction of lectins with blood cell surface glycans, employing several lectins of different origins, led to findings of interest. With regard to Gal-1, homologous Gal-1, CG-16 (chicken galectin-16) and bovine Gal-1 induce the aggregation of neutrophils, thymocytes and platelets [35]. Information about Gal-1–platelet interactions is scarce. Previously, in our laboratory, by immunofluorescence studies on human and porcine neutrophils exposed to pig spleen Gal-1, we detected the presence of endogenous Gal-1 when the PMN cellular suspension was contaminated with platelets [12]; this finding led us to initiate human platelet studies. Moreover, Pacienza et al. [27] showed that recombinant Gal-1 binds to platelets in a carbohydrate-depending manner and synergizes with ADP or thrombin to induce platelet aggregation and ATP release. Furthermore, these authors provide evidence that Gal-1 induces F-actin polymerization, up-regulation of P-selectin and GPIIIa expression, promotes shedding of microvesicles and triggers conformational changes in GPIIb/IIIa. In addition, it was also confirmed [27] the presence of endogenous Gal-1 in platelets by immunoblot analysis, flow cytometry and immunofluorescence staining. However, its low concentration [14] renders it difficult to obtain by standard purification procedures. In this paper we describe for the first time the purification of this lectin from human platelets, and provide evidence suggesting that Gal-1 forms a complex with actin in human platelets.

2 Materials and Methods

2.1 Materials

All reagents were purchased from Sigma Chemical Co. (Saint Louis, Mo, USA) unless otherwise stated. Recombinant Gal-1 was used as control and anti-Gal-1 polyclonal antibodies was obtained as previously described [12].

2.2 Washed Platelet Extract (WPE) Preparation

Human platelet enriched plasma (PRP) obtained from Blood Bank with negative results for serology which included HBsAg, HCV, HBV, HTLV I/II, HIV 1 + 2 among others, was depleted of leukocytes by filtration through polyurethane filters, and centrifuged at 560*g* for 20 min at 4 °C. To eliminate any remaining red blood cells, 1% ammonium chloride was added to the pellet. After 5 min at room temperature, it was washed twice with 0,01 M Tris–HCl buffer, 1 M EDTA, 0.15 M NaCl, 5 mM glucose, 1 mM PMSF, PGE_1 3.5% (w/v), pH 7.4, and once more with the same buffer without EDTA. Protease inhibitors PMSF (0.075 g/L), E64 (1–10 μM) and pepstatin (1 μM) were added to the suspension of 5×10^{11}/L platelets. Two different procedures were assayed to obtain a platelet lysate.

A. Platelets were frozen at −20 °C, thawed three times and centrifuged at 2,000*g* for 2 h at 4 °C to separate the platelet membranes from the washed platelet extract.
B. Tris–glycine buffer, pH 8.7, 1% Triton 100, was added to the platelets, stirred for 30 min at 4 °C, and centrifuged at $2{,}000 \times g$ for 1 h at 4 °C to separate the platelet membranes from WPE.

2.3 Purification of Galectin-1 from WPE

The WPE was submitted to ionic exchange chromatography using DEAE-Sepharosa CL-6B. EP (200 mL) was stirred with 100 mL of the resin, in batch, for 1 h at 4 °C, and then transferred to a column. After extensive washing with ME-PBS buffer (5.8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 7.5 mM NaCl, 4 mM β-mercaptoethanol, 0.2 mM EDTA, pH 7.5), the fractions eluted with MEPBS, 500 m M NaCl were applied to a lactose-agarose column (50 mL) equilibrated and washed with the same buffer. Gal-1 fraction was eluted with the same buffer with the addition of 100 mM lactose.

2.4 Protein Assays

Protein concentration was measured following Bradford [5]. Bovine serum albumin (BSA) solutions were used as standard.

