INS AND OUTS OF PLATELET α-GRANULE PROTEINS

Claudia Zappelli

Cover illustration: representation of platelet exocytosis. This oil on canvas by Gennaro Cicalese shows the City of Naples and the Mount Vesuvius at night. Mount Vesuvius explosion mimics platelets activation and exocytosis. The eruption finally results in the release of the α-granule protein Factor V.

ISBN 978-94-6182-446-2

The work described in this thesis was performed at the Department of Plasma Proteins, Sanquin Research, Amsterdam, The Netherlands.

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

Additional financial support was obtained from Sanquin Research.

INS AND OUTS OF PLATELET α-GRANULE PROTEINS

Ins en outs van α-granule-eiwitten van bloedplaatjes

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op maandag 7 juli 2014 des middags te 2.30 uur

door

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geboren op 18 april 1983, te Napels, Italië

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CHAPTER 1

General Introduction

SCOPE OF THE THESIS

Platelets are critical for effective bleeding arrest at sites of vessel injury by contributing to the formation of the haemostatic plug. In addition to strong adhesion to the damaged site, platelets tune this process by secreting several critical proteins of which most are stored in the platelet α-granules. The protein cargo of the α-granule mainly originates from biosynthesis by the platelet progenitor cells the megakaryocytes. However, the α-granules also comprise proteins that are taken up from plasma via largely unknown receptor-mediated processes. Besides the uptake and storage of α-granule proteins, the mechanisms involved in the regulated release of α-granules have also remained poorly understood. Elucidating these mechanisms will greatly enhance our understanding of bleeding disorders that are related to defects in α-granule protein loading and release.

In the present thesis, the focus is on the mechanism behind endocytosis of factor V (FV) and fibrinogen. It has previously been proposed that the platelet glycoprotein (GP) IIbIIIa (integrin αIIbβ3) mediates uptake of fibrinogen. Employing platelets of Leukocyte Adhesion Deficiency type III (LAD-III) patients with a defective integrin activation machinery, and platelets of Glanzmann's thrombasthenia (GT) patients, which lack GPIIbIIIa, the role of integrins in α-granule protein cargo enrichment of FV, fibrinogen and other α-granule proteins is investigated by mass spectrometry analysis. Pull-down experiments have previously suggested that glycan binding protein Galectin-8 (Gal8) may bind both FV and GPIIbIIIa and GPIb-IX-V. The role of Gal8 for FV endocytosis as well as its putative interaction with the platelet glycoproteins is assessed employing megakaryocyte-like cells and protein binding studies. Furthermore, it has previously been proposed that mammalian uncoordinated-18 (Munc18-2) is critical for α-granule release. Yet, bleedings are inconsistently observed in Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL-5) patients that exhibit a functional absence of Munc18-2. Employing the platelets of FHL-5 patients, the role of this protein for the release of α-granule proteins is re-evaluated utilizing flow cytometry, mass spectrometry analysis and electron microscopy studies. In the following paragraphs, an overview of the current knowledge of platelet structure, as well as description of diseases associated with platelet dysfunctions is reported. A summary of the questions addressed in this thesis will follow at the end of this chapter.

GENERAL INTRODUCTION

The first description of particles in blood smaller than leukocytes and erythrocytes is dated at the end of the nineteenth century. Osler described in 1873 disk-like structures that circulate throughout the blood stream and aggregate upon removal[1]. The question whether these particles are blood-derived biological components or an exogenous "organism" remained open. It is now commonly accepted that in the biennium 1881-1882 Giulio Bizzozzero was the first to describe the central role of these blood elements in thrombosis and haemostasis. Bizzozzero named these elements «piastrine», i.e. small plates and in 1882 a translation into German as «blut plättchen» and into French as «petites plaques» was established[1,2]. Thanks to these outstanding pioneers, we can nearly 140 years later confidently describe blood platelets as discoid anucleated blood cells that are produced in the bone marrow by the megakaryocytes[3,4].

In spite of their small size, platelets are highly specialized effector cells comprising a high quantity of receptors that act in concert to achieve the primary physiological function of platelets i.e. to arrest bleeding at the site of vascular injury. Platelets are not only simple components of the haemostatic plug. They actively participate in the initiation and support of the coagulation cascade, and are carriers of many critical factors necessary to restore a normal haemostatic balance[4,5]. Platelets also participate in the regulation of other critical biological processes beyond haemostasis and thrombosis[6]. Platelets have been suggested to support inflammatory and immune responses by recruiting leukocytes and progenitor cells to sites of injury. They further play a role in essential steps required for tissue repair by inducing changes in cell permeability and to promote chemotaxis and cell proliferation[7,8].

Given the central and multiple roles played by platelets, it is of critical importance to unravel platelet function. Yet, in spite of the significant progress made in understanding platelet biology, many questions have remained unanswered.

Platelet structure and functions

Platelet structure

With a reported size between 2-4 µm platelets represent the smallest circulating cellular element. A schematic representation of platelet structure is shown in Figure 1. Although appearing smooth, the platelet membrane comprises numerous invaginations to the interior of the cell. These invaginations form the so-called open canalicular system (OCS), which provides a route for the release of platelet proteins upon platelet stimulation[9]. The platelet intracellular space itself is loaded with three types of secretory granules, i.e. α-granules, dense granules, and lysosomes. With approximately 50–80 granules per platelet, the α-granules are the most abundant among the three types of secretory granules[5,10]. They occupy about 10% of the platelet volume, which is about 10-fold more than the dense granules[5].

The α-granules are the major storage organelles for more than hundred distinct proteins. These proteins can be subdivided on the basis of the biological function in 9 subgoups[5,11], i.e. adhesive proteins, clotting factors and their inhibitors, fibrinolytic factors and their inhibitors, proteases and inhibitors, growth and mitogenic factors, chemokines, cytokines, anti-microbial proteins and membrane glycoproteins[5,11]. Dense granules contain platelet agonists such as adenine nucleotides (ADP), Ca^{2+}, and signaling molecules such as histamine, serotonin, and epinephrine[12].

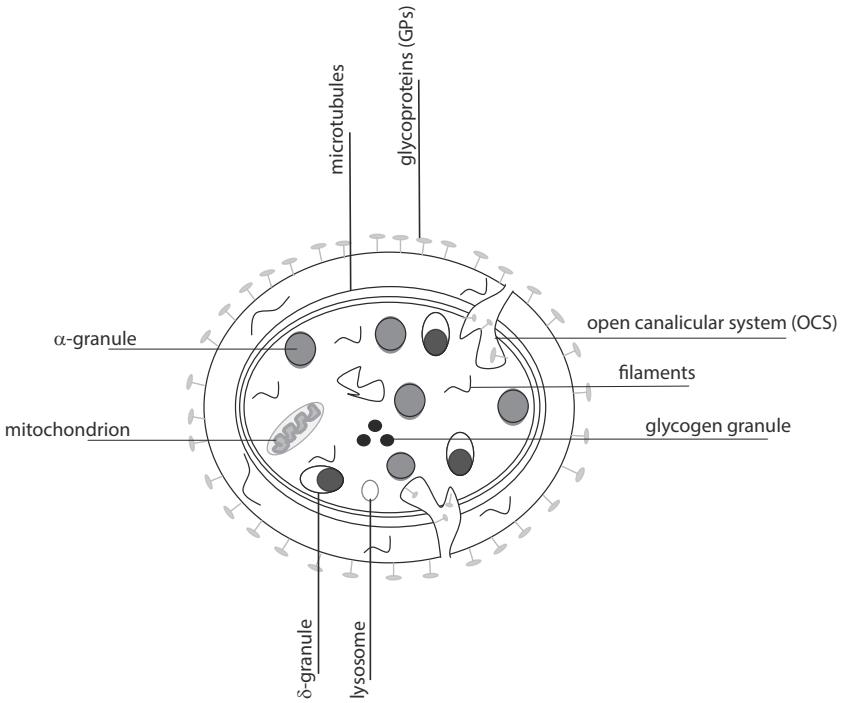

Figure 1. Schematic diagram of a platelet. Platelets are 2–4 μm discoid cells containing α-granules, dense granules, and lysosomes. Platelets also contain mitochondria and glycogen granules. Platelet membrane is rich in glycoproteins that act as critical receptors for platelet function. Invaginations of the plasma membrane form a complex membrane network, named open canalicular system.

Lysosomes contain a mixture hydrolases and glycohydrolases, e.g. β-glucuronidase, cathepsins, β-hexosaminidase, β-galactosidase, endoglycosidase, elastase, and collagenase.

Platelet activation and clot formation

Platelets show no interaction with the endothelial cells of the inner vascular lining under normal conditions. However, when the sub-endothelial matrix becomes exposed to the blood stream at sites of vessel injury, a coordinated series of molecular events take place leading to platelet clot formation[13–15]. A summary of specific events that can be identified is given below and schematically illustrated in Figure 2.

1- Platelet tethering

Upon vessel injury, the endothelial cells of the vascular lining become activated leading to the local release of high concentrations of von Willebrand factor (VWF). The disruption of the endothelial layer integrity also causes the exposure to the circulation of collagen and other sub-endothelial matrix proteins. Collagen and the released VWF are key players in the initial recruitment of platelets to the site of injury. In arteries, the local shear forces induced by the

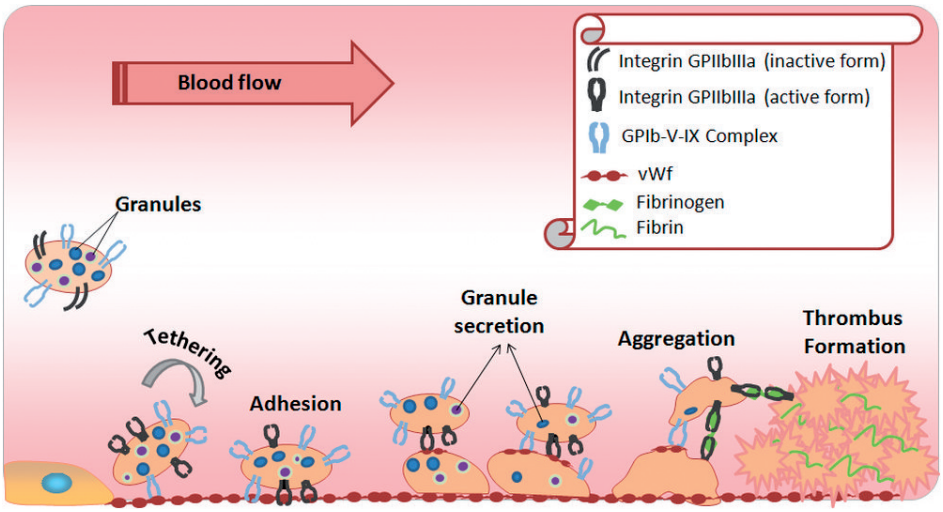

Figure 2. Illustration of the multi-step platelet activation and thrombus formation mechanism. Upon vascular injury, platelets rapidly tether to subendothelial-bound VWF through GPIb/V/IX. Tethered platelets subsequently roll over the damaged vessel surface and then form stationary adhesion contacts through one or more platelet integrins (e.g. the GPIIbIIIa integrin). Once adherent, platelets release their granule contents and expose endogenous and plasma VWF on their surface membrane. The immobilized VWF on the surface of platelets serves to recruit additional platelets via GPIb/V/IX. Stable platelet–platelet adhesion contacts are mediated by fibrinogen molecules through integrin GPIIbIIIa. Fibrinogen is then cleaved by thrombin generating fibrin molecules, which in turn, can cross-link leading to protofibrils. Thus a blood clot consists of a plug of platelets enmeshed in a network of insoluble fibrin molecules.

high blood flow rate assist in the tethering of platelets to VWF and collagen[16,17]. This is, however, not a tight interaction and results in rolling of platelets over the site of injury. A key platelet receptor that plays an indispensable role in this interaction is the complex of the glycoproteins Ib, IX and V (GPIb-IX-V)[18,19]. This complex mediated effective binding to VWF via the GPIb. In the venous system, in which the shear stress is markedly reduced compared to the arterial system, platelets can directly bind collagen and other matrix proteins such as fibronectin and laminin[19].

2- Platelet activation and aggregation

Firm and stable platelet adhesion require additional receptor–ligand interactions. Of critical importance is the interaction of collagen to the platelet collagen receptor GPVI[19]. This triggers potent intracellular signals (inside-out signaling) leading to a conformational change of the platelet integrins. Integrins are heterodimeric proteins consisting of α and β subunits and include α2β1, α5β1, α6β1, αVβ3 and αIIbβ3[20]. αIIbβ3 is also referred to as GPIIbIIIa. The conformational change that takes place upon integrin activation leads to high affinity interaction with proteins of the subendothelial matrix. This assists in tight binding of the loosely tethered platelets to the site of injury[21]. The key regulatory event in the formation of platelet aggregates is the activation of GPIIbIIIa. Activated GPIIbIIIa can bind circulating fibrinogen as well as VWF and contributes to the formation of cross-linked structures responsible of the platelet plug stability.

3- Spreading

Outside-in signaling via GPIIbIIIa also induces a reorganization of the platelet cytoskeleton. As a consequence, the disk-like platelets change in morphology via a process that is referred to as platelet spreading. This provides an increased surface area and a solid base for additional recruitment of platelets, and facilitates the growing platelet plug (thrombus).

4- Secretion and signaling

Activated platelets rapidly release the content of the dense and α-granules to the extracellular space. In addition, the platelets release thromboxane (Tx), which is synthesized from the arachidonic acid molecules via phospholipase A2-mediated hydrolysis of membrane phospholipids. ADP and TxA2 are the two major feedback mediators that reinforce platelet activation. Granule release supports the propagation of platelet activation by mediating inside-out signaling, which in turn leads to activation of additional GPIIbIIIa receptors. Recruitment of more platelets occurs through a continued cycle of tethering and activation (via ADP and TxA2).

5- Procoagulant activity

Activation of platelets also leads to the exposure of the pro-coagulant lipid phosphatidylserine (PS) on platelet membrane. This generates a platform for the assembly of coagulation factor complexes. It allows the formation of the activated factor X-generating complex comprising activated factor VIII and activated factor IX, as well as the thrombin-generating complex, which consists of activated factor X and activated factor V (FVa)[22]. These complexes ultimately lead to the generation of a large amount of thrombin. Thrombin is, in turn, a potent activator of platelets and enhances therefore the rate of platelet plug formation[23]. To this end, thrombin cleaves an extracellular loop of a subset of G protein-coupled receptors known as protease-activated receptors (PARs)[24,25]. This generates a new N-terminus that serves as a tethered ligand for the receptor itself[26]. It has been shown that PAR-1 and PAR-4 are the critical G protein-coupled receptors on human platelets that mediated platelet activation upon proteolytic cleavage by thrombin. Although stimulation by either PAR1 or PAR4 can initiate platelet secretion and aggregation, PAR1 is the major thrombin receptor[27].

Platelet biogenesis and granule formation

The platelet producing megakaryocytes (MKs) are rare myeloid cells (representing less than 1% of these cells) that reside primarily in the bone marrow but are also found in the lung and peripheral blood. A constant cycle of generation of new MKs (megakaryopoiesis) is required to maintain the plasma concentration of platelets ranging between $1.5–4x10^8$/ml[28,29]. Failure in maintaining a sufficient platelet count has been associated with prolonged and spontaneous bleeding.

MKs originate from hematopoietic stem cells (HSC). Four intermediate cell types can be identified during MK differentiation from HSC, i.e. megakaryoblast, basophilic megakaryocyte, granular megakaryocyte, and the platelet-producing megakaryocyte. Thrombopoietin (TPO) is the primary regulator of thrombopoiesis[30] and is thought to act in synergy with other factors, including IL-3, IL-6, and IL-1β. Platelet specific proteins including platelet integrins,

glycoproteins and α-granule proteins are synthesized in a differentiation stage-dependent manner. The integrin GPIIbIIIa and the glycoprotein GPIb-V-XI complex are targeted to MK plasma membrane, and proteins including VWF, platelet factor IV (PF4) and β-thromboglobulin are loaded into the secretory α-granule[31,32].

Not all α-granule proteins originate from MK biosynthesis. In 1993, Handagama *et al*.[33] employed the disintegrin barbourin and a specific antagonist of GPIIbIIIa to show that GPIIbIIIa is the primary receptor mediating fibrinogen uptake and storage by MK. It has been suggested that also platelets support the uptake and α-granule storage of fibrinogen[33]. A crucial question is how the non-activated GPIIbIIIa can mediate the uptake of fibrinogen[34]. Other α-granule proteins that are taken up via receptor-mediated endocytosis uptake are factor V, insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3)[35–37]. It has further been suggested that the α-granule proteins albumin and IgG are taken up from the plasma by both megakaryocytes and platelets via pinocytosis[10].

Platelet generation is initiated when the MK transform into pro-platelets. This ultimately leads to the release of 2000–5000 new platelets per MK cell[29]. Newborn platelets are released into the circulation where they reside approximately 10 days after which they are cleared by the liver and spleen[10,38].

The α-granule protein factor V

Factor V (FV) is a cofactor for the serine protease activated factor X that converts prothrombin to thrombin. FV is produced in the liver as a large-single-chain polypeptide of about 330 kD. FV has a mosaic-like structure comprising three homologous A-type domains, a B domain and two homologous C-type domains. FV requires limited proteolytic cleavage by either thrombin or activated Factor X (FXa) to be converted into the active form FVa. The activated cofactor can subsequently assemble with activated factor X on the activated platelets to form the thrombin-generating complex[35,39,40]. To prevent unlimited thrombin generation, the FVa cofactor function is down-regulated through proteolytic cleavage by activated protein C (APC).

