Novel Role for Galectin-8 Protein as Mediator of Coagulation Factor V Endocytosis by Megakaryocytes*

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Background: Galectin-8 has been suggested to bind platelet FV, but the function is unknown.

Results: Functional absence of galactin-8 in megakaryocytic-like cells impairs the cellular uptake of FV.

Conclusion: Galactin-8 is part of the mechanism involved in the endocytosis of FV.

Significance: Galectin-8 may be a novel regulator of platelet function by mediating the uptake of platelet proteins by megakaryocytes.

Galectin-8 (Gal8) interacts with β-galactoside-containing glycoproteins and has recently been implicated to play a role in platelet activation. It has been suggested that Gal8 may also interact with platelet coagulation factor V (FV). This indispensable cofactor is stored in α-granules of platelets via a poorly understood endocytic mechanism that only exists in megakaryocytes (platelet precursor cells). In this study, we now assessed the putative role of Gal8 for FV biology. Surface plasmon resonance analysis and a solid phase binding assay revealed that Gal8 binds FV. The data further show that β-galactosides block the interaction between FV and Gal8. These findings indicate that Gal8 specifically interacts with FV in a carbohydrate-dependent manner. Confocal microscopy studies and flow cytometry analysis demonstrated that megakaryocytic DAMI cells internalize FV. Flow cytometry showed that these cells express Gal8 on their cell surface. Reducing the functional presence of Gal8 on the cells either by an anti-Gal8 antibody or by siRNA technology markedly impaired the endocytic uptake of FV. Compatible with the apparent role of Gal8 for FV uptake, endocytosis of FV was also affected in the presence of β-galactosides. Strikingly, thrombopoietin-differentiated DAMI cells, which represent a more mature megakaryocytic state, not only lose the capacity to express cell-surface bound Gal8 but also lose the ability to internalize FV. Collectively, our data reveal a novel role for the tandem repeat Gal8 in promoting FV endocytosis.

The human galectin protein family includes 15 members that specifically recognize β-galactosides (1, 2). Depending on their structural organization, galectins can be subdivided into three groups, i.e. the prototypical group comprising a single carbohydrate recognition domain (CRD), the chimeric group containing one CRD and a non-lectin-binding domain, and the tandem repeat group, which consists of two CRDs (3). Galectins have been implicated in a range of biological processes including cell differentiation, cell adhesion, growth regulation, and apoptosis (4). It has been proposed that galectins mainly exert their role by cross-linking specific glycoproteins, thereby triggering intracellular signaling cascades (5).

Originally identified as cytosolic proteins, galectins can also be secreted via an atypical and poorly understood secretory mechanism (6). Secreted galectins are retained at the cell surface or are released into the environment for interaction with the surrounding cells or extracellular proteins (1). Once secreted, galectins may be reinternalized by the cells (7, 8). Through this mechanism, galectins have been suggested to modulate the composition of the extracellular matrix (9, 10). For galectin-3 (Gal3), it has been suggested that it directly contributes to the endocytosis of advanced glycation end products and modified LDL particles (11).

Recent evidence indicates that galectins may also play a role in thrombosis and hemostasis. It has been shown that galectin-8 (Gal8) and galectin-1 (Gal1) can activate platelets (12, 13). Employing mass spectrometry approaches, Romaniuk et al. (12) identified several putative binding partners of Gal8 in platelet lysates. Surprisingly, the list of potential ligands included coagulation factor V (FV), which is critical for proper functioning of the coagulation cascade. FV acts in this cascade as a cofactor of activated factor X (FXa) in the prothrombinase complex (14, 15). A role of Gal8 in FV biology, however, has not been investigated so far.

About 20% of total FV pool in whole blood is stored in a partially activated state in the α-granules of platelets (16, 17). As activated platelets release their protein content at sites of vascular injury, platelets represent a unique source of FV for effec-

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2 The abbreviations used are: CRD, carbohydrate recognition domain; FV, factor V; Gal, galectin; PS, phosphatidylserine; TDG, D-galactopyranosyl-β-D-thiogalactopyranoside; TPO, thrombopoietin.
Galectin-8 Mediates Factor V Endocytosis

The source of platelet FV has been debated for years. Although it was initially suggested that megakaryocytes synthesize FV (19), it has now been established that platelet FV is taken up from plasma by megakaryocytes via receptor-mediated endocytosis (16, 20). Megakaryocytes appear to transiently express the proteins that contribute to FV internalization as the uptake process is absent in platelets (21). The actual mechanism behind the endocytic uptake of FV, however, is still unclear. It has been proposed that FV first binds to the cell surface via an unknown cellular component, after which it is internalized via low density lipoprotein receptor-related protein 1 (LRP-1) (22).