2.5 Hemagglutination Assays

To test the specific activity in the eluted fraction, hemagglutination of neuraminidase treated rabbit erythrocytes was carried out as previously described [13]. Hemagglutination titer was defined as the reciprocal of the highest dilution capable of giving visible agglutination. Inhibition assays with 100 mM lactose were also run and inhibition specificity was tested employing 100 mM saccharose. Activity measurements were simultaneously run with controls of recombinant human Gal-1 obtained as previously described [19], in presence or absence of muscle actin. In these assays, Gal-1 was used at a constant concentration of 10 μM and actin from 200 μM up to 0.1 μM. These proportions were selected taking into account that the physiological concentrations for both molecules measured in resting platelets were included in this experimental range.

2.6 Western Blot

Protein samples were separated on 12.5% SDS-PAGE gels. Samples containing 0.005–0.010 μg of protein were loaded per lane and after running, the gel was fixed with 100 mL of 50% methanol, 12% acetic acid, 50 μL of 37% formaldehyde, for 1 h or overnight to prevent diffusion of protein and decrease the background. After washing with 30% ethanol to remove acetic acid, gels were treated with 0.02% S_2O_3Na for exactly 1 min. Later, gels were washed three times with bidistilled water for 20 s each time to remove excess of the pretreatment solution. Then, a staining solution of 0.2% $AgNO_3$, 75 μL of 37% formaldehyde, was added for 20 min. After washings, the gel was immersed in a developing solution for about 2 min, then in a stopping solution for 10 min.

Unstained gels were electroblotted onto nitrocellulose membranes. The membrane was blocked with 5% cow milk in 10 mM Tris–HCl with 150 mM NaCl, pH 8.0 and 0.1% Tween 20 for 2 h at room temperature. The membrane was then incubated with anti-Gal-1 antibody (1:100 diluted), or with anti-actin antibody (diluted 1:1,000) both obtained in rabbits. After washing three times, the membrane was incubated with anti-rabbit IgG-peroxidase secondary antibody for 1 h. The membrane was again washed three times and the specific protein bands were visualized using 4-chloro-1-naphthol as a substrate. In parallel, in order to improve the assay, a more sensitive method was employed using the avidin–biotin system. The same protocol was followed but biotinylated IgG anti rabbit was used as second antibody.

2.7 Protein Molecular Mass Determination

Proteins were analyzed by RP-HPLC–MS using a 1.0 mm × 30 mm Vydac C_8 column, operating at 40 μL/min, connected to a Surveyor HPLC System on-line with an LCQ Duo (ESI Ion Trap) mass spectrometer (Thermo Fisher, San José, CA, USA). Proteins were eluted using a 15 min gradient from 10 to 100% solvent B (Solvent A: 2% acetic acid and 2% ACN, Solvent B:2% acetic acid and 96% ACN. Protein characterization was performed by Full Scan 300–2000 amu and ProMass deconvolution program.

2.8 Protein identification from MS/MS Data

The purified Gal-1 fraction was reduced, carbamidomehylated and digested with trypsin at a 1:20 enzyme/substrate ration in 2 M urea, 0.1 M ammonium bicarbonate (pH 8.0) at 37 °C for 20 h [30]. Peptides were separated by reverse phase liquid chromatography. The procedure was performed in a C_{18}, 250 × 1 mm Vydac column (76 min gradient) interfaced to an LCQ Duo ESI-IT Mass Spectrometer (ThermoFisher, San José, CA, USA). The detector was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition, and dynamic exclusion selecting the three most intense precursor ions for fragmentation using collision induced dissociation (CID).

Peptide masses and MS/MS data were used to identify proteins by comparing the experimental data to theoretical data calculated from protein sequence databases, using tandem MS search algorithms: Mascot and Sequest.

2.9 Preparation of Washed Human Platelets for Confocal Microscopy (CM) Assay

Blood samples were obtained from healthy individuals (n: 3) who had not ingested any medication 10 days before the day of bleeding. Sampling was done by venipuncture

directly into plastic tubes containing anticoagulant (3.8 g% sodium citrate, 9:1). Whole blood was centrifuged at $100\times g$ for 10 min. PRP was separated from the top supernatant layer and treated as described in Sect. 2.2. The washed platelets were resuspended at a concentration of 6×10^{11}/L in PBS for CM tests.