During biosynthesis by hepatocytes, FV undergoes post-translational modifications, including extensive O- and N-linked glycosylation[35,41]. The carbohydrate chains, which represent more than 25% of the mass of FV, have been shown to be relevant for the multiple biological activities of FV. Bruin *et al*.[42] were the first to report that deglycosylation results in a loss of cofactor function and an impaired activation of FV by thrombin. It has later been established that part of the circulating FV carries an additional N-linked glycan in the C2-domain leading to a different pro-coagulant and anticoagulant function of FV[43,44]. Of the two circulating isoforms, the glycosylated C2 domain variant appears to be more thrombogenic[44,45].

The source of FV in platelets has been debated for several years. After the initial hypothesis that FV is synthesized by MK[46], it has later been unequivocally established that FV in α-granules derives from receptor-mediated endocytosis by megakaryocytes[36]. The stored FV in the platelets represents approximately 20% of total content of FV in blood where it circulates at a concentration of about 30 nM. Physical and functional differences between stored and circulating FV have been identified[47]. Platelet FV is more efficiently activated by factor Xa and more resistant to inactivation by APC than plasma FVa[47]. The combination of intrinsic biochemical

features and its targeted release at the site of vascular damage make platelet FV particularly effective during the early phases of coagulation. Clinical and experimental evidence show that the stored platelet FV is sufficient to prevent or reduce the severity of bleeding symptoms in patients with neutralizing antibodies directed to circulating FV[48].

FV endocytosis by MK has been proposed to occur via a selective receptor-mediated clathrin-dependent event involving a two-step mechanism[36,49]. According to the proposed model, FV initially binds to a specific unidentified receptor after which FV is transferred to low density lipoprotein receptor-related protein-1 (LRP-1). The latter receptor mediates endocytosis of FV. After its endocytosis, FV undergoes additional biochemical processing prior to storage in the α-granules where it is covalently linked to multimerin[50]. The exact molecular mechanism that contributes to FV uptake and storage is, however, still poorly understood. It is further remarkable that MK seem to exhibit distinct uptake mechanisms for FV and fibrinogen. Whether other α-granule proteins utilize these uptake mechanisms remains to be established as well.

Exocytosis of granules from platelets

Platelet activation leads to the fusion of the secretory granules with the open canalicular system (OCS). The essential granule-to-OCS membrane fusions events are the result of a cascade of protein-protein interactions[51]. Regulated exocytosis is generally mediated by soluble n-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), soluble proteins such as N-ethylmaleimide-sensitive-factor (NSF), and soluble NSF attachment proteins (SNAPs), and SNAREs regulators[52–54]. According to the "SNARE hypothesis", SNAREs that reside on the vesicle are referred to as v-SNAREs (or vesicle-associated membrane proteins) and SNAREs that are located on target membrane are the t-SNAREs. In platelets, v-SNAREs that have been reported to be important for granule-membrane fusion are synaptobrevin/VAMP-2, cellubrevin/VAMP-3 and endobrevin/SNAP-8. Syntaxin 2, 4, 11 and SNAP 23 represent the functional t-SNAREs in platelets[55].

v- and t-SNARE proteins comprise so-called SNARE motifs that include about 65 amino acid residues. SNARE motifs form the v- and t-SNARES can assemble into a tight trans-SNARE complex that bridges the two membranes[54]. Although SNARE complex formation is sufficient in some systems for mediating vesicle-membrane fusion, tight kinetics and timing are required to control platelet secretion exclusively in response to external signals. Several potential regulators of SNARE complex formation have been identified in platelets and include Munc18 family members, Munc13, granuphilin, and tomosyn[53,54-56]. It has been reported that Munc18 proteins may act as both positive and negative regulators of membrane fusion. Overexpression of these proteins in cellular systems has, however, produced conflicting observations[53,54]. The key functional roles of the Munc18 protein family in exocytosis remain, therefore, a topic for further investigation.

Among the seven known Munc18 proteins[57], Munc18-1, -2, and -3 have been suggested to be critical for exocytosis in mammals. For platelets, it has initially been shown that Munc18-1 and Munc18-2 contribute to α-granule release[58,59]. Later studies showed that Munc18-2 is also critical for the regulated release of α-granules and dense-granule[60]. The relative contribution and importance of the individual Munc18 family members for granule release by platelets remains, however, to be established.

Roles of galectins in platelet functions

Galectins are a family of proteins originally identified in extracts of vertebrate tissue as galactoside-binding lectins. Current researche indicates that galectins play important roles in diverse physiological and pathological processes, including immune and inflammatory responses, tumor expansion and development, neural degeneration, atherosclerosis, diabetes, and wound repair[61,62]. To date, fifteen mammalian galectins have been identified comprising either one (Galectin-1,-2, -3, -5, and -7) or two carbohydrate-recognition domains (CRD) (Galectin-4, -6, -8, -9, and -12)[63,64]. Galectins can be found in the cytosol, nucleus, and outside the cell[65]. The individual CRD domains can bind glycans with different affinities. Due to their bivalent and multivalent binding capacity, galectins can act as a cross-linker between two glycoproteins[65].

In platelets Galectin-8 and Galectin-1 have been described to be able to trigger platelet activation[66,67]. Intriguingly, Galectin-8 and Galectin-1 have been suggested to interact with GPIb-V-IX complex and integrin GPIIbIIIa, respectively[66,67]. Moreover, changes of Galectin-8 expression and localization in platelets have been shown to occur upon activation[66]. An altered expression and localization of Galectin-8 has also been observed in megakaryocytes upon maturation[68]. Pull-down experiments in platelets identified FV, multimerin-1 and VWF as putative binders for Galectin-8 in in vitro[66]. However, the physiologic significance of in vivo interaction between Galectin-8 and each of these potential partners still remains to be unraveled. Galectins have previously also been implicated to contribute to ligand internalization[69,70]. This opens the possibility that Galectin-8 plays a role in the mechanism that regulates FV endocytosis by megakaryocytes.

Rare genetic disorders associated with platelet defects

Glanzmann's thrombastenia

Even though rare in the world-wide context, Glanzmann's thrombastenia (GT) is the most common inherited recessive disorder of platelet function. The thrombastenic phenotype is associated with deficiency or dysfunction of the platelet fibrinogen receptor GPIIbIIIa. Platelets from these patients fail to aggregate in response to stimuli that normally trigger activation of GPIIbIIIa. VWF–GPIb interaction is however not affected in these platelets. The GT patients have a lifelong hemorrhagic syndrome typically characterized by episodes of spontaneous muco-cutaneous bleeding. Bleeding severity differs considerably between patients. GT-causing mutations can affect either the GPIIb or IIIa encoding genes. The platelet content of GPIIbIIIa is used to categorize the disease into three subtypes: type I GT with less than 5% residual integrin, type II GT, with 5-20% residual GPIIbIIIa, and type III GT (also named GT variant) with 50% to normal level of a functionally defective GPIIbIIIa[71].

The hypothesis that GPIIbIIIa is responsible for fibrinogen uptake and storage in α-granules[33] was strengthened by the observation that GT type I patients lack granular fibrinogen. However, levels of granular fibrinogen in GT type II and III, seem to be dependent on the reported mutation[72–74]. These observations raise questions about the exact mechanism of granular-fibrinogen uptake that remains enigmatic and leaves gaps in our knowledge on integrin function.

Familial hemophagocytic lymphohistocytosis-5 (FHL-5)

Familial hemophagocytic lymphohistiocytosis (FHL) is a genetically heterogeneous immune disorder of autosomal recessive inheritance[75]. A hallmark of this condition is the occurrence

of uncontrolled proliferation and activation of polyclonal T/NK lymphocytes and macrophages that infiltrate multiple organs including liver and spleen and the central nervous system. It is associated with cytopenia and hyperferritinemia and can have a fatal outcome. The first genetic locus to be described in FHL (FHL-1) was on chromosome 9q21.3 22[76]. However the gene where the disease-causing mutation is located remains to be identified. Later studies identified different disease-causing mutations affecting proteins that contribute to granule release in leucocytes. Mutations in perforin have been associated with FHL-2, Munc13-4 variants with FHL-3, and defects in syntaxin-11 with FHL-4. Mutations in these proteins account for approximately 80% of FHL cases[77,78].

Only recently it has been reported that mutations in the Munc18-2 gene lead to FHL-5[79]. Among the 20 identified mutations, the most frequently observed Munc18-2 variant in FHL-5 patients is the c.1247-1G>C splice-site mutation. The critical role of Munc18-2 for granule release in platelets is, however, discordant with the clinical symptoms observed in FHL-5 patients that carry this variant. Only rare episodes of excessive bleeding have been reported in these patients, and symptoms seem to be highly variable among patients[80,81]. Yet, Al Hawas *et al*.[60] showed that functional absence of Munc18-2 in the platelets of FHL-5 patients leads to a complete secretion defect of α-granules and dense-granules. The exact role of Munc18-2 in α-granule release by platelets still, therefore, remains unclear.

LAD-III syndrome

Three leukocyte adhesion deficiency syndromes (LAD) have been described[82–85]. Clinical observations that are common to LAD are recurrent bacterial infection, which are often life-threatening and difficult to treat. While the genetic defects underlying LAD-I (functional absence of the β2 integrin subunit) and LAD-II (defect in the GDP-L-fucose transporter) have been known for several years, the molecular basis of LAD-III syndrome has only recently been identified[82–85]. Unique to LAD-III is the "Glanzmann-like" bleeding due to impaired platelet adhesive functions caused by integrin activation defects[86].

In recent years, some important aspects of the signaling pathway leading to integrin activation have been resolved. Upon platelet activation, intracellular diacylglycerol and Ca^{2+} activate protein kinase C and/or Rap1 guanine nucleotide exchange factor (CalDAG-GEF1)[87]. These proteins promote activation of Rap1, which in turn associates with Rap1-GTP–interacting adaptor molecule (RIAM) and forms a ternary complex together with talin-1 at the cytoplasmic tail of the integrin. Recently, it has been recognized that kindlin-3 is also involved in integrin activation[87]. Both talin-1 and kindlin-3 have similar FERM (four-point-one/ezrin/radixin/moesin) domain structures. FERM subdomain 3 of these proteins binds the β subunit of integrins. It has been proposed that binding of both talin-1 and kindlin-3 is required for effective integrin activation[88].

It has initially been though that low levels CalDAG-GEFI are the cause for LAD-III. However recent studies employing kindlin-3 knockout mice provided the basis for the finding that absence of kindlin-3 is the underlying defect that causes LAD-III. A breakthrough in the field was the observation that expression of kindlin-3 in immortalized cells of LAD-III patients, and not that of CalDAG-GEFI[89], completely rescued the defective integrin function. Yet, whether

or not absence of kindlin-3 in platelets only affects integrin activation, or that other platelets proteins are affected as well remains a topic for further investigation.

QUESTIONS ADDRESSED IN THIS THESIS

The two opposite distinct mechanisms behind the regulated exocytosis and endocytosis of α-granule proteins still remain enigmatic with many unanswered questions. In the present thesis, we aim to gain insight into the players critical for these two mechanisms by combining classical biochemical methods, mass spectrometry studies and flow cytometry analysis on in vitro model systems and platelets from patients with rare genetic disorders. In **Chapter II**, we employ confocal analysis, RNAi silencing and flow cytometry studies to address the question whether Gal8 is involved in FV endocytosis by megakaryocytes. In **Chapter III**, we analyze platelets of GT and LAD-III patients in a mass spectrometry study to unravel the enigma regarding the significance of integrin activation for fibrinogen endocytosis. Other questions addressed in the chapter are: i) Is GPIIbIIIa integrin involved in the internalization of other plasma proteins beside fibrinogen? ii) Are other proteins differentially expressed in platelets with a single GPIIbIIIa deficiency or a complete integrin activation defect? iii) Does mass spectrometry analysis of platelets provide an alternative to identify rare platelet disorders? In **Chapter IV,** we analyze platelet exocytosis and the role of Munc18-2 therein by means of mass spectrometry and classical biochemical tools. Platelets from FHL-5 patients are analyzed to address the following questions: i) What is the effect of the Munc18-2 splice-site mutation 1247-1G>C, the most frequently observed in FHL-5 patients, on platelet degranulation? ii) What is the protein product, if any, of the 1247-1G>C splice-site mutation. In **Chapter V**, we investigate whether GPIIbIIIa or GPIb-GPV contribute to endocytosis of FV. We addressed the questions: i) Does GPIIbIIIa also contribute in FV uptake? ii) Can Gal8 form a cross-linking bridge between FV and one of these receptors? Finally in **Chapter VI**, the results of our findings are discussed in view of their implications for platelet functions.

REFERENCES

1 Robb-Smith AHT. Why the Platelets were Discovered. *Br. J. Haematol*. 1967;13:618–637.

2 Coller BS. Blood elements at surfaces: platelets. Ann. N. Y. Acad. Sci. 1987;516:362–379.

3 Hartwig J, Italiano J Jr. The birth of the platelet. *J. Thromb. Haemost*. 2003;1(7):1580–1586.

4 Broos K, De Meyer SF, Feys HB, Vanhoorelbeke K, Deckmyn H. Blood platelet biochemistry. *Thromb. Res*. 2012;129(3):245–249.

5 Blair P, Flaumenhaft R. Platelet α-granules: Basic biology and clinical correlates. *Blood Rev*. 2009;23(4):177–189.

6 Smyth SS, McEver RP, Weyrich AS, et al. Platelet functions beyond hemostasis. *J. Thromb. Haemost*. 2009;7(11):1759–1766.

7 McNicol A, Israels SJ. Platelet dense granules: structure, function and implications for haemostasis. *Thromb. Res*. 1999;95(1):1–18.

8 Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil-interactions: linking hemostasis and inflammation. *Blood Rev*. 2007;21(2):99–111.

9 Behnke O. The morphology of blood platelet membrane systems. *Ser. Haematol*. 1970;3(4):3–16.

10 Flaumenhaft R. Molecular Basis of Platelet Granule Secretion. *Arterioscler. Thromb. Vasc. Biol*. 2003;23(7):1152–1160.

11 Nurden AT. Platelets, inflammation and tissue regeneration. *Thromb. Haemost*. 2011;105 Suppl 1:S13–33.

12 Fogelson AL, Wang NT. Platelet dense-granule centralization and the persistence of ADP secretion. *Am. J. Physiol*. 1996;270(3 Pt 2):H1131–1140.

13 Zucker MB, Nachmias VT. Platelet activation. *Arteriosclerosis*. 1985;5(1):2–18.

14 Kamath S, Blann AD, Lip GY. Platelet activation: assessment and quantification. *Eur. Heart J*. 2001;22(17):1561–1571.

15 Jennings LK. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. *Thromb. Haemost*. 2009;102(2):248–257.

16 Roth GJ. Developing relationships: arterial platelet adhesion, glycoprotein Ib, and leucine-rich glycoproteins. *Blood*. 1991;77(1):5–19.

17 Goto S. Understanding the mechanism of platelet thrombus formation under blood flow conditions and the effect of new antiplatelet agents. *Curr. Vasc. Pharmacol*. 2004;2(1):23–32.

18 Ruggeri ZM. Mechanisms of shear-induced platelet adhesion and aggregation. *Thromb. Haemost*. 1993;70(1):119–123.

19 Savage B, Almus-Jacobs F, Ruggeri ZM. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*. 1998;94(5):657–666.

20 Bennett JS, Berger BW, Billings PC. The structure and function of platelet integrins. *J. Thromb. Haemost*. 2009;7:200–205.

21 Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler. Thromb. Vasc. Biol*. 2008;28(3):403–412.

22 Hemker HC, van Rijn JL, Rosing J, et al. Platelet membrane involvement in blood coagulation. *Blood Cells*. 1983;9(2):303–317.

23 Brass LF. Thrombin and platelet activation. *Chest*. 2003;124(3 suppl):18S–25S.

24 Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J. Clin. Invest*. 1999;103(6):879–887.

25 Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J. Biol. Chem*. 2000;275(33):25216–25221.

26 Holinstat M, Voss B, Bilodeau ML, Hamm HE. Protease-activated receptors differentially regulate human platelet activation through a phosphatidic acid-dependent pathway. *Mol. Pharmacol*. 2007;71(3):686–694.

27 Peters T, Henry PJ. Protease-activated receptors and prostaglandins in inflammatory lung disease. *Br. J. Pharmacol*. 2009;158(4):1017–1033.

28 Slayton WB, Wainman DA, Li XM, et al. Developmental differences in megakaryocyte maturation are determined by the microenvironment. *Stem Cells*. 2005;23(9):1400–1408.

29 Bluteau D, Lordier L, Di Stefano A, et al. Regulation of megakaryocyte maturation and platelet formation. *J. Thromb. Haemost*. 2009;7 Suppl 1:227–234.

30 Kaushansky K. The molecular mechanisms that control thrombopoiesis. *J. Clin. Invest*. 2005;115(12):3339–3347.

31 Vainchenker W, Debili N, Norol F, Wendling F. Thrombopoietin and megakaryocyte differentiation]. *Schweiz. Med. Wochenschr*. 1998;128(42):1575–1581.

32 Deutsch VR, Tomer A. Megakaryocyte development and platelet production. *Br. J. Haematol*. 2006;134(5):453–466.

33 Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa). *Blood*. 1993;82(1):135–138.

34 Handagama PJ, Amrani DL, Shuman MA. Endocytosis of fibrinogen into hamster megakaryocyte alpha granules is dependent on a dimeric gamma A configuration. *Blood*. 1995;85(7):1790–1795.

35 Mann KG, Kalafatis M. Factor V: a combination of Dr Jekyll and Mr Hyde. *Blood*. 2003;101(1):20–30.

36 Bouchard BA, Williams JL, Meisler NT, Long MW, Tracy PB. Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event. *J. Thromb. Haemost*. 2005;3(3):541–551.