During biosynthesis by hepatocytes, FV undergoes post-translational modifications including extensive O- and N-linked glycosylation (23). In addition to a role in the biosynthesis and half-life of FV, a carbohydrate moiety on FV has been suggested to contribute to the regulation of FV cofactor function (23, 24). Although the role of glycosylation for FV biology has been investigated, no data are currently available on a possible function of the carbohydrate side chains for FV endocytosis.

The potential endocytic role of galectins and the suggestion that Gal8 may bind FV prompted us to assess the role of Gal8 in the cellular uptake of FV. Employing a biochemical and a cell-based approach, we show that Gal8 binds FV in a carbohydrate-dependent manner, and we demonstrate that Gal8 mediates endocytosis of FV.

**EXPERIMENTAL PROCEDURES**

**Materials**—D-Galactopyranosyl-β-D-thio-galactopyranoside (TDG) was from Carbosynth (Compton, Berkshire, UK). Unless specified, all the other chemicals were from Sigma-Aldrich.

**Proteins and Antibodies**—Recombinant full-length human Gal8 and Gal1 were purchased from R&D Systems (Minneapolis, MN), and bovine lactadherin was from Hematologic Technologies (Essex Junction, VT). Plasma-derived FV was purified as described (25). Mouse monoclonal anti-factor V (sc-130566), anti-Gal1 antibody, and goat polyclonal anti-Gal8 antibody were from Santa Cruz Biotechnology (Bergheim Heidelberg, Germany). FITC-conjugated mouse IgG isotype control was from Dako (Glostrup, Denmark), FV-HRP sheep anti-human FV was from Kordia (Leiden, The Netherlands), AlexaFluor 488- or 568-conjugated secondary antibodies were from Invitrogen (Breda, The Netherlands), and anti-Gal3 was from Abcam (Huisen, The Netherlands).

**Cell Culture and Treatment**—DAMI cells were from the American Type Culture Collection (ATCC CRL 9792). Cells were cultured in DMEM F-12 supplemented with 10% HyClone FCS (Thermo Fisher Scientific), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen) at 37 °C in humidified atmosphere with 5% CO₂. To ensure logarithmic growth, cells were subcultured every 2 days. A cell density of 1 × 10⁶ ml⁻¹ was employed for the experiments. When required, cells were induced to differentiate for 72 h with 10 ng/ml thrombopoietin (TPO) (Sanquin Pelikines, Amsterdam, The Netherlands) in complete medium. Platelets were obtained from healthy donors and collected in EDTA buffer as described before (26) in accordance with Dutch regulations and following approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki.

**Factor V-Galectin Binding Studies**—Surface Plasmon Resonance (SPR) analysis was performed using a BIACoreTM3000 biosensor system (Uppsala, Sweden) essentially as described (27). Plasma-purified FV was coupled to CM5-sensor chip to a density of 1500 response units using the amine-coupling kit as indicated by the manufacturer. Increasing concentrations of Gal8 (1.5–75 nM) were passed over the chip at a flow rate of 20 μl/min at 25 °C in HEPES binding buffer (20 μM HEPES (pH 7.4), 150 mM NaCl, 5 mM CaCl₂, 0.005% (v/v) Tween 20). The sensor chip surface was regenerated by three repeated washes in the same buffer containing 1 m NaCl. Competition experiments were performed by flowing 50 nM Gal8 over immobilized FV (1700 response units) in the presence of increasing concentrations of lactose, sucrose, and TDG dissolved in binding buffer.