2.10 Co-Localization of Gal-1 and Actin in Human Platelets by CM

In order to active platelets, 6×10^{11}/L Plth suspensions were incubated with Tr 0.2 IU/mL from 15 up to 180 s at 37 °C according to manufacturer's instructions (Wiener-Lab., Rosario, Argentina); activation was inhibited by the addition a high volume of a washing solution (PBS) followed by centrifugation. Fifty µL of resting and activated platelets were incubated with anti-Gal-1 or with phalloidin-TRITC separately or simultaneously. Control of nonspecific binding of second antiserum was also performed with PBS. Briefly, platelets were fixed on slides with 5% formaldehyde, pH: 6.5, during 5 min and permeabilized with 0.5% TritonX-100 in PBS-1% BSA during 5 min. After this, slides were incubated with first antibody (goat anti-Gal-1/100 in PBS-0.1% albumin) and/or with phalloidin-TRITC. After three washes with PBS, anti-goat-IgG-FITC (Sigma, St. Louis, USA) (1/400 in PBS-0, 1% BSA) was added. The specimens were observed and photographed in a Olympus FV1000**,** with the $\times 100$ objective and employing two solid state diode lasers at 473 nm (15 mw) for FITC and 635 nm (29 mw) for TRITC.

3 Results

The yield of protein obtained after purification was 7–10 fg/Plth. Figure 1 shows the results achieved by SDS-PAGE of the purified fraction of Gal-1 isolated by affinity chromatography on the lactose-agarose column. Two bands were observed, corresponding to 14.5 and 55 kDa approximately. In some preparations the 14.5 kDa component was only detected by HPLC as only a faint band appeared in SDS-PAGE. No significant differences were apparent when the platelets were fresh or up to 72 h after bleeding. Also there were no noticeable differences when either freeze-thawing or TritonX-100 treatment was used for platelet lysis. Both protein bands reacted with the anti-Gal-1 antibody. In addition, the band of 55 kDa also reacted with anti-actin antibody, thus indicating the presence of both proteins in this band (Fig. 1a).

Consistent with these results, when this fraction was analyzed by HPLC (Fig. 2) two peaks were detected. Electrospray mass spectrometry determination showed three molecular species present in the first peak (Fig. 2), with masses of 14,777, 14,853 and 14,929 respectively, thus corresponding to the mass of Gal-1 and its derivatives. These values differ from each other by exactly 76, compatible with the formation of derivatives with the β-mercaptoethanol used in the purification procedure. The mass of the proteins present in the second peak could not be determined by this procedure due to apparent heterogeneity.

To confirm these results, purified Gal-1 fraction was submitted to trypsin digestion and identification of the proteins present by mass spectrometry. Peptide masses and MS/MS data were used to identify proteins, using Mascot and Sequest algorithms to search the UniProt/Swiss Prot sequence databases. The results, including fragmentation analyses to infer peptide sequences, allowed the identification of Gal-1 (three peptides sequenced with a significant XCorr score) and actin (15 peptides sequenced with a significant XCorr score), both present in this fraction (Table 1).