37 Chan K, Spencer EM. Megakaryocytes Endocytose Insulin-Like Growth Factor (IGF) I and IGF-Binding Protein-3: A Novel Mechanism Directing Them into α Granules of Platelets. *Endocrinology*. 1998;139(2):559–565.

38 Fritz E, Ludwig H, Scheithauer W, Sinzinger H. Shortened platelet half-life in multiple myeloma. *Blood*. 1986;68(2):514–520.

39 Segers K, Dahlbäck B, Nicolaes GAF. Coagulation factor V and thrombophilia: background and mechanisms. *Thromb. Haemost*. 2007;98(3):530–542.

40 Jeimy SB. Review of the Structure and Function of Factor V in Blood Coagulation. *Univ. Tor. Med. J.* 2010;87(2):91–100.

41 Silveira JR, Kalafatis M, Tracy PB. Carbohydrate moieties on the procofactor factor V, but not the derived cofactor factor Va, regulate its inactivation by activated protein C. *Biochemistry*. 2002;41(5):1672–1680.

42 Bruin T, Sturk A, ten Cate JW, Cath M. The function of the human factor V carbohydrate moiety in blood coagulation. *Eur. J. Biochem*. 1987;170(1-2):305–310.

43 Nicolaes GAF, Tans G, Thomassen MCLGD, et al. Peptide Bond Cleavages and Loss of Functional Activity during Inactivation of Factor Va and Factor Va by Activated Protein C. *J. Biol. Chem*. 1995;270(36):21158–21166.

44 Nicolaes GA, Villoutreix BO, Dahlbäck B. Partial glycosylation of Asn2181 in human factor V as a cause of molecular and functional heterogeneity. Modulation of glycosylation efficiency by mutagenesis of the consensus sequence for N-linked glycosylation. *Biochemistry*. 1999;38(41):13584–13591.

45 Fernández JA, Hackeng TM, Kojima K, Griffin JH. The carbohydrate moiety of factor V modulates inactivation by activated protein C. *Blood*. 1997;89(12):4348–4354.

46 Giampaolo A, Vulcano F, Macioce G, et al. Factor-V expression in platelets from human megakaryocytic culture. *Br. J. Haematol*. 2005;128(1):108–111.

47 Gould WR, Simioni P, Silveira JR, et al. Megakaryocytes endocytose and subsequently modify human factor V in vivo to form the entire pool of a unique platelet-derived cofactor. *J. Thromb. Haemost*. 2005;3(3):450–456.

48 Duckers C, Simioni P, Spiezia L, et al. Residual platelet factor V ensures thrombin generation in patients with severe congenital factor V deficiency and mild bleeding symptoms. *Blood*. 2010;115(4):879–886.

49 Bouchard BA, Meisler NT, Nesheim ME, et al. A unique function for LRP-1: a component of a two-receptor system mediating specific endocytosis of plasma-derived factor V by megakaryocytes. *J. Thromb. Haemost*. 2008;6(4):638–644.

50 Hayward CPM, Fuller N, Zheng S, et al. Human platelets contain forms of factor V in disulfide-linkage with multimerin. *Thromb. Haemost*. 2004;92(6):1349–1357.

51 White JG. Platelet secretion during clot retraction. *Platelets*. 2000;11(6):331–343.

52 Duman JG, Forte JG. What is the role of SNARE proteins in membrane fusion? *Am. J. Physiol*. 2003;285(2):C237–C249.

53 Sudhof TC, Rothman JE. Membrane Fusion: Grappling with SNARE and SM Proteins. *Science*. 2009;323(5913):474–477.

54 Rizo J, Südhof TC. The Membrane Fusion Enigma: SNAREs, Sec1/Munc18 Proteins, and Their Accomplices—Guilty as Charged? *Annu. Rev. Cell Dev. Biol*. 2012;28(1):279–308.

55 Reed GL, Fitzgerald ML, Polgár J. Molecular mechanisms of platelet exocytosis: insights into the "secrete" life of thrombocytes. *Blood*. 2000;96(10):3334–3342.

56 Gagescu R. SNARE hypothesis 2000. *Nat. Rev. Mol. Cell Biol*. 2000;1(1):5–5.

57 Gordon DE, Bond LM, Sahlender DA, Peden AA. A targeted siRNA screen to identify SNAREs required for constitutive secretion in mammalian cells. *Traffic*. 2010;11(9):1191–1204.

58 Houng A, Polgar J, Reed GL. Munc18-syntaxin complexes and exocytosis in human platelets. *J. Biol. Chem*. 2003;278(22):19627–19633.

59 Schraw TD, Lemons PP, Dean WL, Whiteheart SW. A role for Sec1/Munc18 proteins in platelet exocytosis. *Biochem. J.* 2003;374(Pt 1):207–217.

60 Al Hawas R, Ren Q, Ye S, et al. Munc18b/STXBP2 is required for platelet secretion. *Blood*. 2012;120(12):2493–2500.

61 Hughes RC. Galectins as modulators of cell adhesion. *Biochimie*. 2001;83(7):667–676.

62 Liu F-T, Rabinovich GA. Galectins as modulators of tumour progression. *Nat. Rev. Cancer*. 2005;5(1):29–41.

63 Barondes SH, Castronovo V, Cooper DN, et al. Galectins: a family of animal beta-galactoside-binding lectins. *Cell*. 1994;76(4):597–598.

64 Cooper DNW, Barondes SH. God must love galectins; He made so many of them. *Glycobiology*. 1999;9(10):979–984.

65 Rabinovich GA, Toscano MA, Jackson SS, Vasta GR. Functions of cell surface galectin-glycoprotein lattices. *Curr. Opin. Struct. Biol.* 2007;17(5):513–520.

66 Romaniuk MA, Tribulatti MV, Cattaneo V, et al. Human platelets express and are activated by galectin-8. *Biochem. J.* 2010;432(3):535–547.

67 Romaniuk MA, Croci DO, Lapponi MJ, et al. Binding of galectin-1 to $\alpha IIb\beta_3$ integrin triggers "outside-in" signals, stimulates platelet activation, and controls primary hemostasis. *FASEB*. 2012;26(7):2788–2798.

68 Zappelli C, van der Zwaan C, Thijssen-Timmer DC, Mertens K, Meijer AB. Novel role for galectin-8 protein as mediator of coagulation factor V endocytosis by megakaryocytes. *J. Biol. Chem.* 2012;287(11):8327–8335.

69 Furtak V, Hatcher F, Ochieng J. Galectin-3 mediates the endocytosis of beta-1 integrins by breast carcinoma cells. *Biochem. Biophys. Res. Commun*. 2001;289(4):845–850.

70 Schneider D, Greb C, Koch A, et al. Trafficking of galectin-3 through endosomal organelles of polarized and non-polarized cells. *Eur. J. Cell Biol*. 2010;89(11):788–798.

71 Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood*. 2011;118(23):5996–6005.

72 Grimaldi CM, Chen F, Scudder LE, Coller BS, French DL. A Cys374Tyr homozygous mutation of platelet glycoprotein IIIa (beta 3) in a Chinese patient with Glanzmann's thrombasthenia. *Blood*. 1996;88(5):1666–1675.

73 Chen P, Melchior C, Brons NH, et al. Probing conformational changes in the I-like domain and the cysteine-rich repeat of human beta 3 integrins following disulfide bond disruption by cysteine mutations: identification of cysteine 598 involved in alphaIIbbeta3 activation. *J. Biol. Chem.* 2001;276(42):38628–38635.

74 Coller BS, Shattil SJ. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood*. 2008;112(8):3011–3025.

75 Filipovich AH. Hemophagocytic lymphohistiocytosis (HLH) and related disorders. *Hematology Am. Soc. Hematol. Educ. Program*. 2009;127–131.

76 Ohadi M, Lalloz MR, Sham P, et al. Localization of a gene for familial hemophagocytic lymphohistiocytosis at chromosome 9q21.3-22 by homozygosity mapping. *Am. J. Hum. Genet*. 1999;64(1):165–171.

77 Cetica V, Pende D, Griffiths GM, Aricò M. Molecular basis of familial hemophagocytic lymphohistiocytosis. *Haematologica*. 2010;95(4):538–541.

78 Sieni E, Cetica V, Mastrodicasa E, et al. Familial hemophagocytic lymphohistiocytosis: a model for understanding the human machinery of cellular cytotoxicity. *Cell. Mol. Life Sci*. 2012;69(1):29–40.

79 Côte M, Ménager MM, Burgess A, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J. Clin. Invest*. 2009;119(12):3765–3773.

80 Rohr J, Beutel K, Maul-Pavicic A, et al. Atypical familial hemophagocytic lymphohistiocytosis due to mutations in UNC13D and STXBP2 overlaps with primary immunodeficiency diseases. *Haematologica*. 2010;95(12):2080–2087.

81 Pagel J, Beutel K, Lehmberg K, et al. Distinct mutations in STXBP2 are associated with variable clinical presentations in patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL5). *Blood*. 2012;119(25):6016–6024.

82 Bunting M, Harris ES, McIntyre TM, Prescott SM, Zimmerman GA. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands. *Curr. Opin. Hematol*. 2002;9(1):30–35.

83 Alon R, Etzioni A. LAD-III, a novel group of leukocyte integrin activation deficiencies. *Trends Immunol*. 2003;24(10):561–566.

84 Kuijpers TW, van de Vijver E, Weterman MAJ, et al. LAD-1/variant syndrome is caused by mutations in FERMT3. *Blood*. 2009;113(19):4740–4746.

85 Svensson L, Howarth K, McDowall A, et al. Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat. Med*. 2009;15(3):306–312.

86 Harris ES, Smith TL, Springett GM, Weyrich AS, Zimmerman GA. Leukocyte adhesion deficiency-I variant syndrome (LAD-Iv, LAD-III): molecular characterization of the defect in an index family. *Am. J. Hematol*. 2012;87(3):311–313.

87 Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood*. 2004;104(6):1606–1615.

88 Moser M, Legate KR, Zent R, Fässler R. The Tail of Integrins, Talin, and Kindlins. *Science*. 2009;324(5929):895–899.

89 Pasvolsky R, Feigelson SW, Kilic SS, et al. A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets. *J. Exp. Med*. 2007;204(7):1571–1582.

CHAPTER 2

Galectin-8 mediates factor V endocytosis
A novel role for galectin-8 as a mediator of coagulation factor V endocytosis by megakaryocytes

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The Journal of Biological Chemistry
vol. 287, no. 11, pp. 8327-35, Mar 9, 2012

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ABSTRACT

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 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, TPO 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.

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

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

Conclusion: Galectin-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.

Key words: endocytosis, platelet factor V, galectin-8, megakaryocytes

INTRODUCTION

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 haemostasis. 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 effective blood coagulation. The relevance of platelet FV has been demonstrated by clinical observations. It has, for instance, been reported that patients lacking platelet FV exhibit a bleeding diathesis despite of the presence of normal levels of plasma FV (18).

The source of platelet FV has been debated for years. Although it was initially been 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 (St. Louis, MO, USA).

Proteins and Antibodies – Recombinant full length human Gal8 and Gal1 were purchased from R&D Systems (Minneapolis, USA) and bovine lactadherin was from Hematologic Technologies (Essex Junction, USA). 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 (Bergheimer Heidelberg, Germany). FITC-conjugated mouse IgG isotype control was from Dako (Glostrup, Denmark), FV-HRP sheep anti-human FV from Kordia (Leiden The Netherlands), Alexafluor 488- or 568-conjugated secondary antibodies were from Invitrogen (Breda, The Netherlands), and anti-Gal3 from Abcam (Huissen, 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, Waltham, USA), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Breda, Nederland) at 37°C in humidified atmosphere with 5% CO_2. To ensure logarithmic growth, cells were sub-cultured every two days. A cell density of $1x10^6$ ml^{-1} was employed for the experiments. When required, cells were induced to differentiate for 72 hours 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 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 RU 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 nM HEPES (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$, 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 RU) 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 hour at 37°C with 2% human serum albumin (HSA) (Cealb) in Tris-HCl binding buffer (TBS) (20 mM Tris-HCl

(pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.05% (v/v) Tween-20). Appropriate dilutions of FV were subsequently incubated with Gal8 for 1 hour 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 SpectramaxPlus 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 hour at 37°C. The mixture was then incubated with immobilized Gal8 on the plate for 1 hour 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 FV : 3 FITC) (FluoReporter® FITC Protein Labeling Kit, Invitrogen, Breda, The Netherlands) according to 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 pre-incubated for 30 minutes at 37°C in presence of 4 µg/ml of 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 ultrapure methanol-free p-formaldehyde (Polysciences, Eppelheim, Germany) and re-suspended in TBS, 0.5% human serum albumin (HSA) and analyzed by flow cytometry.

Galectin expression by DAMI cells or purified platelets was assessed by fixing the cells as described above followed by an incubation for one hour 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) HSA, and 0.05 % (w/v) saponin. After removal of the unbound anti-galectin antibody by washing with PBS, the cells were incubated for one hour at room temperature with the secondary Alexafluor 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 Alexafluor 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 minutes at 4°C in 4% p-formaldehyde. When required, whole cells were stained with carboxyfluorescein-succinimidyl ester (CFSE) employing 1 µM carboxyfluoresceindiacetate-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) HSA, and 0.05 % (w/v) saponin. After washing with PBS, the cells were incubated with the appropriate secondary antibody (Alexafluor 488-conjugated conjugated antibody for galectin staining and Alexafluor 568-conjugated 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 63x/1.3 Oil immersion lens (Zeiss LSM 510, Carl Zeiss, Heidelberg, Germany).

Gal8 siRNA gene silencing – Three Gal8-specific (sense and anti-sense) 20-25 nt siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The sense strands are: 5'-GAACU GACAGAGAUAAGUA-3', 5-CAACACUUAUAGCCAGUUA-3'and5'-CCAGUAUGUUCCCUGUUCU-3'. The non-targeting control siRNA pools were from Dharmacon (Thermo Fisher Scientific, Waltham, USA). 6 µl of 50 µM Gal8 siRNA or non-targeting control siRNA were added to $3x10^6$ DAMI cells in serum-free medium. Cells were then pulsed at 250 V, 150 µF and ∞ Ω in a BioradGenepulser (Bio-Rad, Hercules, USA) as described (27). Gal8 knockdown was confirmed after 48 h by incubating the cells for 30 minutes 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 leukaemia (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 hours 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 (Fig. 1B). The results revealed that the internalized FV was localized in a punctate pattern inside the cell. We did not observe any staining of FV when the DAMI cells were not incubated with FV (Fig. S1). The uptake of FITC-FV was also monitored at 4°C and in the presence of unlabeled FV. Uptake was reduced by more than 50% in the 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. Figure 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 non permeabilized 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 figures 2B and 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, 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. Figure 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 binding 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 β-galactoside containing compounds effectively block the association of Gal8 to FV. In figure 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 employing siRNA technology (Fig. 6A,B). The results show that cells transfected with Gal8 siRNA, but not those with non-targeting siRNA, have a markedly reduced ability to endocytose FV. This observation provided additional evidence that Gal8 contributes

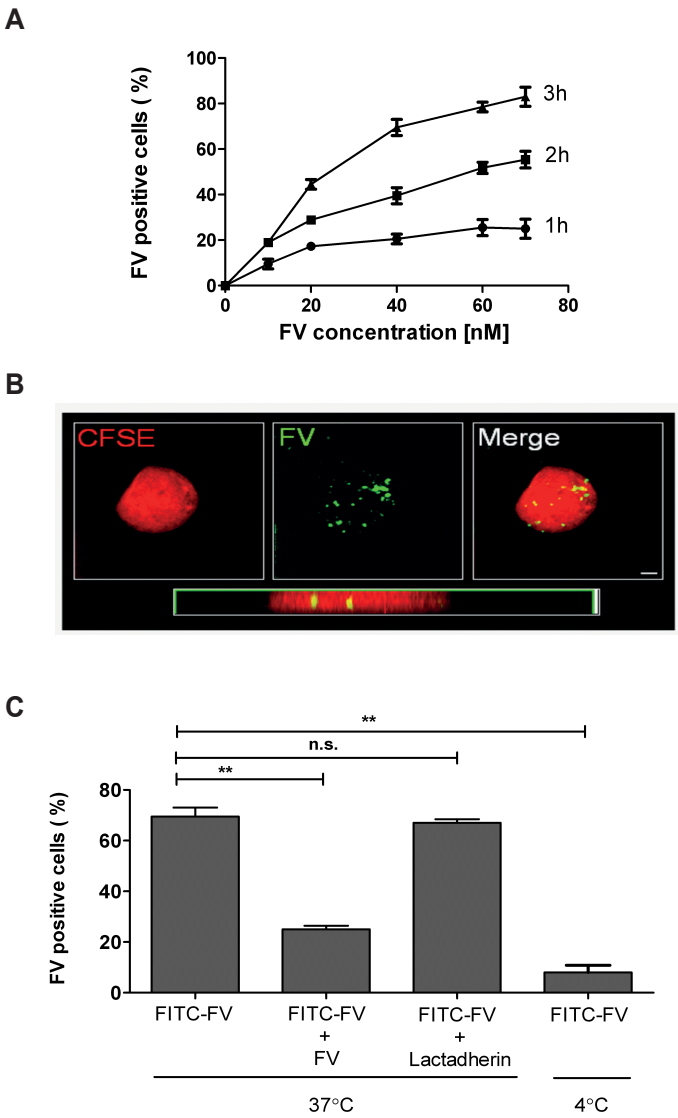

Figure 1. FV is internalized by the megakaryocytic DAMI cells. (A) Increasing concentrations of FITC-FV were incubated with DAMI cells in serum-free medium at 37°C for the indicated times. The percentage of FITC-FV positive cells was assessed by flow cytometry as described in the experimental procedures. Data represent the mean values ± SD of three independent experiments. (B) 40 nM unlabeled FV was incubated with the cells in serum free medium for 3 hours at 37°C. Cells were analyzed by confocal microscopy after immunofluorescence staining of FV as described in the experimental procedures. The top three panels show the whole cell in red (CFDA-SE staining) and FV in green. The white scale bar represents 5 µm. The bottom panel shows a side view of the cell which was obtained employing z-stack analysis of the cell displayed in the panel (cell height: 4 µm). (C) Flow cytometry analysis of cells that were incubated with 40 nM FITC-FV in serum free medium for 3 hours at 4° or at 37°C in the presence of 0.3 µM unlabeled FV or 1.2 µM lactadherin. Data represent the mean values ± SD of three independent experiments and are expressed as percentage of FITC-FV positive cells.