Enzyme-linked immunoassay (ELISA) was performed in 96-well microtiter plates (Nunc, Roskilde, Denmark). Wells were coated overnight at 4 °C with recombinant human Gal8 (5 μg/ml) in 50 mM sodium carbonate-bicarbonate buffer (pH 9.8). Plates were then blocked for 1 h at 37 °C with 2% human serum albumin (Celab) in Tris-HCl binding buffer (TBS) (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM CaCl₂, 2 mM MgCl₂, and 0.05% (v/v) Tween 20). Appropriate dilutions of FV were subsequently incubated with Gal8 for 1 h at 37 °C. After washing with binding buffer to remove unbound FV, binding was assessed by utilizing anti-human FV-HRP. The absorbance at 540 nm/450 nm was measured on a SpectraMax Plus 384 (Molecular Devices). For the competition experiment, 15 nM FV was incubated with increasing concentrations of lactose, sucrose, and TDG in binding buffer for 1 h at 37 °C. The mixture was then incubated with immobilized Gal8 on the plate for 1 h at 37 °C. Residual binding was assessed as described above.

**Assessment of Cellular Uptake of Factor V and Expression of Galectins**—FV was labeled with FITC dye (mole ratio 1:3 FITC) (FluoReporter® FITC protein labeling kit, Invitrogen) according to the manufacturer’s instructions. To assess FV uptake, DAMI cells were incubated with FITC-FV in serum-free medium at 37 °C. For blocking experiments with antibodies, DAMI cells were first preincubated for 30 min at 37 °C in the presence of 4 μg/ml anti-Gal8 or anti-Gal1 antibody in serum-free medium. After the incubation with FITC-FV, cells were washed with ice-cold TBS, fixed with 1% freshly dissolved formaldehyde in PBS, and collected in EDTA buffer as described before (26) in accordance with Dutch regulations and following approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki.

Galectin expression by DAMI cells or purified platelets was assessed by fixing the cells as described above followed by an incubation for 1 h at 4 °C with antibodies directed against Gal8, Gal1, or Gal3. These antibodies were diluted 500-fold in phosphate-buffered saline (PBS), 1% (w/v) human serum albumin,
and 0.05% (w/v) saponin. After removal of the unbound antigalactin antibody by washing with PBS, the cells were incubated for 1 h at room temperature with the secondary Alexa Fluor 488-conjugated antibody. The cells and platelets were subsequently analyzed by flow cytometry.

**Flow Cytometry Analysis**—Flow cytometry analysis was performed on a FACS LSRII (BD Biosciences, Uppsala, Sweden) as described (27). Appropriate negative controls with Alexa Fluor 488-conjugated or FITC-conjugated isotype IgG were included in all the experiments. The analysis regions to obtain the percentage of FITC-FV or galectin-positive cells were set such that less than 2% of the control cells were considered positive. When required, the significance of the difference between positive cells was assessed employing a Student's *t* test. *p* values < 0.05 are indicated in Figs. 1, 3, 6, and 7 with a single asterisk; *p* values < 0.01 are indicated with double asterisks, and *p* values < 0.001 are indicated with triple asterisks.

**Immunofluorescence Analysis**—Cells were washed with ice-cold PBS and fixed for 30 min at 4 °C in 4% *p*-formaldehyde. When required, whole cells were stained with carboxyfluorescein succinimidyl ester employing 1 μM carboxyfluorescein diacetate succinimidyl ester before fixation as indicated by the manufacturer. FV, Gal1, Gal3, and Gal8 were visualized by incubating the cells with the appropriate antibodies, which were diluted 500-fold in PBS, 1% (w/v) human serum albumin, and 0.05% (w/v) saponin. After washing with PBS, the cells were incubated with the appropriate secondary antibody (Alexa Fluor 488-conjugated antibody for galectin staining and Alexa Fluor 568-conjugated antibody for FV staining) that was diluted 500-fold in the same buffer. The cell suspension was then covered with a coverslip to allow cell settlement overnight at 4 °C. Subsequently, samples were analyzed by confocal microscopy using the appropriate filter settings and using a Plan-Neofluar 63×/1.3 oil immersion lens (Zeiss LSM 510, Carl Zeiss, Heidelberg, Germany).