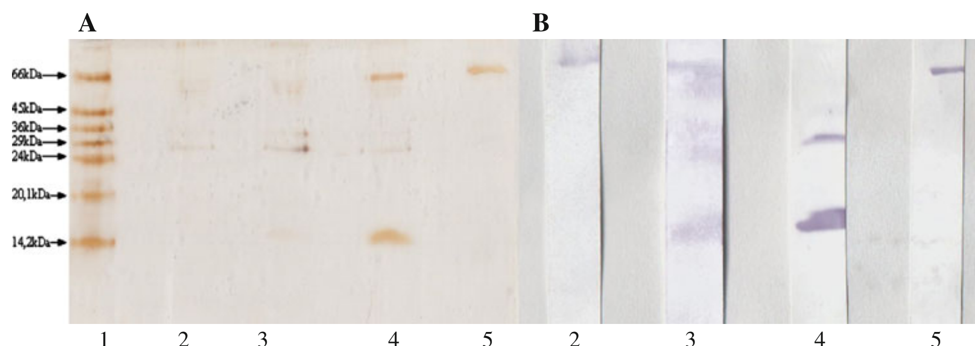

Fig. 1 SDS-PAGE of platelet Gal-1. **a** *Lane 1*: Molecular mass markers: Bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3 phosphate-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.2 kDa); *lanes 2* and *3*, platelet Gal-1; *lane 4*, recombinant Gal-1 (control); *lane 5*, actin (control). **b** Western blot of platelet Gal-1. SDS-PAGE gels were electroblotted onto nitrocellulose membranes. *Lanes 2* and *3*, platelet Gal-1 developed with anti-actin antibody and polyclonal anti-Gal-1 antibody respectively; *lane 4*, recombinant Gal-1 developed with polyclonal anti-Gal-1 antibody (control); *lane 5*, actin developed with anti-actin antibody (control)

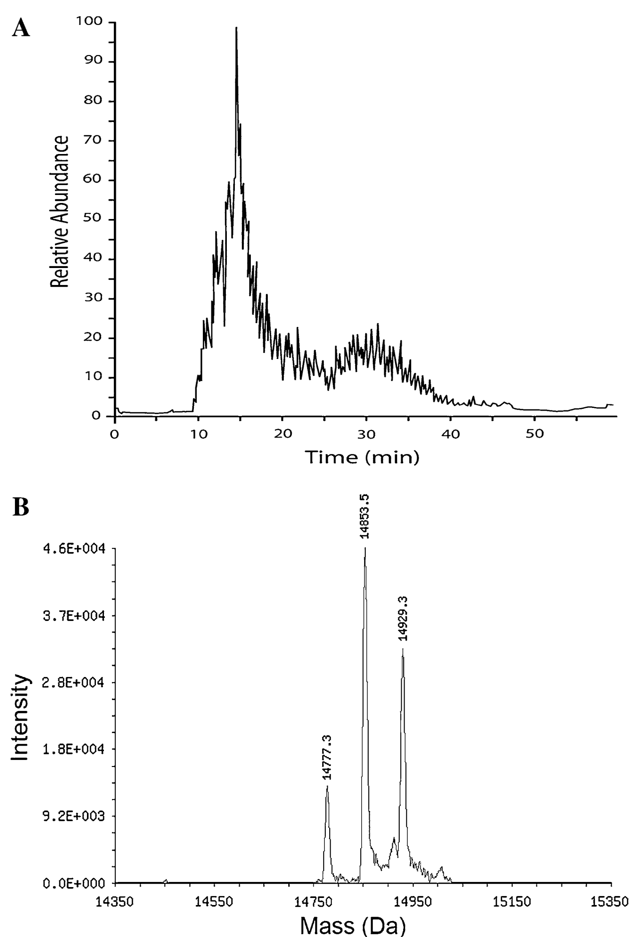

Fig. 2 **a** Reverse-phase chromatography (HPLC) of platelet Gal-1. Chromatography was performed on a C_8 column, operating at 40 μL/min, connected to a Surveyor HPLC System on-line with an LCQ Duo (ESI Ion Trap) mass spectrometer (Thermo Fisher, San José, CA, USA). Proteins were eluted using a 15 min gradient from 10 to 100% solvent B (Solvent A: 2% acetic acid and 2% ACN, Solvent B: 2% acetic acid and 96% ACN. **b** Mass spectrometry analysis of the first peak obtained by HPLC (Fig. 3) on an LCQ Duo (ESI Ion Trap) mass spectrometer (Thermo Fisher, San José, CA, USA. Protein characterization was performed by Full Scan 300–2000 amu and the ProMass deconvolution program

Table 1 Protein identification by mass spectrometry

Protein	Access no.	Mass (kDa)	Peptides sequenced	Coverage (%)
Gal-1	gi 227920	14.6	3	30.3
Actin	gi 112956	42	15	36.5

The specific hemagglutination activity measured in the eluted fraction that contained the Gal-1-actin complex was 10 times lower than that of porcine spleen Gal-1 and 15 times lower than rGal-1 employed in this work. However, the carbohydrate-binding specificity was the same, since hemagglutination was inhibited by lactose and its derivatives but not by a carbohydrate such as saccharose (data not shown).