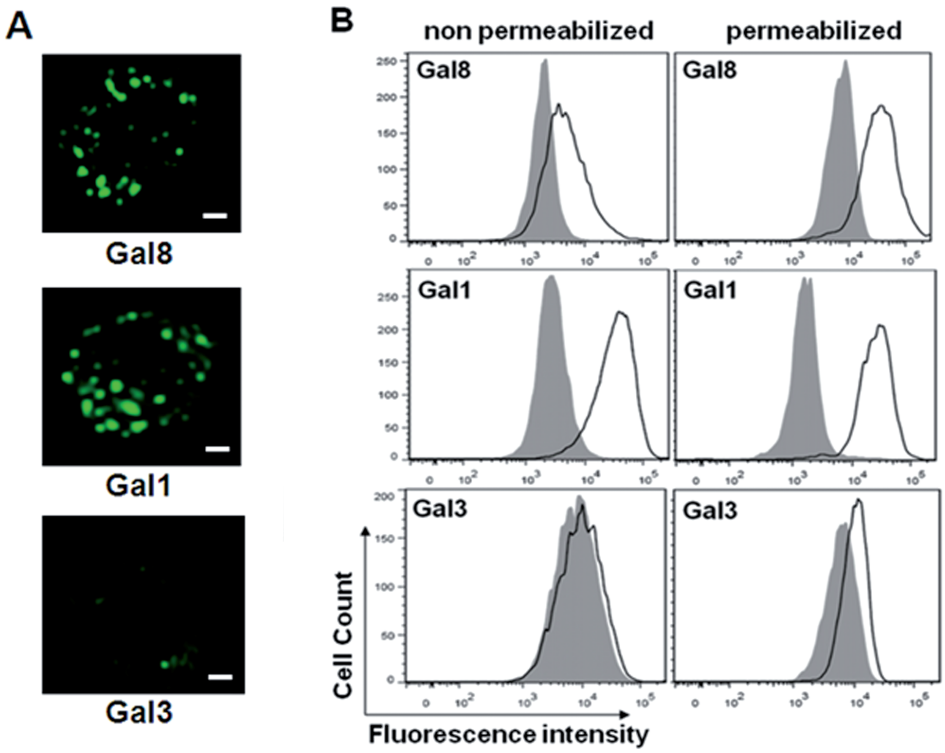

Figure 2. Gal8 and Gal1 are expressed by DAMI cells. (A) Confocal images of DAMI cells in which Gal8, Gal1 and Gal3 are stained in green as described in the experimental procedures. White scale bars represent 5 µm. (B) Flow cytometry analysis of the presence of Gal1, Gal3 and Gal8 on or in DAMI cells by staining these galectins in non permeabilized and permeabilized cells. Shown are representative histograms of one out of three experiments. The grey histograms represent the background fluorescence of the cells in the absence of the primary anti-galectin antibody.

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. 6C,D). The data revealed that FV uptake was blocked by about 70% in the presence of anti-Gal8 but remained unchanged in the presence of the anti-Gal1 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). Since 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. Figure 7A reveals that Gal8 was hardly detected on non permeabilized 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

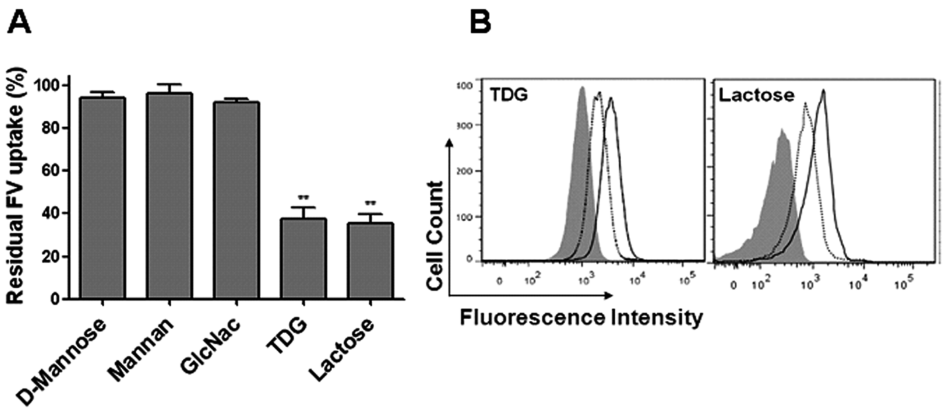

Figure 3. FV is internalized in a carbohydrate-dependent manner. (A) DAMI cells were incubated with 40 nM FITC-FV for 3 hours at 37°C in the absence and in the presence of 5 mM D-mannose, GlcNAc, lactose, TDG or 1 mg/ml mannan. Data are the mean values ± SD of three independent experiments, and are expressed as the percentage of residual FITC-FV uptake relative to the uptake of FITC-FV in the absence of the carbohydrates. (B) FACS histograms of FITC-FV uptake by DAMI cells in the absence (*solid line*) and in the presence (*dotted line*) of 5 mM TDG or 5 mM lactose.

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 β-galactosides (Fig. 3). Second, we demonstrate with SPR analysis and a solid phase binding assay that Gal8 binds FV in a carbohydrate-dependent manner (Fig. 4A,B, Fig. 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. 6C,D).

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 β-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

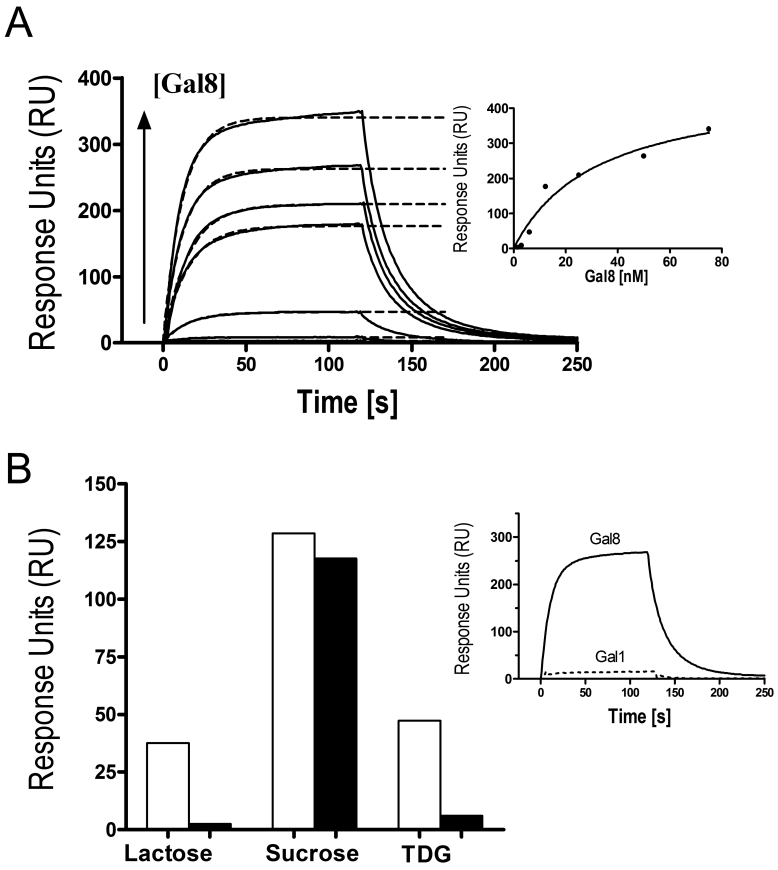

Figure 4. SPR analysis of carbohydrate-dependent Gal8–FV complex formation. (A) Increasing concentrations of Gal8 (1.5, 3, 6, 12, 25, 50 and 75 nM) were passed over immobilized FV in 20 mM HEPES (pH 7.4), 150 nM NaCl, 5 mM $CaCl_2$, and 0.005% (v/v) Tween-20 as described in the experimental procedures. Association and dissociation are indicated in Response Units (RU). The broken line represents the fit to the association phase of a one-site association model. *The inset* shows the estimated equilibrium binding response as a function of the Gal8 concentration. (B) 50 nM Gal8 was associated to immobilized FV in the presence (white bars) 0.2 mM and (black bars) of 2 mM lactose, TDG or sucrose. After 120 s of association, the buffer comprising the carbohydrates and Gal8 was replaced with the same buffer without these compounds. The binding response obtained 10 s after the start of the dissociation phase is shown at the indicated carbohydrate concentrations. The data are representative for the results obtained from at least three independent experiments. *The inset* shows the interaction with immobilized FV of 50 nM Gal8 and 50 nM Gal1.

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

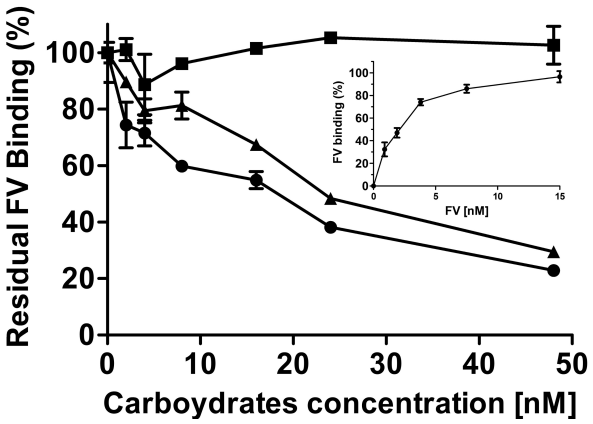

Figure 5. Enzyme-linked immunosorbent assay of carbohydrate-dependent Gal8–FV complex formation. 15 nM FV was incubated with Gal8 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 2 mM MgCl2 and 0.05% (v/v) Tween-20 for 1 hour at 37 °C. Binding was assessed as described in experimental procedures in 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 ± SD 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.

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.

Gal8 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 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*. 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 (22). 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.

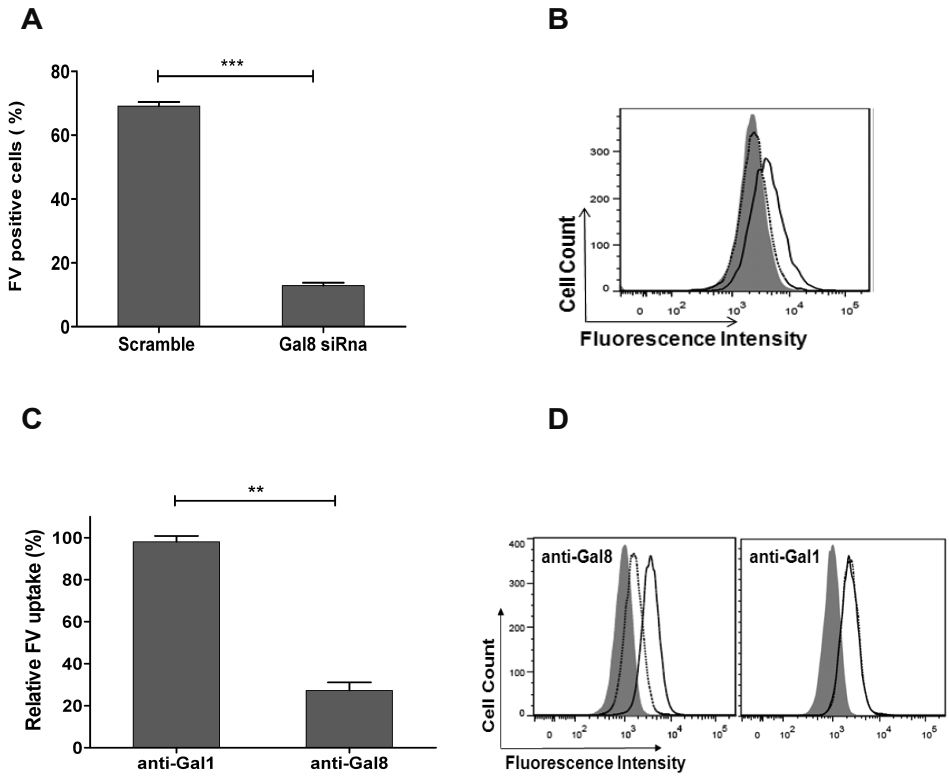

Figure 6. Gal8 mediates endocytosis of FV. (A) 40 nM FITC-FV was incubated for 3 hours at 37°C with DAMI cells transfected with non-targeting (scramble) siRNA, or Gal8 siRNA as described in the experimental procedures. The uptake of FITC-FV was assessed by flow cytometry analysis. Shown is the percentage of FITC-FV positive cells. (B) A representative histogram of Gal8 positive cells after transfection of the cells with Gal8 siRNA (dotted line) or non-targeting siRNA (solid line). Grey 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 hours at 37°C with DAMI cells that were pre-incubated for 30 minutes 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. Panel D shows representative histograms of FITC-FV uptake in control (solid line) and antibody-pretreated (dotted line) cells. Grey histograms represent the background fluorescence of the cells. All data reported are the means ± SD of at least three experiments.

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

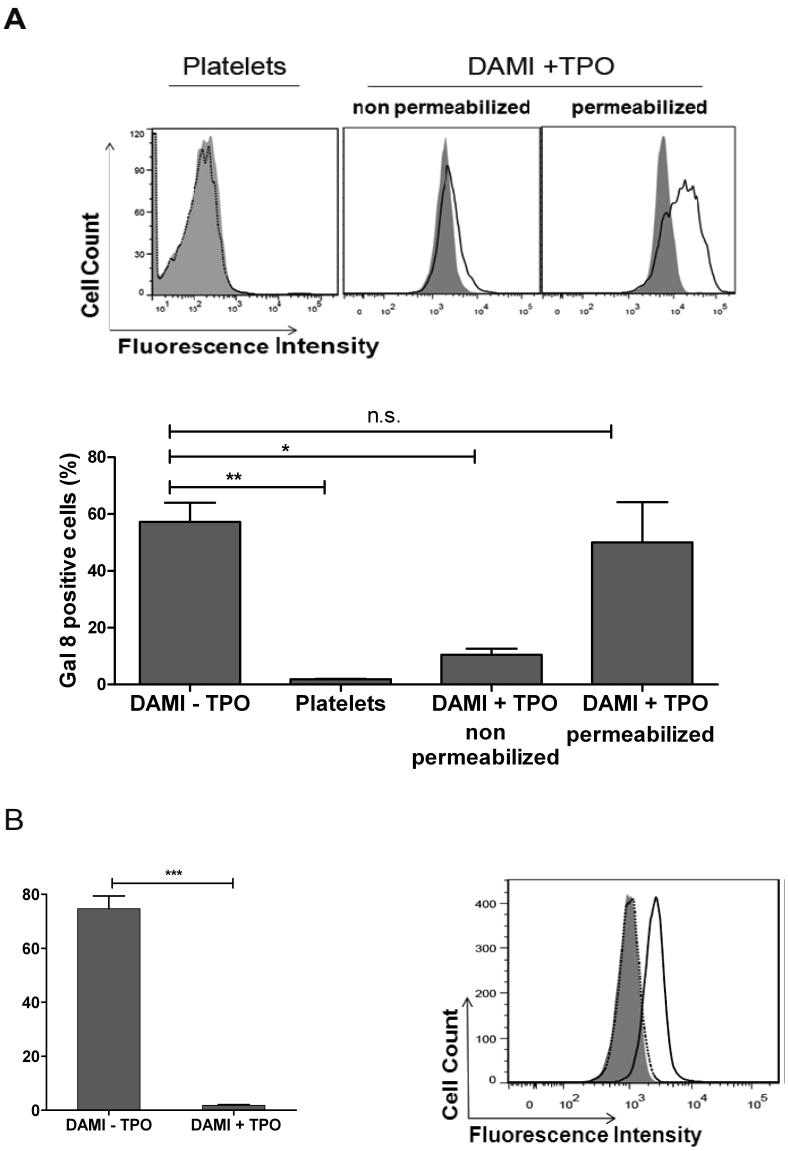

Figure 7. Gal8 surface expression and FV uptake is impaired in TPO-differentiated DAMI cells. (A) The upper panels show representative FACS histograms of the extracellular staining of Gal8 in non permeabilized platelets and TPO-treated DAMI cells (DAMI + TPO). Intracellular Gal8 staining in TPO-treated DAMI cells (permeabilized) was included as control. Grey 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 non permeabilized TPO-differentiated DAMI cells (DAMI + TPO). Data reported are the means ± SD 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 hours at 37°C in serum free medium. The data represent mean ± SD 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., non significant.

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. We further 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.

FOOTNOTES

Abbreviations: factor V (FV); human serum albumin (HSA); galectin (Gal); N-acetylglucosamine (GlcNAc); lipoprotein related protein 1 (LRP-1); phosphatydylserine (PS); Surface Plasmon Resonance (SPR); D-Galactopyranosyl-b-D-thiogalactopyranoside (TDG); thrombopoietin (TPO); non significant (n.s.)

*This work was financially supported by the PPOC-10-012.