**Gal8 siRNA Gene Silencing**—Three Gal8-specific (sense and antisense) 20–25-nucleotide siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The sense strands are: 5’-GAAUCUGACAGAUAGUA-3’, 5’-CAACACUUAGCAGAGUAU-3’, and 5’-CCAGUAUGUUCGUCUCGUUC-3’. The nontargeting control siRNA pools were from Dharmacon (Thermo Fisher Scientific). 6 μl of 50 μM Gal8 siRNA or nontargeting control siRNA were added to 3 × 10⁶ DAMI cells in serum-free medium. Cells were then pulsed at 250 V, 150 microfarads, and infinite resistance in a Bio-Rad Gene Pulser (Bio-Rad) as described (27). Gal8 knockdown was confirmed after 48 h by incubating the cells for 30 min at 4 °C with anti-Gal8 as described above. The fluorescence intensity of the cells was assessed by flow cytometry analysis.

**RESULTS**

**DAMI Cells Efficiently Endocytose FV**—The megakaryoblastic cell line DAMI is derived from the peripheral blood of a patient with megakaryoblastic leukemia (28). These cells have characteristics of immature megakaryocytes and have recently been shown to generate platelet-like particles after differentiation (29). We now used these cells as an experimental model to study FV endocytosis. DAMI cells were incubated with increasing concentrations of FITC-FV for 1, 2, and 3 h at 37 °C, and FV uptake was monitored by flow cytometry (Fig. 1A). The result showed that the percentages of FITC-FV-positive cells increased dose- and time-dependently. The uptake seemed to reach saturation, especially at the highest FV concentration. The ability of the cells to internalize FV was confirmed by confocal microscopy analysis employing immunofluorescence staining of FV as described under “Experimental Procedures.” The top three panels show the whole cell in red (carboxyfluorescein succinimidyl ester staining) and FV in green. The white scale bar represents 5 μm. The bottom panel shows a side view of the cell that was obtained employing z-stack analysis of the cell displayed in the upper panel (cell height: 4 μm).

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presence of a 7-fold excess of the unlabeled protein and almost completely ablated at 4 °C (Fig. 1C). These data seem to be consistent with an active receptor-mediated saturable process (20). As FV is known to bind with high affinity phosphatidylserine (PS)-containing phospholipids (30), we investigated whether PS might also contribute to the uptake of FV by the cells. Fig. 1C shows that the cellular uptake of FV was not affected by the presence of a 30-fold excess of lactadherin, which binds PS with a high affinity. This result excludes any contribution of PS to the endocytic uptake of FV. Taken together, the data show that FV is internalized by DAMI cells.

Gal8 and Gal1 Are Expressed on Surface of DAMI Cells—DAMI cells were characterized for the expression of galectins. In particular, we monitored the expression of Gal1, Gal3, and Gal8. Confocal microscopy analysis showed that DAMI cells are positive for Gal8 and Gal1 (Fig. 2A). Gal3, which is mainly expressed by epithelial and immune cells (31), was poorly detectable in DAMI cells. The expression of Gal8, Gal1, and Gal3 was further assessed by FACS analysis. To this end, galectin detection was performed with anti-galectin antibodies on nonpermeabilized or permeabilized cells. This approach allows for the detection of extracellular cell surface-bound galectins and the total amount of galectins (extracellular and intracellular). The histograms in Fig. 2B and supplemental Fig. S2 show that there is an extracellular pool of Gal8 and Gal1 on DAMI cells. In agreement with confocal analysis, hardly any Gal3 was detected within or on the cells. The observation that DAMI cells internalize FV and express Gal8 on their surface implies that these cells represent a good model system to study the role of Gal8 for FV uptake.

β-Galactosides Inhibit FV Endocytosis and FV Binding to Gal8—As β-galactosides are typical ligands for galectins, like Gal8, we evaluated the endocytic uptake of FV in the presence of N-acetylglucosamine (GlcNAc), d-mannose, mannan, and lactose, and the lactose analog TDG. The latter two compounds are β-galactosides and are known to block carbohydrate-dependent binding of galectins to their ligands. Fig. 3 shows that the uptake of FV by DAMI cells is markedly reduced in the presence of TDG and lactose. However, mannan, d-mannose and GlcNAc did not affect FV internalization by the cells. Taken together, these findings suggest a specific role of the FV glycans in promoting FV uptake by cells.