In relation to the rGal-1/actin interaction studied by hemagglutination, we observed that pre-incubation with actin at concentrations greater than 100 μM inhibited 10 μM rGal-1 (titer 8) hemagglutinating activity (data not shown).

CM studies gave very interesting results. The actin, being one the most abundant molecule of the platelets, was present in extended red areas of the cytoplasm, as shown by phalloidin-TRICT staining, taking advantage of the property of phalloidin to bind specifically at the interface between F-actin subunits, locking adjacent subunits together. For resting platelets, the patterns of Gal-1/actin co-localisation fluorescence (yellow) were not uniform from the qualitative and quantitative point of view. So, some present homogenous patterns centered in the cytoplasm while others showed a scarce and spickled distribution (Fig. 3). On the other hand, in activated platelets which present many pseudopods, an important segregation of Gal-1 seems to occur as a green polarized region appears. In these activated platelets co-localized Gal-1 and abundant actin were also detected.

4 Discussion

In order to extend our studies where we previously detected the presence of ligands for pig spleen Gal-1 in human platelets [15–17], we intended to purified endogen Gal-1 from human platelets. The present work is the first report on the purification of Gal-1 from this source and it was a very difficult and time consuming goal as the amount of protein was very low. Moreover, our results show that Gal-1 co-purifies with actin and forms an actin-Gal-1 complex which is difficult to dissociate, even in the presence of SDS. Other experimental approaches to support the finding of the complex such as HPLC, mass spectrometry, confocal microscopy and hemagglutination assays as mentioned in Results, gave more evidence on this way.

Another factor that may contribute to the low yield of Gal-1 obtained from human platelets could be that upon actin polymerization, a significant amount of Gal-1 may remain as part of an insoluble Gal-1-F actin complex. Proteomic studies did not include Gal-1 among the 50 most abundant proteins of human platelets [24], consistent with the small amount present and the low yield obtained in its purification. Besides, the low specific hemagglutinating activity of the protein obtained suggests that when Gal-1 is in complex with actin, it cannot dimerize and, in consequence, can not hemagglutinate erythrocytes, so that all the activity observed corresponds only to free Gal-1.