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REFERENCES

1 Elola, M. T., Wolfenstein-Todel, C., Troncoso, M. F., Vasta, G. R., and Rabinovich, G. A. (2007) *Cell Mol Life Sci* **64**, 1679-1700

2 Zick, Y., Eisenstein, M., Goren, R. A., Hadari, Y. R., Levy, Y., and Ronen, D. (2002) *Glycoconjugate Journal* **19**, 517-526

3 Yang, R. Y., Rabinovich, G. A., and Liu, F. T. (2008) *Expert Rev Mol Med* **10**, e17

4 Leffler, H. (2001) *Results Probl Cell Differ* **33**, 57-83

5 Rabinovich, G. A., Toscano, M. A., Jackson, S. S., and Vasta, G. R. (2007) *Curr Opin Struct Biol* **17**, 513-520

6 Hughes, R. C. (1999) *Biochim Biophys Acta* **1473**, 172-185

7 Stechly, L., Morelle, W., Dessein, A. F., Andre, S., Grard, G., Trinel, D., Dejonghe, M. J., Leteurtre, E., Drobecq, H., Trugnan, G., Gabius, H. J., and Huet, G. (2009) *Traffic* **10**, 438-450

8 Merlin, J., Stechly, L., de Beauce, S., Monte, D., Leteurtre, E., van Seuningen, I., Huet, G., and Pigny, P. (2011) *Oncogene* **30**, 2514-2525

9 Delacour, D., Cramm-Behrens, C. I., Drobecq, H., Le Bivic, A., Naim, H. Y., and Jacob, R. (2006) *Curr Biol* **16**, 408-414

10 Mishra, R., Grzybek, M., Niki, T., Hirashima, M., and Simons, K. (2010) *Proc Natl Acad Sci U S A* **107**, 17633-17638

11 Zhu, W., Sano, H., Nagai, R., Fukuhara, K., Miyazaki, A., and Horiuchi, S. (2001) *Biochem Biophys Res Commun* **280**, 1183-1188

12 Romaniuk, M. A., Tribulatti, M. V., Cattaneo, V., Lapponi, M. J., Molinas, F. C., Campetella, O., and Schattner, M. (2010) *Biochem J* **432**, 535-547

13 Pacienza, N., Pozner, R. G., Bianco, G. A., D'Atri, L. P., Croci, D. O., Negrotto, S., Malaver, E., Gomez, R. M., Rabinovich, G. A., and Schattner, M. (2008) *FASEB J* **22**, 1113-1123

14 Segers, K., Dahlback, B., and Nicolaes, G. A. (2007) *Thromb Haemost* **98**, 530-542

15 Dahlback, B. (1980) *J Clin Invest* **66**, 583-591

16 Suehiro, Y., Veljkovic, D. K., Fuller, N., Motomura, Y., Massé, J. M., Cramer, E. M., and Hayward, C. P. (2005) *Thromb Haemost* **94**, 585-592

17 Gould, W. R., Silveira, J. R., and Tracy, P. B. (2004) *J Biol Chem* **279**, 2383-2393

18 Tracy, P. B., Giles, A. R., Mann, K. G., Eide, L. L., Hoogendoorn, H., and Rivard, G. E. (1984) *J Clin Invest* **74**, 1221-1228

19 Giampaolo, A., Vulcano, F., Macioce, G., Mattia, G., Barca, A., Milazzo, L., Ciccarelli, C., and Hassan, H. J. (2005) *Br J Haematol* **128**, 108-111

20 Bouchard, B. A., Williams, J. L., Meisler, N. T., Long, M. W., and Tracy, P. B. (2005) *Journal of Thrombosis and Haemostasis* **3**, 541-551

21 Camire, R. M., Pollak, E. S., Kaushansky, K., and Tracy, P. B. (1998) *Blood* **92**, 3035-3041

22 Bouchard, B. A., Meisler, N. T., Nesheim, M. E., Liu, C. X., Strickland, D. K., and Tracy, P. B. (2008) *J Thromb Haemost* **6**, 638-644

23 Kim, S. W., Ortel, T. L., Quinn-Allen, M. A., Yoo, L., Worfolk, L., Zhai, X., Lentz, B. R., and Kane, W. H. (1999) *Biochemistry* **38**, 11448-11454

24 Nicolaes, G. A. F., Villoutreix, B. O., and Dahlbäck, B. (1999) *Biochemistry* **38**, 13584-13591

25 Bos, M. H., Meijerman, D. W., van der Zwaan, C., and Mertens, K. (2005) *J Thromb Haemost* **3**, 522-530

26 Phillips, J. E., Lord, S. T., and Gilbert, G. E. (2004) *Journal of Thrombosis and Haemostasis* **2**, 1806-1815

27 Meems, H., van den Biggelaar, M., Rondaij, M., van der Zwaan, C., Mertens, K., and Meijer, A. B. (2011) *Int J Biochem Cell Biol* **43**, 1114-1121

28 Greenberg, S., Rosenthal, D., Greeley, T., Tantravahi, R., and Handin, R. (1988) *Blood* **72**, 1968-1977

29 Lev, P. R., Goette, N. P., Glembotsky, A. C., Laguens, R. P., Meckert, P. M., Salim, J. P., Heller, P. G., Pozner, R. G., Marta, R. F., and Molinas, F. C. (2011) *Platelets* **22**, 26-36

30 Majumder, R., Quinn-Allen, M. A., Kane, W. H., and Lentz, B. R. (2008) *Blood* **112**, 2795-2802

31 Yang, R. Y., Hsu, D. K., and Liu, F. T. (1996) *Proceedings of the National Academy of Sciences* **93**, 6737-6742

32 Carlsson, S., Carlsson, M. C., and Leffler, H. (2007) *Glycobiology* **17**, 906-912

33 Patnaik, S. K., Potvin, B., Carlsson, S., Sturm, D., Leffler, H., and Stanley, P. (2006) *Glycobiology* **16**, 305-317

34 Ideo, H., Seko, A., Ishizuka, I., and Yamashita, K. (2003) *Glycobiology* **13**, 713-723

35 Stowell, S. R., Arthur, C. M., Slanina, K. A., Horton, J. R., Smith, D. F., and Cummings, R. D. (2008) *J Biol Chem* **283**, 20547-20559

36 Graham, J. E., Glaser, R., Loving, T. J., Malarkey, W. B., Stowell, J. R., and Kiecolt-Glaser, J. K. (2009) *Health Psychol* **28**, 621-630

37 Furtak, V., Hatcher, F., and Ochieng, J. (2001) *Biochem Biophys Res Commun* **289**, 845-850

38 Nishi, N., Shoji, H., Seki, M., Itoh, A., Miyanaka, H., Yuube, K., Hirashima, M., and Nakamura, T. (2003) *Glycobiology* **13**, 755-763

39 Handagama, P., Scarborough, R. M., Shuman, M. A., and Bainton, D. F. (1993) *Blood* **82**, 135-138

40 Bax, M., Garcia-Vallejo, J. J., Jang-Lee, J., North, S. J., Gilmartin, T. J., Hernandez, G., Crocker, P. R., Leffler, H., Head, S. R., Haslam, S. M., Dell, A., and van Kooyk, Y. (2007) *J Immunol*. **179**, 8216-8224.

41 Duckers, C., Simioni, P., Spiezia, L., Radu, C., Dabrilli, P., Gavasso, S., Rosing, J., and Castoldi, E. (2010) *Blood* **115**, 879-886

42 Rosendaal, F. R., Koster, T., Vandenbroucke, J. P., and Reitsma, P. H. (1995) *Blood* **85**, 1504-1508.

SUPPLEMENTAL DATA

This manuscript includes two supplemental figures.

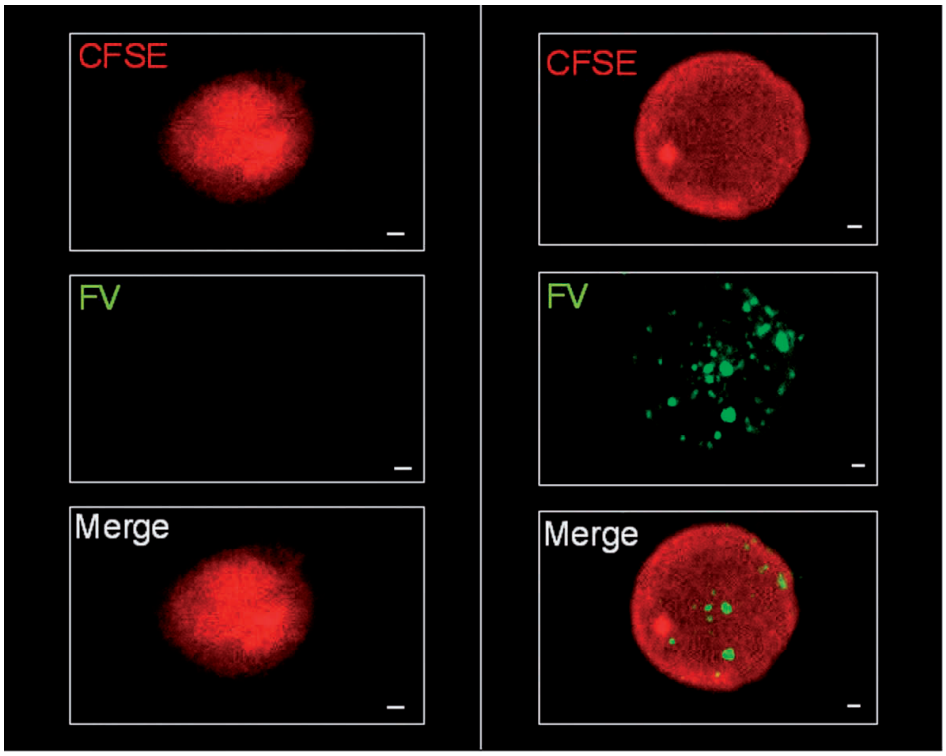

Figure S1. DAMI cells do not express FV. DAMI cells were incubated in absence (left panels) or presence (right panels) of 40 nM FV in serum free medium for 3 hours at 37°C. FV was detected via immunofluorescence staining as described in the experimental procedures. FV is shown in green and the cell is shown in red (CFSE staining). The white scale bar represents 5 µm.

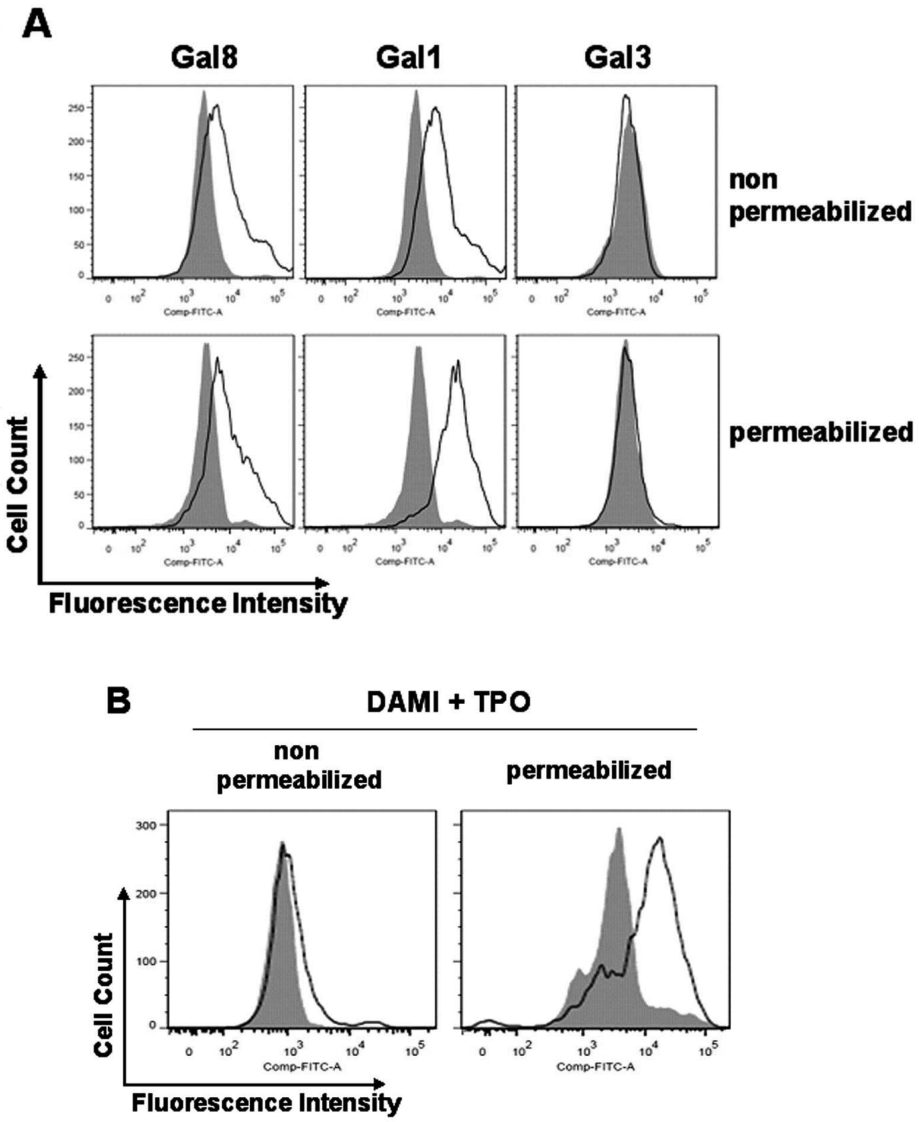

Figure S2. Gal8, Gal1, and Gal3 expression by DAMI cells. The expression of galectins was analyzed by flow cytometry as described in the experimental procedures. Panel A shows the histograms of Gal8, Gal1 and Gal3 expression in non permeabilized and permeabilized DAMI cells. Panel B shows the histograms of Gal8 expression in DAMI cells that were differentiated with TPO. . The grey histograms represent the cells in which Goat IgG isotype (Gal8 and Gal1) control antibody (GeneTex, Irvine, USA) or mouse IgG isotype (Gal3) control antibody (Pelicluster, Sanquin, The Netherland) were employed in the staining procedure.

CHAPTER 3

Single αIIbβ3 deficiency or a complete integrin activation defect in rare platelet disorders identified by mass spectrometry analysis

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Submitted for publication

ABSTRACT

Mass spectrometry (MS) analysis may be a powerful alternative to identify rare platelet disorders using only a limited amount of platelets. We therefore employed MS to analyze platelets of Glanzmann's thrombastenia (GT) type I (GT-I) patients lacking integrin αIIbβ3, and of leukocyte-adhesion-deficiency type III (LAD-III) patients with an impaired integrin activation mechanism caused by absence of fermitin-family-homolog-3 (FERMT3). MS analysis revealed a decreased level of, amongst others, β1-comprising integrins next to the almost complete absence FERMT3 in LAD-III platelets. These platelets may therefore not only exhibit an integrin activation defect but also a reduced level of β1-integrins. The GT-I platelets lacked fibrinogen and αIIbβ3 complying with the observation that αIIbβ3 contributes to α-granule protein loading of fibrinogen. In contrast, neurobeachin-like 2, which is absent in gray platelet syndrome, was among the up-regulated proteins. The underlying genetic defect of an unidentified GT patient was identified by MS as a R214W mutation in β3. Results showed that this causes GT type III with αIIbβ3 expression of about 40% of normal values and normal fibrinogen content. Our findings show that MS analysis has the potential to identify rare platelet disorders and to provide insight in the differential expression of previously unidentified proteins.

INTRODUCTION

The platelet adhesion and aggregation capacity is tightly restrained in the bloodstream, and becomes promptly available upon platelet activation at sites of vessel injury[1]. Activated platelets further release proteins from secretory α-granules leading to high local concentrations of a variety of distinct proteins, including von Willebrand Factor (VWF) and fibrinogen[1,2]. These mechanisms are critical for platelet plug formation and effective bleeding arrest. The activated platelet integrins play an indispensable role therein as impaired integrin functioning has been associated with bleedings. Although many studies have addressed platelet adhesion defects caused by dysfunctional integrins, the impact on the platelet protein level is hardly investigated. Mass spectrometry (MS) may provide, however, a unique and powerful tool to assess without bias the changes in protein content of platelets from patients. Moreover, it has recently been shown that there are only minor variations in platelet protein expression patterns in the platelets of healthy individuals. It has therefore been proposed that MS may also serve as a diagnostic tool to identify rare platelets disorders[3]. In the present study, we therefore analyzed by MS the putative change in protein content of platelets with rare defects in integrin functioning.

Five distinct platelet integrins have been identified. These integrins include either a β1 (α2β1, α5β1, α6β1) or a β3 subunit (αIIbβ3, α5β3). αIIbβ3 is the most abundant platelet integrin, and acts as the main receptor for fibrinogen and VWF[1,4]. The other integrins have been suggested to bind exposed ligands in the damaged vessel wall. Integrin α2β1 has, for instance, been shown to act as a receptor for collagens[4,5]. Critical proteins that contribute to integrin activation in platelets are talin-1 and fermitin-family-homolog-3 (FERMT3). These FERM-domain-containing proteins have been suggested to synergistically contribute to integrin activation by binding to distinct sites within the cytoplasmic tail of the β subunit of the integrin[1,6,7]. Functional absence of FERMT3 is best known for causing leukocytes-adhesion-deficiency type III (LAD-III), which is a rare immune disorder associated with impaired bacterial killing due to a severe integrin activation defects in leucocytes[8-10]. Absence of FERMT3 has also been reported to affect the erythrocytes. It has been suggested to be a cause of poikilocytosis and anemia[11]. LAD-III patients have further been described to exhibit bleedings due to dysfunctional integrin activation in platelets[8,12,13].

Next to their adhesive function, integrins have been proposed to contribute to cell migration and remodeling of the extracellular matrix in cellular systems[14]. This process is mediated by internalization of ligand-bound integrins from the cell surface. The FERM-domain-containing protein sortin-nexin-17 (SNX17) has recently been suggested to prevent lysosomal degradation of the internalized β1 integrin by binding the β1-tail upon FERMT3 dislodgement on early endosomes. This mechanism may facilitate recycling of the unbound integrin to the cell surface[15]. Although absence of FERMT3 has been proposed to lead to integrin activation defects, this issue raises the question whether absence of FERMT3 may affects the protein level of integrins and associated proteins in the platelets as well.