To assess whether Gal8 binds FV in a carbohydrate-dependent manner, we investigated the binding of Gal8 to FV in the presence of β-galactosides with surface plasmon resonance analysis (SPR) analysis. We first passed increasing concentrations of Gal8 over immobilized FV (Fig. 4A). The data revealed a reversible and dose-dependent binding of Gal8 to immobilized FV. The binding curves revealed, however, complex kinetics. This result is not unexpected as Gal8 may bind multiple carbohydrates on the heavily glycosylated FV. This finding does, however, preclude a straightforward assessment of the equilibrium dissociation constant ($K_D$) of the Gal8-FV complex. To still gain insight into the $K_D$, we estimated the binding response at equilibrium for each Gal8 concentration by fitting the association phase to a one-site association model.
The obtained estimated binding response at equilibrium was subsequently plotted as a function of the employed Gal8 concentration (Fig. 4A, inset). The results revealed that the apparent $K_{D}$, which is reflected by the Gal8 concentration at which half-maximum binding is reached, is about 30 nM for the FV-Gal8 complex. This value is in the same range as the plasma concentration of FV, suggesting that this interaction may occur under physiological conditions.

We subsequently assessed the binding of Gal8 to FV in the presence of lactose, TDG, and sucrose (Fig. 4B). As the presence of these carbohydrates produces unwanted buffer effects during the association phase, we plotted the residual binding of Gal8 to FV at the start of the dissociation phase. The data revealed that the $\beta$-galactoside-containing compounds effectively block the association of Gal8 to FV. In Fig. 4B, the inset also shows the association to FV of 50 nM Gal8 and 50 nM Gal1. The results showed that there was hardly any association of Gal1 to FV.

In a complementary setup, we evaluated the carbohydrate dependence of the interaction between Gal8 and FV in a solid phase binding assay. We first assessed the maximum binding response for the association of FV with immobilized Gal8 (Fig. 5, inset). Next, we demonstrated by competition experiments that both TDG and lactose prevent the interaction between Gal8 and FV in a dose-dependent manner (Fig. 5). In agreement with the results obtained with SPR analysis, these data support the observation of a specific and glycan-dependent binding of Gal8 to FV.

**Surface-bound Gal8 Is Crucial for FV Uptake**—The blocking effects of lactose and TDG on FV internalization by cells as well as on the direct binding of Gal8 to FV suggest a role for Gal8 as a mediator of FV uptake. This hypothesis was verified by reducing the functional presence of Gal8 in DAMI cells. First, we reduced Gal8 expression by employing siRNA technology (Fig. 6, A and B). The results show that cells transfected with Gal8 siRNA, but not those with nontargeting siRNA, have a markedly reduced ability to endocytose FV. This observation provided additional evidence that Gal8 contributes to FV endocytosis. Subsequently, we evaluated FV uptake by DAMI cells in the presence of a blocking antibody directed against Gal8. As a
control, we monitored FV uptake in the presence of an antibody against Gal1 (Fig. 6, C and D). The data revealed that FV uptake was blocked by about 70% in the presence of anti-Gal1 but remained unchanged in the presence of the anti-Gal8 antibody. The above data together demonstrate that Gal8 is of principle importance for mediating endocytosis of FV.

Differentiation of DAMI Cells with TPO Leads to Loss of Surface-bound Gal8—Previous studies reported that platelets express Gal8 on their surface only after thrombin stimulation (12). Because differentiated DAMI cells are able to produce platelet-like particles (29), we assessed whether these cells also lack cell surface expression of Gal8. To this end, we monitored the presence of cell surface-bound Gal8 in TPO-differentiated DAMI cells employing flow cytometry. Fig. 7A reveals that Gal8 was hardly detected on nonpermeabilized platelets and non-permeabilized TPO-differentiated DAMI cells. In contrast, Gal8-positive cells were identified in TPO-differentiated DAMI cells upon permeabilization of these cells. This finding shows that, similar to platelets, Gal8 is not present at the cell surface after differentiation of the DAMI cells with TPO. Intriguingly, the loss of surface-bound Gal8 correlated with a markedly reduced uptake of FV by the cells (Fig. 7B). The data together demonstrate that reducing the functional presence of extracellular Gal8 markedly impairs the cellular uptake of FV. This finding may also provide an explanation why platelets do not support the uptake of FV from plasma.