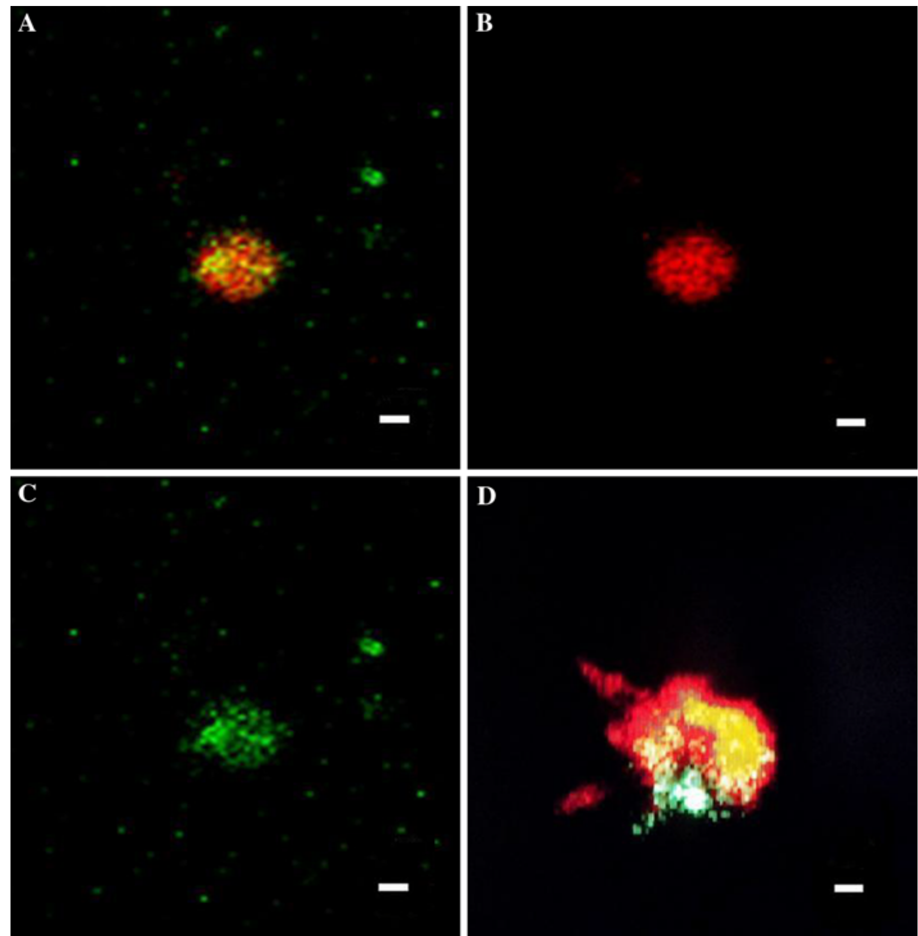

Fig. 3 Localisation of endogenous Gal-1 and actin molecules in resting and activated platelets observed by confocal microscopy. Platelets were fixed and permeabilized as described in Sect. 2. Resting control platelets showing fluorescence for: **a** co-localisation of actin-Gal-1 (*yellow*), **b** actin (*red*), **c** Gal-1 (*green*), **d** thrombin activated platelet showing co-localised area for actin and Gal-1 and areas for both molecules in isolated patterns (*scale bar* = 1 μm) (Color figure online)

On the other hand, actin is a globular protein of 42-kDa molecular weight found in all eukaryotic cells where it may be present at concentrations of over 100 μM. It is also one of the most highly-conserved proteins, differing by no more than 20% in species as diverse as algae and humans. Actin is the monomeric subunit of two types of filaments in cells: microfilaments, one of the three major components of the cytoskeleton and thin filaments. In agreement with these findings, the interaction of lectin–actin has been observed previously. Firstly, in muscle cells indirect evidence was obtained that suggested a relationship between a galactoside-binding protein and actin [8]. Later, employing a biotinylated probe for detecting endogenous ligands for a soluble brain lectin, actin was identified as one of the reacting proteins in a protein–protein recognition pattern and coincidentally both are co-localised in postsynaptic structures [20]. In addition, in MOLT-4 T cells, actin has also been shown to bind to Gal-1 in a carbohydrate-dependent manner [26]. In addition, in human neoplastic astrocytes, Gal-1 modulates gliobastoma cell migration through modifications of the organization of the actin cytoskeleton [6]. The increase in the motility and the reorganization of the cytoskeleton is associated with an increase of the expression of RhoA, a protein that modulates the polymerization and depolymerization of actin [7]. Furthermore, Gal-1-mediated tumor invasion and metastasis involves reorganization of the actin cytoskeleton [37]. Actin has many types of interactions with many structural proteins such as the example of cadherin-based adherens junction proteins. Actin filaments are linked to α-actinin and to the membrane through vinculin. In the process of nucleation, the growth of actin filaments can be regulated by thymosin and profilin. Thymosin binds to G-actin to prevent it from polymerizing while profilin binds to G-actin to promote monomeric addition to one of the ends. In summary, actin has many functions in different cells and subcellular compartments, interacting with many proteins. Gal-1 could be a new member of these actin-binding proteins. In platelets, since Pacienza et al. [27] described that the addition of recombinant Gal-1 to human platelets induces the polymerization of F actin, it can be hypothesized that this polymerization process starts with the formation of the actin-Gal-1 complex. The results presented here suggest that endogenous Gal-1 forms an intracellular complex with monomeric actin and that, after platelet activation, Gal-1 plays a role in the polymerization-depolymerization process

of actin, which concludes in platelet aggregation. Further spectrometric studies to elucidate the nature of the complex bindings are in course.

Lectins from different species produce dose-dependent platelet aggregation. The action of exogenous Gal-1 in heterologous assays on platelets and other cells provides evidence that these cells have receptors for carbohydrate-binding proteins that could induce different biological responses [31–35]. Considering that platelets have marked roles in thrombosis and inflammation, the knowledge of Gal-1 and its possible implications in those pathological conditions is relevant.

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