In platelets, an integrin recycling mechanism is involved in α-granule protein cargo enrichment. αIIbβ3 has been demonstrated to mediate the uptake and storage of plasma-derived fibrinogen in α-granules[16]. Employing mice models with a fibrinogen deficiency, it has been proposed that fibrinogen is, in turn, required for the maintenance of the level of P-selectin in platelets[17]. The critical role of αIIbβ3 as an endocytic receptor for fibrinogen is signified by Glanzmann's thrombasthenia

type I (GT-I) patients who exhibit an αIIbβ3 level of less than 5% in their platelets. Next to impaired αIIbβ3-mediated platelet aggregation, the platelets of these patients exhibit a markedly reduced content of fibrinogen in the α-granule. However, normal expression of a functional defective αIIbβ3, as found in Glanzmann's thrombasthenia type III (GT-III) patients, appears to have a limited influence on fibrinogen storage in platelets[18]. It has therefore been proposed that the uptake of fibrinogen may not depend on the activation state of αIIbβ3. The full impact of functional absence of αIIbβ3 on α-granule proteins other than fibrinogen remains unclear.

The platelets of LAD-III and GT-I patients provide the unique opportunity to assess the effect of a complete integrin activation defect on the one hand, and absence of only αIIbβ3 on the other hand on the level of the platelet integrins and associated proteins. In addition, the value of MS as a potential diagnostic tool for the identification of rare platelet disorders can be evaluated. In the present study, we have therefore performed a comparative MS analysis of the proteome of the platelets of three LAD-III patients, and two GT-I patients. We further analyzed a GT patient of which the type of GT remained to be established. MS analysis was used to identify the genetic defect that is associated with the platelet disorder of the latter patient.

EXPERIMENTAL PROCEDURES

Platelet isolation – The study was approved by the Amsterdam Medical Center Institutional Medical Ethics Committee in accordance with the Declaration of Helsinki. Venous blood was collected from healthy donors and from LAD-III and GT patients after obtaining informed consent. The GT-I patients have been previously described[13,19]. Two of the three LAD-patients are from families that have been described earlier[8]. A third LAD-III patient carries a previously unidentified mutation in FERMT3, i.e. c.1173delT, p.Asp393ThrfsX29, on both alleles. The collected blood was anti-coagulated with heparin. Washed platelets were obtained as previously described [19]. The collected platelets contained less than 1 leukocyte per 10^6 platelets and less than 1 erythrocyte/10^4 platelets.

Western blot analysis and flow cytometry aggregation of the platelets - All fine chemicals used in this study were from Sigma-Aldrich (Zwijdrecht, The Netherlands) unless otherwise specified. 1x10^8 platelets were lysed in 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS supplemented with Halt protease inhibitors cocktail (Thermo scientific, Breda, The Netherlands) and kept on ice for 15 min. The lysates were centrifuged 5min at 13,000xg to remove insoluble particles and were boiled for 5 min at 100°C in sample loading buffer under reducing conditions. Platelet proteins were separated employing a NuPAGE® Bis-Tris Precast Gels with NuPAGE® MOPS SDS running buffer (Life Technologies, Blieswijk, The Netherlands). Proteins were blotted to PVDF membrane which was blocked with 5% milk powder, 0.05% Tween-20 in PBS for 1 h. Proteins were stained employing enhanced chemiluminescence (General Electric Healthcare, Milwaukee, USA) utilizing the primary monoclonal antibodies MB9 against αIIb[20] and β3 (Transduction Laboratories™) and an HRP-conjugated antibody (GE Healthcare). Glyceraldehyde 3-phospate dehydrogenase, which was utilized as a loading control was stained employing monoclonal anti-GAPDH (Chemicon #MAB374). Platelet aggregation tests employing flow cytometry was performed as previously described[19]. Briefly carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes,

Eugene, OR) and PKH26-labeled platelets were mixed 1:1 and preincubated with agonists: 100 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich) or 1.5 mg/ml ristocetin (Biopool; Trinity Biotech). Samples were taken in time and were fixed in 0.5% formaldehyde, PBS. Double-colored events were assessed employing a LSRII HTS flow cytometer and data were analyzed by FACSDiva Version 6.1 software.

In-gel digestion of platelet proteins and LC-MS/MS analysis – The proteins in the lysates of equal amounts of platelets were separated on SDS-PAGE (4-12% acrylamide). The proteins in the gel bands were processed for MS analysis as described[21,22]. Peptides were separated using a reverse-phase C18 Acclaim PepMap RSLC (75 µm × 150 mm, 2 µm particles) at a flow rate of 300 nl/min using a one-hour linear gradient from 0.05% acetic acid (v/v) to 0.05% (v/v) acetic acid and 35% (v/v) acetonitrile. As a nanoLC pump, we used a Dionex Ultimate 3000 RSLC. Eluted peptides were sprayed directly into the LTQ Orbitrap XL mass spectrometer using a nanoelectrospray source with a spray voltage of 1.9 kV. The mass spectrometer was operated in a data-dependent mode as previously described[22]. The mass spectrometer, the nanoelectrospray source and emitters, the C18 column, and the nanoLC system were from Thermo Fisher Scientific Inc, (Bremen, Germany).

Peptide and protein identification - MS/MS samples were analyzed using Sequest (XCorr Only) (Thermo Fisher Scientific, San Jose, CA, USA; version 1.2.0.208) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1). Sequest and X! Tandem was set up to search uniprot-organism_9606_AND_keyword_kw-0181.fasta (20234 entries) assuming the digestion enzyme trypsin. Fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20 ppm were used. Carbamidomethyl of cysteine was specified in Sequest and X! Tandem as fixed modification. Oxidation of methionine was specified as a variable modification. Scaffold (version Scaffold_4.0.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm [23] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [24]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Assessment of differentially expressed proteins and identification of the genetic defect of the unclassified GT patient - Quantitative Q-Q scatter plots shown in figure 1 and 2 as well as normalized spectral counts were obtained via Scaffold_4.0.4. To identify the genetic mutation of the unclassified GT patient, the PEAKS Studio 6.0 software suite (Waterloo, ON Canada) was employed [25]. For the initial search, a protein database, comprising only the αIIb and β3 subunit, was employed using a parent mass error tolerance of 30 ppm and a fragment error tolerance of 0.8 Da. Carbamidomethylation was set as a fixed modification. The peptides were filtered using peptide scores of -10lgP ≥15, and De Novo TLC ≥3. The identified mutated peptide sequence according to PEAKS PTM search algorithm was subsequently included in the protein database uniprot-organism_9606_AND_keyword_kw-0181.fasta. The PEAKS search engine was utilized again with the same peptide filter criteria as mentioned above allowing a false discovery rate of 0.2%.

RESULTS

Analysis of differential protein expression in GT-I platelets

To assess with MS analysis the differential protein expression in the GT-I platelets, the average normalized spectral counts (NSC) of the proteins derived from healthy control platelets were plotted as a function of those obtained from GT-I platelets (Fig. 1). Data points that are on or close to the diagonal in figure 1 represent proteins that are equally present in healthy and GT-I platelets. Proteins are considered up or down regulated when the corresponding data points are above or below the indicated dashed lines in figure 1. The result shows 5 distinct data points that are well below the dashed lines (see Fig. 1A, B). These data points represent the α, β and γ chain of fibrinogen and the αIIb and β3 integrin subunits (Table I). This confirms that fibrinogen and the αIIbβ3 integrin are markedly down-regulated in the platelets of the GT-I patients. No other α-granule proteins could be classified as significantly reduced in the GT-I platelets. This suggests no specific involvement of αIIbβ3 or fibrinogen in the cargo enrichment of other α-granule proteins in human platelets. Differentially expressed proteins further include HLA type proteins revealing that control platelets and GT-I platelets partially differ in HLA haplotype. The reason behind the reduction of the levels of the other proteins remains to be established (Table I). The guanine nucleotide-binding protein G(i) subunit α-2, and cGMP-specific 3',5'-cyclic phosphodiesterase may be involved in intracellular signaling mediated by integrins. Rho GTPase-activating protein, and 1 Rho-associated protein kinase 2 may, in turn, contribute to the dynamics of the cytoskeleton[26,27]. Next, to down-regulated proteins, we identified significantly up-regulated proteins (Fig. 1A,C, Table I). The up-regulated protein coactosin-like protein may also contribute to regulation of the cytoskeleton[28]. Also neurobeaching-like protein 2 (NBEAL2) is among the up-regulated protein. Absence of this protein has recently been implicated in Gray platelet syndrome[29-31]. The results show that the MS approach is particularly effective in identifying the absence of proteins that have been previously associated with GT-I. In addition, other differentially expressed proteins were identified that may be related to GT-I as well.

Analysis of differential protein expression in LAD-III platelets

Platelets lysates of three LAD-III patients were processed for mass spectrometry analysis to assess the differential proteins expression compared to healthy control platelets (Fig. 2). The analysis revealed that the data point, which represents FERMT3, was markedly below the lower dashed line (Fig 2A,B). Figure 2C shows the obtained NSC of FERMT3 for the individual controls, the GT-I patients and the LAD-III patients. The data together illustrate the almost complete absence of FERMT3 in the platelets of the LAD-III patients only. An overview of the other proteins that are significantly up- or down-regulated is shown in Table II. The result shows that most of the up-regulated proteins are derived from erythrocytes (e.g. spectrin, ankyrin-1, and hemoglobin). This may be related to the observation that LAD-III patients exhibit instable erythrocytes which may cause enhanced hemolysis during platelet isolation[11]. The identified erythrocyte proteins are therefore most likely co-purified with the platelets. Among the down-regulated proteins are the α-granule proteins factor V (FV), VWF and latent transforming growth factor beta-binding protein 1 (LTBP1), and proteins involved in the processing of N-linked glycans (neutral alpha-glucosidase AB) and energy metabolism (i.e. hexokinase-1, glycogen phosphorylase , ATP citrate synthase) [2,32].

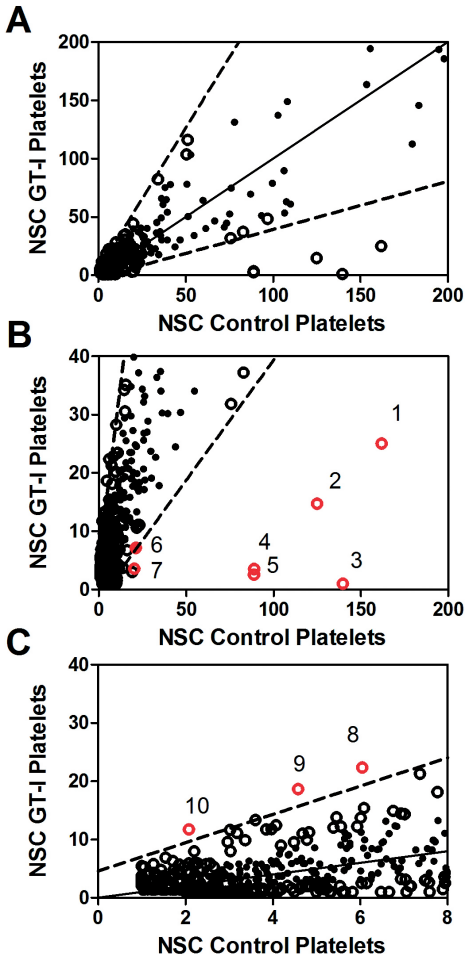


Figure 1. Differential protein expression between the platelets of healthy controls and GT-I patients. (A) The average normalized spectral count (NSC) of proteins derived from 4 unrelated healthy donor platelets is displayed as a function of that of the GT-I platelets. Data points close to or on the indicated diagonal represent proteins that are about equally present in the platelets. Open circles represent proteins that more than 2-fold differ in expression level between the healthy and GT-I platelets. Proteins are considered up- or down-regulated when the data points are above or below the dashed lines. These proteins are more than two standard deviations away from being equally present in both healthy and GT-I platelets. Panel (B) and (C) show zoomed-in sections of panel A. The identities of data points that are indicated with the numbers 1 to 10 are shown in Table I. This table also shows the other up- and down- regulated proteins.

The down-regulated staphylococcal nuclease domain-contain protein 1 is part of the RNA-induced silencing complex and may be involved in gene regulation[33]. The relevance, if any, of the down-regulation of these proteins for LAD-III remains to be established. Remarkably, integrin subunits α2, α6 and β1 are also among the down-regulated proteins. These integrin subunits can together form the α2β1 and α6β1 integrins. This suggests that absence of FERMT3 may not only affect integrin activation but also the levels of the β1-subunit comprising integrins. The observations together confirm that mass spectrometry analysis is a powerful tool to identify the rare platelet disorder observed in LAD-III. In addition, the data provide novel information about the differential expression of proteins that may also contribute to the clinical phenotype associated with LAD-III.

Characterization of the platelets of the putative GT patient

We employed MS analysis of the platelets of a patient of which the GT type remained to be established. Figure 3 shows the NSC of the integrin subunits and fibrinogen from platelets of four

Table I. Up- and down-regulated proteins in GT-I platelets. Proteins are displayed of which the data points in figure 1 are below or above the dashed lines in figure 1. These proteins are considered down or up-regulated. Indicated are the numbers by which the involved proteins are shown in figure 1. Next to the protein name, the database entry name is displayed (www.uniprot.org). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone are indicated with an *.

Shown in Fig.1	Entry name	Fold change	NSC control platelets	NSC GT-I platelets	Protein
1	FIBA_HUMAN	7	169	25	Fibrinogen alpha chain
2	FIBB_HUMAN	9	129	15	Fibrinogen beta chain
3	ITA2B_HUMAN	145	145	1	Integrin alpha-IIb
4	FIBG_HUMAN	25	90	4	Fibrinogen gamma chain
5	ITB3_HUMAN	36	92	3	Integrin beta-3
6	ILEU_HUMAN	6	22	4	Leukocyte elastase inhibitor
7	IDHP_HUMAN	7	22	3	Isocitrate dehydrogenase
8	COTL1_HUMAN	0.2	4	19	Coactosin-like protein
9	PRDX2_HUMAN	0.2	5	22	Peroxiredoxin-2
10	NBEL2_HUMAN	0.1	1	12	Neurobeachin-like protein 2
Other differentially expressed proteins					
	GNAI2_HUMAN	8	8	1	Guanine nucleotide-binding protein G(i) subunit alpha-2
	PDE5A_HUMAN	8	9	1	cGMP-specific 3'-5'-cyclic phosphodiesterase
	RHG01_HUMAN	7	11	2	RHO GTPase-activating protein 1
	ROCK2_HUMAN	8	12	2	RHO-associated protein kinase 2
	CO3_HUMAN	5	11	2	Complement C3
	TRFE_HUMAN	3	13	4	Serotransferrin
	1A01_HUMAN	11	11	1	HLA class I histocompatibility antigen, A-1 alpha chain*
	1A03_HUMAN	11	11	1	HLA class I histocompatibility antigen, A-3 alpha chain*
	1A24_HUMAN	10	10	1	HLA class I histocompatibility antigen, A-24 alpha chain*
	1A11_HUMAN	10	10	1	HLA class I histocompatibility antigen, A-11 alpha chain*

Table II. Up- and down-regulated proteins in LAD-III platelets. Proteins are displayed of which the data points in figure 2 are below or above the dashed lines in figure 2. These proteins are considered down or up-regulated. The protein name and the uniprot accession number is displayed (www.uniprot.org). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone are indicated with an *.