DISCUSSION

Gal8 has previously been implied to contribute to cell adhesion, cell growth, and apoptosis (2, 3). Recently, it has been suggested that Gal8 may interact with platelet FV (12). Employing independent approaches, we now reveal a novel role for Gal8 as a mediator for the endocytic uptake of FV. First, we show that the endocytic uptake of FV is reduced by 1/H9252-galactosides (Fig. 3). Second, we demonstrate with SPR analysis and a solid phase binding assay that Gal8 binds FV in a carbohydrate-dependent manner (Figs. 4, A and B, and 5). Third, reducing the expression of Gal8 employing siRNA technology impairs FV uptake (Fig. 6A). Fourth, FV internalization is effectively inhibited by an antibody directed against Gal8 (Fig. 6, C and D).

FIGURE 5. Enzyme-linked immunosorbent assay of carbohydrate-dependent Gal8-FV complex formation. 15 nM FV was incubated with Gal8 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 2 mM MgCl2, and 0.05% (v/v) Tween 20 for 1 h at 37 °C. Binding was assessed as described under “Experimental Procedures” in the presence of increasing concentrations of lactose (circles), sucrose (squares), or TDG (triangles). Data represent residual FV binding to Gal8 and are the mean values ± S.D. of three experiments. The inset shows increasing concentrations of FV that were incubated with immobilized Gal8. FV binding is expressed as the percentage of the maximum binding response.

FIGURE 6. Gal8 mediates endocytosis of FV. A, 40 nM FITC-FV was incubated for 3 h at 37 °C with DAMI cells transfected with nontargeting (scramble) siRNA or Gal8 siRNA as described under “Experimental Procedures.” The uptake of FITC-FV was assessed by flow cytometry analysis. Shown is the percentage of FITC-FV-positive cells. B, a representative histogram of Gal8-positive cells after transfection of the cells with Gal8 siRNA (dotted line) or nontargeting siRNA (solid line). The gray histogram represents the background fluorescence of control cells when the primary antibody was omitted from the staining procedure. C, 40 nM FV in serum-free medium was incubated for 3 h at 37 °C with DAMI cells that were preincubated for 30 min with 4 μg/ml anti-Gal8 antibody or 4 μg/ml anti-Gal1 antibody. The percentage of FITC-FV-positive cells was assessed by flow cytometry. D, representative histograms of FITC-FV uptake in control (solid line) and antibody-pretreated (dotted line) cells. The gray histograms represent the background fluorescence of the cells. All data reported are the means ± S.D. of at least three experiments.
Gal8 and Gal1, which have been suggested to contribute to platelet function (12, 13), are also expressed on the surface of DAMI cells. Although Gal1 and Gal8 are both known to bind $\beta$-galactosides, our findings remarkably demonstrate that only Gal8 effectively interacts with FV (Fig. 4B, inset). On the other hand, it has been shown that modifications in a CRD domain affect the fine specificity of the galectins for complex carbohydrates (32). Apparently, FV comprises a glycan structure that can be effectively bound by Gal8 and only poorly bound by Gal1. A different specificity for a particular glycan has also been demonstrated for the two CRDs within Gal8 itself (33). The N-terminal CRD has been reported to exhibit a high affinity for 3'-O-sulfated or 3'-O-sialylated glycoconjugates (34), whereas the C-terminal CRD domain preferentially binds poly-$N$-acetyl-lactosamine glycans (33). It therefore seems feasible that one CRD of Gal8 specifically binds FV, whereas the other CRD may have a preference for binding glycans expressed on the cell surface.

The reported ability of Gal8 to induce PS exposure on the surface of cells (35, 36) and the known affinity of FV for PS (30) raised the possibility that the uptake mechanism may be dictated by PS exposure. However, we found that lactadherin, which binds PS with a high affinity and shares homology to the PS-binding domains of FV (30), completely failed to prevent FV uptake (Fig. 1C). This finding demonstrates that exposure of PS is not part of the mechanism of Gal8-mediated FV uptake at all.