Accession number	Fold change	NSC control platelets	NSC LAD-III platelets	Protein
URP2_HUMAN	188	188	1	Fermitin family homolog 3 (FERMT3)
SND1_HUMAN	14	14	1	Staphylococcal nuclease domain-containing protein 1
GANAB_HUMAN	11	22	2	Neutral alpha- glucosidase AB
ACLY_HUMAN	10	10	1	ATP–citrate synthase
ITA2_HUMAN	10	10	1	Integrin alpha-2
vWF_HUMAN	10	33	3	Von Willebrand factor
ESYT1_HUMAN	9	17	2	Extended synaptotagmin-1
FA5_HUMAN	7	17	3	Coagulation factor V
AT2A2_HUMAN	7	16	2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2*
AT2A3_HUMAN	5	33	7	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3*
LTBP1_HUMAN	5	25	5	Latent-transforming growth factor beta-binding protein 1
ITB1_HUMAN	5	15	3	Integrin beta-1
ITA6_HUMAN	5	14	3	Integrin alpha-6
HXK1_HUMAN	5	30	6	Hexokinase-1
PYGB_HUMAN	5	22	4	Glycogen phosphorylase, brain form
UN13D_HUMAN	5	26	6	Protein unc-13 homolog D
A1AG1_HUMAN	0.3	1	3	Alpha-1-acid glycoprotein 1
HBB_HUMAN	0.3	101	397	Hemoglobin subunit beta*
HBD_HUMAN	0.2	33	152	Hemoglobin subunit delta*
HBG2_HUMAN	0.2	6	40	Hemoglobin subunit gamma-2*
HBG1_HUMAN	0.1	6	41	Hemoglobin subunit gamma-1*
SPTB1_HUMAN	0.2	6	41	Spectrin beta chain, erythrocyte
HBA_HUMAN	0.2	74	353	Hemoglobin subunit alpha
ANK1_HUMAN	0.1	3	25	Ankyrin-1
B3AT_HUMAN	0.04	3	73	Band 3 anion transport protein
SPTA1_HUMAN	0.03	2	53	Spectrin alpha chain, erythrocyte

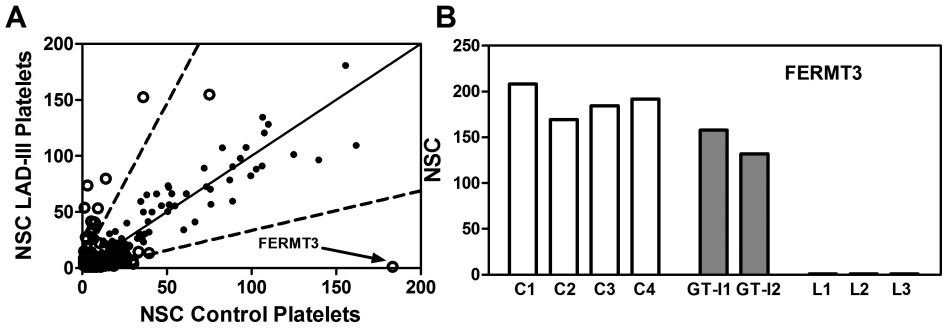


Figure 2. Differential protein expression between the platelets of healthy controls and LAD-III patients. (A) The average normalized spectral count (NSC) of proteins derived from 4 unrelated healthy donor platelets is displayed as a function of that of the three LAD-III patients. Open circles represent proteins that more than 2-fold differ in expression level between the healthy and LAD-III platelets. Proteins are considered up- or down-regulated when the data points are above or below the dashed error bar line. Proteins are considered up- or down-regulated when the data points are above or below the dashed lines. These proteins are more than two standard deviations away from being equally present in both healthy and LAD-III platelets. The data point that represents FERMT3 is indicated (B) NSC of FERMT3 of the platelets of the 4 individual controls, the two GT-I (GT-I1 and GT-I2) patients, and the three LAD-III patients (L1, L2, L3). (C) A zoom-in of the scatter plot shown in panel A. The proteins represented by the open circles that are above or below the dashed lines are shown in Table II.

controls, the GT-I patients and the putative GT patient. For the latter platelets, the data reveals the same spectral counts for fibrinogen with respect to the control platelets, and an about 40% reduction of the αIIb and β3 subunits. As also shown in figure 1, the platelets of the established GT-I patients show a marked reduction of fibrinogen, and an almost complete absence of the integrin subunits. This finding was confirmed by western blot analysis of the αIIbβ3 integrin (inset Fig 3A). Evaluation of platelet aggregation revealed a markedly impaired αIIbβ3-mediated platelet aggregation induced by PMA for the platelets of the GT-I and the putative GT patients (Fig. 4A). Aggregation triggered by ristocetin was, however, comparable to normal platelets (Fig. 4B). Flow cytometry analysis of the platelets employing the antibody PAC-1, which predominantly binds the activated αIIbβ3, demonstrated that the residual level of the integrin in the platelets in the putative GT patient is functionally defective (supplemental figure S1). Similar to the platelets of the GT-I patients, MS analysis of the platelets of the putative GT patient revealed elevated levels of NBEAL2 as well (Fig. 3C). This implies that absence of integrin function may cause up-regulation of NBEAL2. The findings suggest that the patient suffers from GT-III, which is characterized by a functionally defective, but expressed, αIIbβ3 integrin, and near normal levels of fibrinogen.

Identification of the mutation that is associated with GT-III

To identify the potential genetic defect in one of the integrin subunits of the GT-III patient, we re-analyzed the MS data employing PEAKS studio analysis software. Next to peptide identification assisted by a database with known proteins, this program uses algorithms to identify peptides and mutations thereof independent of a protein database. We initially searched against

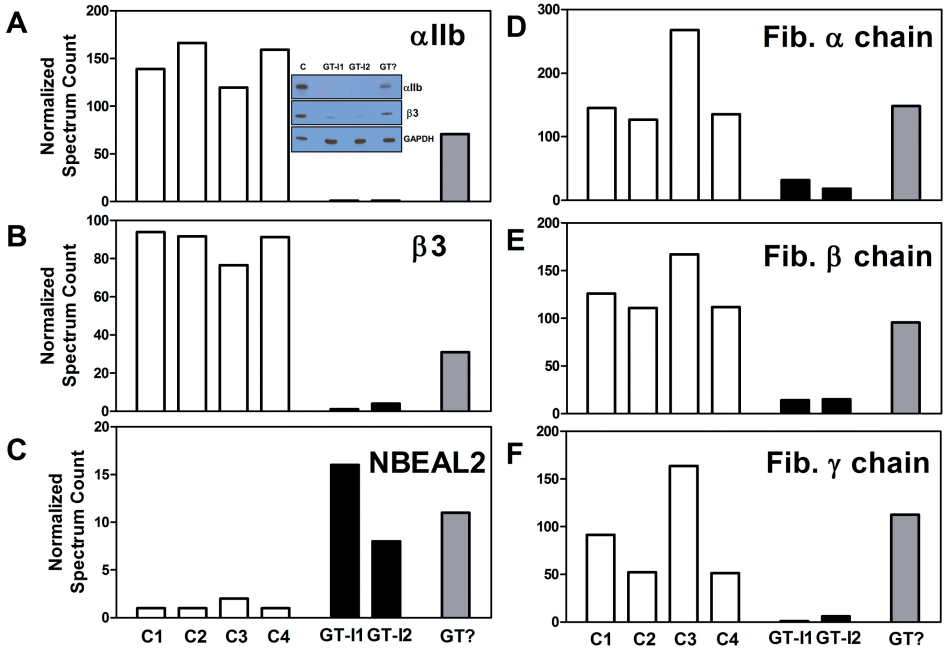


Figure 3. Characterization of the platelets of the putative GT patient. The spectral counts of the proteins were obtained of the platelets from 4 healthy controls (C1-C4), the two GT-I patients (GT-I1 and GT-I2) and the putative GT patient (GT?). Panels A-D show the normalized spectral counts (NSC) of the indicated proteins. The inset in panel A shows a western blot of the integrin subunits αIIb and β3 for the GT platelets and healthy control platelets. Staining of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as a loading control.

a protein database that included only the β3 and αIIb subunit of the integrin. The resulting identified peptides covered 60% of the sequence of the αIIb and β3 subunits (Fig. 5A). The post-translational modification (PTM) module of PEAKS assisted by de novo sequencing identified a spectrum that was uniquely matched to the peptide sequence "QSVSWNR" implying that R214 in the wild type β3 sequence "210-QSVSRNR-216" has been substituted for a tryptophan (Fig. 5B). We next performed search against a human protein database that also included the mutated sequence of the β3 subunit. The identified peptides of the β3 subunit now covered 57% of the primary sequence thereof (Supplemental figure S2). Among the other identified peptides of the platelet proteins (15607 identified peptide matches), the analysis identified the mutated peptide of the β3 subunit with high confidence. This confirms the observation of the initial PEAKS PTM search that the GT-III patient carries a p.R214W mutation in the β3 subunit. Noteworthy, this mutation has been previously associated with GT-III[34]. DNA sequencing confirmed the presence of the mutation in the β3 subunit of the patient. These findings demonstrate the potential of MS analysis to evaluate not only differential protein expression in rare platelet disorders but also to identify the genetic defect that underlies the disorder.

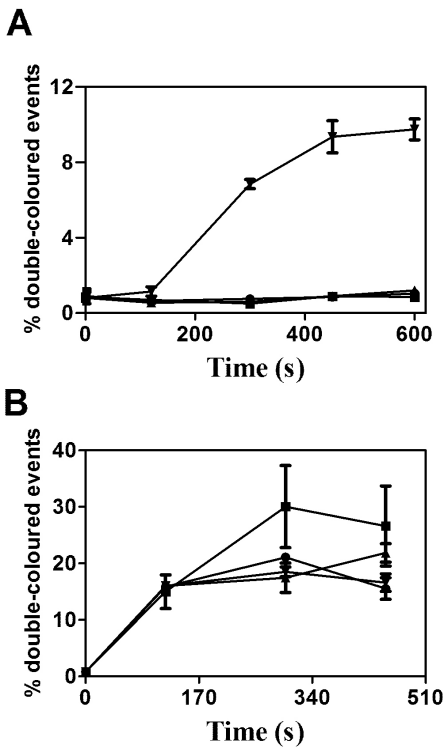


Figure 4. Aggregation analysis of the GT platelets. Agonist-induced aggregation in whole blood of control and GT platelets was followed in time employing flow cytometry. (A) αIIbβ3-mediated platelet aggregation was assessed in time upon stimulation with 100 ng/ml phorbol myristate acetate (PMA). (B) GPIb-mediated platelets aggregation was followed in time in response to 1.5 mg/ml ristocetin. Aggregation is expressed as % of double-colored events versus time corrected for unlabelled events. The two GT-I patients are represented by *diamonds* and *circles*, the putative GT patient in *squares* and the control platelets are indicated by *reversed triangles*.

DISCUSSION

It has been proposed that platelet biogenesis is a highly regulated and reproducible process resulting in only minor variations in protein expression patterns between individuals[3]. Proteomic analysis of platelets could be a powerful approach to identify and study rare platelet disorders. In the present study, we identified quantitative protein differences between normal and dysfunctional platelets from three GT and three LAD-III patients. Besides confirming a marked reduction of αIIbβ3 and fibrinogen in GT-I platelets and FERMT3 in LAD-III platelets, we have identified variations in the level of other proteins associated with platelet disorders (Figs.1-3, Table I and II). These proteins represent attractive targets for future investigations to unravel their role in platelet function. MS analysis has also allowed us to identify the disease-causing R214W mutation in the β3 subunit of the platelets of the GT-III patient (Fig.5, S2). The mutation is located in the ligand-binding regulatory site adjacent to the metal-ion-dependent adhesion site (MIDAS). The region comprising the mutation is referred to as ADMIDAS[18]. Both MIDAS and ADMIDAS as well as the synergistic-metal-binding-site (SyMBS) act in concert to control ligand binding and outside-in signaling[18,35]. The R214W mutation has been suggested to lead to instability of the integrin, which may explain its partially reduced expression (Fig. 3), and to directly affect the interaction between the activated integrin and soluble fibrinogen[18].

The marked reduction of fibrinogen in the platelets of the GT-I patients conclusively confirms αIIbβ3 as the unique and specific platelet receptor responsible of fibrinogen uptake

and storage by megakaryocytes and platelets (Fig. 1,3). It remains therefore remarkable that the R214W mutation in the β3 subunit does not affect the content of fibrinogen in the platelets of the GT-III patient at all (Fig. 3). It agrees with the suggestion that high affinity interaction between fibrinogen and the integrin is not required for fibrinogen endocytosis by megakaryocytes and platelets[36]. The observation that fibrinogen is not among the differentially expressed proteins in the platelets of the LAD-III patients also confirms this hypothesis. These findings do add fuel to the debate whether or not integrins may also bind their ligands prior to activation. In the so-called switchblade model of integrin activation, full extension of the extracellular domain and opening of the head domain of the integrin is required to mediate ligand-binding. The deadbolt model, however, assumes that low affinity ligand binding already occurs in the inactive bend form of the integrin[37,38]. Our result that the platelets of the LAD-III and GT-III patients still exhibit normal levels of fibrinogen are in favor of the latter binding model suggesting that low affinity binding is sufficient to drive the endocytosis of fibrinogen. Although the level of fibrinogen was normal in the GT-I and LAD-III platelets, we did observe a reduction of the α-granule proteins FV, VWF and LTBP1 in only the LAD-III platelets. The reason for this observation is at present unknown. It seems conceivable, however, that an integrin activation dependent mechanism contributes to the regulation of α-granule protein loading of these proteins.

It has been shown that the presence of the α or β integrin subunits dependents on the expression of its respective counterpart [39]. This explains why the GT-I and GT-III patients exhibit reduced levels of both integrin subunits in their platelets (Figs. 1,3). Remarkably, the platelets of the three LAD-III patients showed a decreased expression of the β1, α2 and α6 subunits. Our finding implies, however, that the level of the α2β1 and α6β1 integrin is reduced in the platelets of the investigated LAD-III patients. Interestingly, a modest reduction of the β1 subunit has also been observed in the platelets of FERMT3 knock-out mice[40]. Not only impaired integrin activation may therefore lead to defective platelet adhesion to cellular matrix components in LAD-III patients. Reduced levels of α2β1 and α6β1 may contribute to this defect as well. One possible explanation for the reduced level of β1-integrins is that FERMT3 contributes to an integrin recycling mechanism in platelets and/or megakaryocytes. The FERM-domain-containing protein sortin-nexin-17 (SNX17) has recently been proposed to contribute to integrin recycling by preventing lysosomal degradation of the β1-comprising integrins. It has been suggested that SNX17 protects β1 from degradation by binding this integrin subunit after complex dissociation of the internalized FERMT3 - β1 complex[15]. Possibly, the recycling mechanism is disturbed in the absence of FERMT3 leading to reduced expression levels of the β1-comprising integrins in platelets.

Our data surprisingly revealed a specific up-regulation of NBEAL2 in the GT patients. NBEAL2 has recently been identified as the genetic defect causing the Gray platelet syndrome which is associated with impaired α-granule formation[29-31]. The exact function of NBEAL2 in megakaryocytes remains unknown. It has been suggested that NBEAL2 may be involved in the development of membrane compartments in megakaryocytes or in the structuring of the cytoskeletal organization of megakaryocytes, proplatelets and platelets[41,42]. Our data now suggest that there is a possible direct or indirect mechanistic link between NBEAL2 and αIIbβ3. Notably, absence of αIIbβ3 leads to reduction of Rho GTPase activating protein-1 and Rho-associated kinase-2 both of which may contribute to cytoskeletal organization as well (Table I). The reduction of these proteins may be compensated by up-regulation of NBEAL2. However,

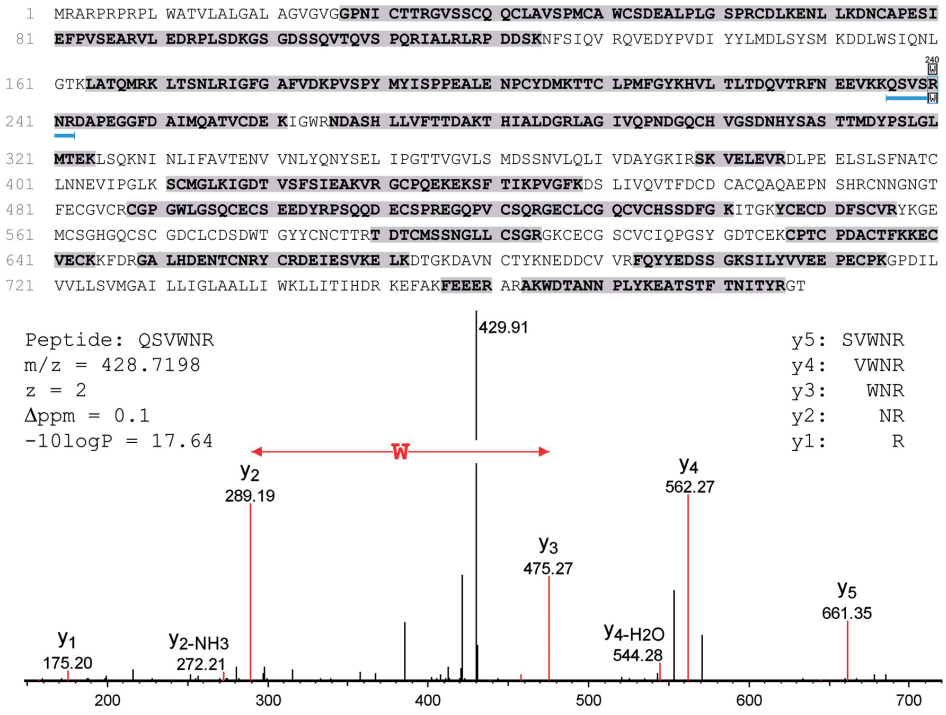


Figure 5. Sequence coverage and mutation of the β3 subunits. (A) Primary sequence of the β3 subunit with in bold the peptide regions that were identified by PEAKS 6.0 using a database comprising only the αIIb and β3 subunit. Underlined is the identified peptide by PEAKS PTM with the R214W mutation. As the signal sequence in included in the amino acid numbering, the mutation in the sequence is indicated at position R240. (B) MS/MS spectrum of peptide QSVWNR identified by the PEAK PTM search algorithm. m/z is the mass to charge ratio, z represents the charge, ppm is the deviation from the theoretical mass of the peptide in parts per million and the term -10logP represents the peptide reliability score. Indicated in the spectrum are the y-ion series of the MS/MS fragments of the peptide. The mass difference between the y2 and y3 fragment that represents the loss of the tryptophan is displayed with the double arrow.

as the role of NBEAL2 is unclear, this remains highly speculative. Further studies are required to unravel the potential link, if any, between NBEAL2 and αIIbβ3.

In this study, proteomic analysis of control and patient-derived proteins showed that the calculated relative amounts for several known proteins reflected the expected relative protein abundance in normal and GT or LAD-III platelets, thereby validating the proteomic approach. Moreover, the approach can provide information for the identification of other proteins implicated in the disease. Our study provides direct evidence that an ambitious patient-oriented database could be considered feasible for characterized platelet defects in a near future.

ACKNOWLEDGMENT

This work was supported by Sanquin Blood Supply, grant number PPOC-10-012 (A.B.M).

AUTHOR CONTRIBUTIONS

C.Z. performed experiments, analyzed the data and wrote the paper; C. vd Z., I.M.D.C. M.vd B. performed experiments. T.vd B., L.G. and K.M. provided critical contribution to discussions; T.W.K. provided platelets samples and critical contribution to discussions. A.B.M. designed and performed experiments, analyzed the data and wrote the paper. We disclose that there is no conflict of interest.

REFERENCES

1 Broos K, De Meyer SF, Feys HB, Vanhoorelbeke K, Deckmyn H. Blood platelet biochemistry. *Thromb Res*. 2012;129(3):245-249.

2 Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev*. 2009;23(4):177-189.