**FIGURE 7.** Gal8 surface expression and FV uptake are impaired in TPO-differentiated DAMI cells. **A**, the upper panels show representative FACS histograms of the extracellular staining of Gal8 in nonpermeabilized platelets and TPO-treated DAMI cells (DAMI + TPO). Intracellular Gal8 staining in TPO-treated DAMI cells (permeabilized) was included as control. The gray histogram represents the background fluorescence of the platelets and cells. The lower panel displays the percentage of Gal8-positive DAMI cells (DAMI - TPO), platelets, and permeabilized and nonpermeabilized TPO-differentiated DAMI cells (DAMI + TPO). Data reported are the means ± S.D. of at least three experiments. **B**, left panel, 40 nM FITC-FV was incubated with DAMI cells and with TPO-differentiated DAMI cells for 3 h at 37 °C in serum-free medium. The data represent mean ± S.D. of three independent experiments as assessed by flow cytometry. Right panel, representative histograms of FITC-FV uptake in DAMI cells (solid line) and in TPO-differentiated DAMI cells (dotted line). The gray histograms represent the background fluorescence of the cells. n.s., nonsignificant.
Galectin-8 Mediates Factor V Endocytosis

Galectin-8 does not comprise an endocytic signal sequence to directly mediate endocytosis of FV. It has, however, been suggested that purified Gal8 can be internalized by CHO cells via an unknown mechanism classified as non-clathrin-dependent and non-caveolae-dependent (33). Further research is required to assess whether the same is true for the uptake of FV by DAMI cells. Most galectins, however, exert their role by cross-linking counter receptors on or in the cell (5). This suggests that Gal8 may also require a counter receptor to mediate the uptake of FV. Interestingly, Bouchard et al. (22) previously proposed that FV uptake by megakaryocytes involves a two-step mechanism in which FV first binds an unidentified cell surface element, after which it is transferred to LRP-1 for receptor-mediated endocytosis. If so, our data suggest that the initial binding event may involve Gal8. Alternatively, it has been suggested that galectins may bind integrin ectodomains to exert their biological function (1, 2). It has, for instance, been demonstrated that Gal3 can regulate the content of the extracellular matrix via β-1 integrin-mediated endocytosis (37). Gal8 has further been shown to interact on neutrophils with the integrin αM (38). Intriguingly, Romaniuk et al. (12) recently demonstrated that Gal8 binds directly to the platelet integrin αIIbβ3, which is involved in the endocytic uptake and storage of fibrinogen (39). Possibly, Gal8 may act as a bridging molecule between FV and αIIbβ3, thereby driving the uptake and storage of FV by megakaryocytes. As galectins have also been suggested to play a role in intracellular targeting of proteins (32), Gal8 may not only be involved in the endocytic uptake of FV but also in the direct delivery of FV to the α-granules.

Unlike fibrinogen, FV is internalized by the megakaryocytes only during a specific stage of their differentiation. Human CD34+ bone marrow cells have been shown to endocytose FV only from day 7 to day 10 of the differentiation process into megakaryocytes (20). Compatible with this observation is the notion that platelets completely lack the ability to internalize FV (21). This implies that one or more critical structural elements, which contribute to the uptake of FV, are transiently expressed during megakaryocyte maturation. In this view, it is an intriguing finding that Gal8 is no longer detected at the cell surface after differentiation of DAMI cells with TPO (Fig. 7A). This correlated with the impaired ability of these cells to internalize FV (Fig. 7B). Gal8 was, however, still present inside the differentiated cells (Fig. 7A). The same was observed for platelets. Although Gal8 is still present inside platelets, it is not detected at the platelet surface (Fig. 7A). The differentiation-regulated presence of Gal8 at the cell surface is not unique to Gal8. It has been demonstrated that maturation of dendritic cells results in pronounced changes in glycan expression at the cell surface, which affects recognition by galectins (40).

A potential pathophysiological role of Gal8 in disorders that relate to FV deserves further investigation. Intriguingly, patients with a functional absence of FV in plasma rarely exhibit a severe bleeding tendency. It has been suggested that the level of platelet FV is a better predictor for bleeding severity in these patients than the level of plasma FV (41). The reasons underlying different levels of FV in platelets are, however, still unclear. Our findings demonstrating the requirement of Gal8 to promote FV uptake by megakaryocytes might provide a new perspective for understanding this aspect of FV biology. Further, we cannot exclude the possibility that Gal8 may play a role in modulating the risk for thrombosis in carriers of the FV Leiden mutation (42).

Taken together, our observations show that Gal8 contributes to endocytosis of FV. This study reveals a putative novel role of Gal8 as a regulator of platelet function by mediating the uptake of platelet proteins by megakaryocytes.

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