3 Burkhart JM, Vaudel M, Gambaryan S, et al. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120(15):e73-82.

4 Bennett JS, Berger BW, Billings PC. The structure and function of platelet integrins. *J Thromb Haemost*. 2009;7 Suppl 1:200-205.

5 Emsley J, Knight CG, Farndale RW, Barnes MJ, Liddington RC. Structural basis of collagen recognition by integrin alpha2beta1. *Cell*. 2000;101(1):47-56.

6 Malinin NL, Zhang L, Choi J, et al. A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans. *Nat. Med.* 2009;15(3):313-318.

7 Moser M, Legate KR, Zent R, Fassler R. The tail of integrins, talin, and kindlins. *Science*. 2009;324(5929):895-899.

8 Kuijpers TW, van de Vijver E, Weterman MA, et al. LAD-1/variant syndrome is caused by mutations in FERMT3. *Blood*. 2009;113(19):4740-4746.

9 Mory A, Feigelson SW, Yarali N, et al. Kindlin-3: a new gene involved in the pathogenesis of LAD-III. *Blood*. 2008;112(6):2591.

10 Svensson L, Howarth K, McDowall A, et al. Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat Med*. 2009;15(3):306-312.

11 Meller J, Malinin NL, Panigrahi S, et al. Novel aspects of Kindlin-3 function in humans based on a new case of leukocyte adhesion deficiency III. *J Thromb Haemost*. 2012;10(7):1397-1408.

12 Pasvolsky R, Feigelson SW, Kilic SS, et al. A LAD-III syndrome is associated with defective expression of the Rap-1 activator CalDAG-GEFI in lymphocytes, neutrophils, and platelets. *J Exp Med*. 2007;204(7):1571-1582.

13 van de Vijver E, De Cuyper IM, Gerrits AJ, et al. Defects in Glanzmann thrombasthenia and LAD-III (LAD-1/v) syndrome: the role of integrin beta1 and beta3 in platelet adhesion to collagen. *Blood*. 2012;119(2):583-586.

14 Caswell PT, Norman JC. Integrin trafficking and the control of cell migration. *Traffic*. 2006;7(1):14-21.

15 Bottcher RT, Stremmel C, Meves A, et al. Sorting nexin 17 prevents lysosomal degradation of beta1 integrins by binding to the beta1-integrin tail. *Nat Cell Biol*. 2012;14(6):584-592.

16 Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa). *Blood*. 1993;82(1):135-138.

17 Yang H, Lang S, Zhai Z, et al. Fibrinogen is required for maintenance of platelet intracellular and cell-surface P-selectin expression. *Blood*. 2009;114(2):425-436.

18 Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood*. 2011;118(23):5996-6005.

19 De Cuyper IM, Meinders M, van de Vijver E, et al. A novel flow cytometry-based platelet aggregation assay. *Blood*. 2013;121(10):e70-80.

20 Simsek S, Heyboer H, de Bruijne-Admiraal LG, Goldschmeding R, Cuijpers HT, von dem Borne AE. Glanzmann's thrombasthenia caused by homozygosity for a splice defect that leads to deletion of the first coding exon of the glycoprotein IIIa mRNA. *Blood*. 1993;81(8):2044-2049.

21 Piersma SR, Broxterman HJ, Kapci M, et al. Proteomics of the TRAP-induced platelet releasate. *J Proteomics*. 2009;72(1):91-109.

22 van Breevoort D, van Agtmaal EL, Dragt BS, et al. Proteomic screen identifies IGFBP7 as a novel component of endothelial cell-specific Weibel-Palade bodies. *J Proteome Res*. 2012;11(5):2925-2936.

23 Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem*. 2002;74(20):5383-5392.

24 Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem*. 2003;75(17):4646-4658.

25 Ma B, Zhang K, Hendrie C, et al. PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2003;17(20):2337-2342.

26 Gong H, Shen B, Flevaris P, et al. G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling. *Science*. 2010;327(5963):340-343.

27 Shen B, Delaney MK, Du X. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Curr Opin Cell Biol*. 2012;24(5):600-606.

28 Provost P, Doucet J, Stock A, Gerisch G, Samuelsson B, Radmark O. Coactosin-like protein, a human F-actin-binding protein: critical role of lysine-75. *Biochem J*. 2001;359(Pt 2):255-263.

29 Albers CA, Cvejic A, Favier R, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. *Nature Genet*. 2011;43(8):735-737.

30 Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet alpha-granules. *Nat Genet*. 2011;43(8):732-734.

31 Kahr WH, Hinckley J, Li L, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. *Nat Genet*. 2011;43(8):738-740.

32 Grinna LS, Robbins PW. Glycoprotein biosynthesis. Rat liver microsomal glucosidases which process oligosaccharides. *J Biol Chem*. 1979;254(18):8814-8818.

33 Caudy AA, Ketting RF, Hammond SM, et al. A micrococcal nuclease homologue in RNAi effector complexes. *Nature*. 2003;425(6956):411-414.

34 Lanza F, Stierlé A, Fournier D, et al. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa complexes and a glycoprotein IIIa 214Arg----214Trp mutation. *J Clin Invest*. 1992;89(6):1995-2004.

35 Springer TA, Zhu J, Xiao T. Structural basis for distinctive recognition of fibrinogen gammaC peptide by the platelet integrin alphaIIbbeta3. *J Cell Biol*. 2008;182(4):791-800.

36 Arnaout MA, Mahalingam B, Xiong JP. Integrin structure, allostery, and bidirectional signaling. *Annu Rev Cell Dev Biol*. 2005;21:381-410.

37 Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annu Rev Immunol*. 2007;25:619-647.

38 Zhu J, Boylan B, Luo BH, Newman PJ, Springer TA. Tests of the extension and deadbolt models of integrin activation. *J Biol Chem*. 2007;282(16):11914-11920.

39 Heino J, Ignotz RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. *J Biol Chem*. 1989;264(1):380-388.

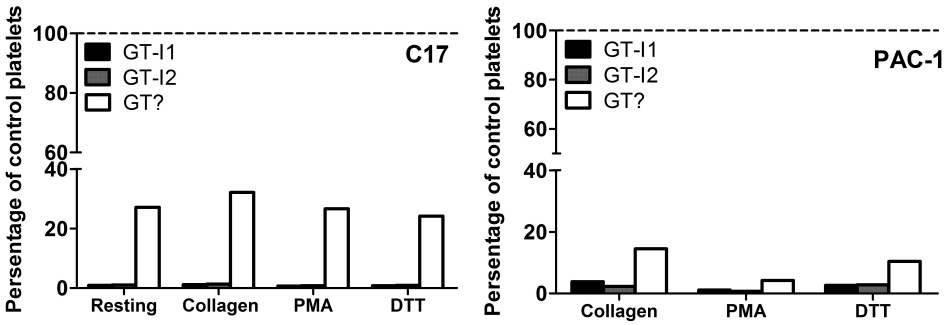
40 Kruger M, Moser M, Ussar S, et al. SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell*. 2008;134(2):353-364.

41 Deppermann C, Cherpokova D, Nurden P, et al. Gray platelet syndrome and defective thrombo-inflammation in Nbeal2-deficient mice. *J Clin Invest*. *2013;123(8):3331-3342*.

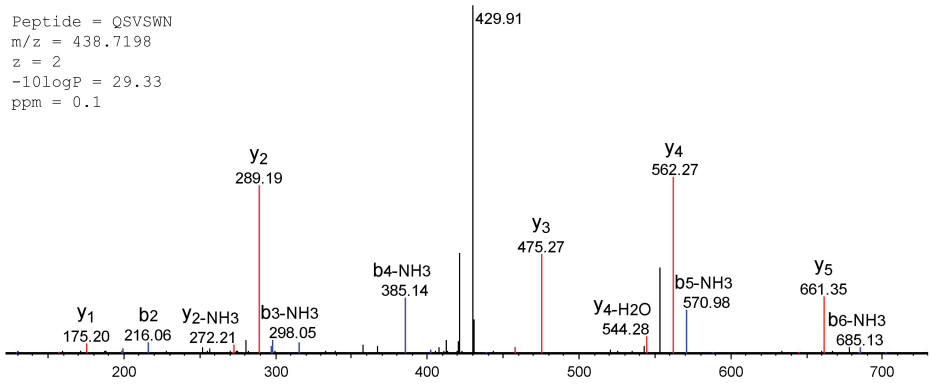
42 Kahr WHA, Lo RW, Li L, et al. Abnormal megakaryocyte development and platelet function in Nbeal2-/- mice. *Blood*. 2013;122(19):3349-3358.

SUPPLEMENTAL DATA

This manuscript includes two supplemental figures.



Supplemental figure S1. Flow cytometry analysis of αIIbβ3 expression and activation profile of the GT platelets. Staining of the αIIbβ3 was performed essentially as previously described[13]. Briefly, resting washed platelets and platelets were stimulated 5 min with agonists (10 g/mL collagen, 100 ng/mL phorbol myristate acetate (PMA), or 10 mM dithiotreithol (DTT). (A) Staining with antibody C17-FITC was employed to assess the total level of αIIbβ3 on the platelet surface. (B) Staining with antibody PAC-1–FITC was employed to detect the active αIIbβ3 integrin on the platelet surface. All data are expressed as percentage of mean fluorescence intensity (MFI) values of control platelets. The results obtained from the two GT-I patients are shown as GT-I1 and GT-I2. The putative GT patient is represented by GT?. The results show that both GT-I patients exhibit no expression of the αIIbβ3 integrin. The GT? patient shows residual expression of the integrin. All GT patients further reveal a markedly impaired activation of the integrin after stimulation with the indicated agents.



Supplemental figure S2. Sequence coverage of the β3 subunits employing a database comprising all known human proteins. (A) Primary sequence of the β3 subunit including the R to W mutation at position 240 (R214W excluding amino acid numbering of the signal sequence). In bold is shown the regions that were identified by PEAKS 6.0 software using a database comprising uniprot-organism_9606_AND_keyword_kw-0181.fasta. (B) MS/MS spectrum of peptide QSVWNR identified by the PEAK 6.0 search engine. m/z is the mass to charge ratio, z represents the charge, ppm is the deviation from the theoretical mass of the peptide in parts per million and the term -10logP represents the peptide reliability score. Indicated in the spectrum are the y-ion and b ion series.

CHAPTER 4

Limited effect of the Munc18-2 c.1247-1G>C splice-site variant on platelet degranulation

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Submitted for publication

ABSTRACT

It has been proposed that mammalian-uncoordinated-18-2 (Munc18-2) is indispensable for platelet degranulation. Bleedings are, however, inconsistently observed in familial hemophagocytic lymphohistiocytosis type-5 (FHL-5) patients with a Munc18-2 c.1247-1G>C splice-site mutation. We have now employed mass spectrometry (MS), flow cytometry and electron microscopy (EM) studies to identify the protein product of the splice-site variant and the effect thereof on α-granule degranulation of platelets. The results revealed that activated platelets of patients, who are homozygous for the splice-site mutation, release α-granules. MS analysis showed that the mutation leads to replacement of 17 amino acids by 19 amino acids derived from intron 14. The platelets displayed a decreased expression of Munc18-2 and its binding partner syntaxin-11. A patient comprising the splice-site and a c.1621G>A (p.Gly541Ser) mutation demonstrated a further decrease in expression of Munc18-2 but not of syntaxin-11. The results showed that also these platelets degranulate upon stimulation. EM studies revealed that especially the platelets of the heterozygous patient exhibit a decreased number of granules correlating with a reduced expression P-selectin on the activated platelet surface. We propose that platelet degranulation is largely intact in FHL-5 patients carrying the Munc18-2 splice-site variant. This may explain the inconsistency of bleeding symptoms associated with FHL-5.

INTRODUCTION

Mammalian uncoordinated-18-2 (Munc18-2), which is also known as Munc18b and syntaxin binding protein-2, is a member of the Sec1/Munc18 family. The family members are key regulators of the universal machinery for secretory granule trafficking, membrane fusion and exocytosis.[1,2] The machinery involves the interaction between the so-called "soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptors" (SNAREs). SNAREs can be divided into vesicular SNAREs, which are incorporated in the membranes of vesicles, and target-localized (t-) SNAREs located in the membranes to which these vesicles dock. Munc18 family members have been described to act as chaperones of the t-SNARE syntaxins, and have been proposed to regulate the specificity and timing of SNARE complex assembly and disassembly. The exact function of the individual Munc18 proteins has remained, however, a topic for investigation.[2-4]

Munc18-2 has initially been identified as a non-neuronal Sec1/Munc18 family member that regulates apical membrane trafficking in epithelial cells and exocytosis of granules from mast cells.[5-10] In recent years, it has become clear that functional absence of Munc18-2 is associated with the disorder Familial Hemophagocytic Lymphohistiocytosis type-5 (FHL-5).[11-17] The disease presents itself with uncontrolled inflammation, lymphoproliferation and tissue infiltration of activated T lymphocytes and macrophages. FHL-5 patients can also display a spectrum of other symptoms including hypogammaglobulinemia, colitis and Hodgkin's Lymphoma.[12-15,17-21] It has now been established that the underlying defect involves impaired degranulation of neutrophils, cytotoxic natural killer (NK) cells, and cytotoxic T lymphocytes (CTL).[12,14,20-22]

It has previously been demonstrated that Munc18 family members contribute to platelet degranulation.[23,24] Based on studies on the platelets of FHL-5 patients, the conclusion has been drawn that Munc18-2 is critical for the regulated release of the α- and δ- granules from platelets.[25,26] Complete absence of Munc18-2 in the platelets from FHL-5 patients with biallelic mutations in Munc18-2 has been shown to lead to an impaired degranulation upon stimulation. Heterozygous patients that have reduced expression of functional Munc18-2 revealed a partially impaired degranulation. It has therefore been proposed that Munc18-2 could be a limiting component of the secretory machinery.[25] In spite of the suggested critical role of Munc18-2 for platelet degranulation, there is no clear evidence that bleedings are directly linked to FHL-5.[13,15,25,26]

To date, up to twenty Munc18-2 variants have been associated with FHL-5. A c.1247-1G>C splice-site mutation in the Munc18-2 gene is the most frequently observed mutation in the patients.[12-15,17] The functional consequence of this variant is, however, poorly understood. It can be causative of a late onset of the disease that might eventually evolve into severe clinical symptoms and death.[13-15,18,20] Whether or not a platelet degranulation defect may contribute to the disease remains to be established. It is also unclear how the splice-site mutation affects the resulting Munc18-2 protein itself. Based on cDNA analysis, Cote *et al*. have proposed that this mutation leads to a protein in which 17 amino acids of exon 15 are replaced with 19 new amino acids derived from intron 14.[12] Based on a similar analysis, two other groups have identified a transcript that results in a truncated protein containing a large stretch of 126 amino acid residues that are not encoded by the Munc18-2 gene.[15,17]

In the present study, the role of the c.1247-1G>C splice-site variant in the release of α-granule proteins from platelets was addressed employing a combination of flow cytometry, mass

spectrometry (MS) and electron microscopy (EM) studies. In addition, we assessed the identity of the resulting protein product in platelets. To this end, we employed the platelets from two FHL-5 patients that are homozygous for the splice-site mutation, and from one FHL-5 patient comprising the splice-site mutation on one allele and a c.1621G>A (p.Gly541Ser) mutation on the second allele. The latter variant results in a replacement of a conserved residue among the Munc18 family members and leads to a complete absence of the protein in NK cells and CTL.[11]

The MS study revealed that the splice-site mutation leads to the protein product comprising the 19 amino acids derived from intron 14, and that its expression level is reduced compared to platelets from healthy individuals. In spite of these findings, our MS, flow cytometry and EM analysis demonstrate that the platelets from the analyzed FHL-5 patients are still able to degranulate. This may explain the inconsistency in clinical bleeding symptoms associated with FHL-5, and suggests other roles for Munc18-2 than merely promoting granule exocytosis.

MATERIALS AND METHODS

Reagents - All fine chemicals were from Merck (Merck, Darmstadt, Germany) unless otherwise stated. The antibodies CD62P-APC, CD42b-APC, and CD41-PerCPCy5.5 were purchased from BD Biosciences (San Jose, USA). CD31-Pacific Blue and CD36-APCCy7 from Biolegend (San Diego, USA), and CD61-FITC from R&D (Basel, Switzerland). The PAR1-activating peptide (SFLLRN-NH2; Par-3676-PI) was purchased from Peptides International (Louisville, USA).

Collection of platelet lysate and releasate - Heparinized blood was collected from three FHL-5 patients and of healthy controls. The study was approved by the academic medical center (AMC) institutional medical ethics committee in accordance to the declaration of Helsinki 1964. Platelets were isolated as described before.[27] Briefly, $3x10^8$ platelets from two healthy donors and the three FLH5 patients were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail (Halt Thermoscientific, Rockford, USA) and kept on ice for 30 min. Platelet lysates were centrifuged at 4°C to remove insoluble material. To collect the releasate, $2x10^8$ platelets were split into two aliquots. One aliquot served as non-stimulated control. The other aliquot was stimulated employing 10µM of the PAR1-activating peptide for 5 min at 37°C under constant stirring at 1110 rpm. Platelet releasate/supernatant were collected by two sequential centrifugations at 2,000xg and 16,000xg, for 10 min at room temperature and then concentrated down to 20 µl in a vacuum centrifuge (Thermoscientific, Breda, The Netherlands).

Analysis of platelet degranulation by flow cytometry and platelet aggregation assessed by flow cytometry - Washed human platelets were activated with increasing concentrations of PAR1-activating peptide (10, 100 and 1000µM), at 37°C while shaking at 1000 rpm. 0.5% paraformaldehyde fixed samples were stained with Allophycocyanin (APC) labeled anti-CD62P antibody (CD62P/APC). Aggregation assessed by flow cytometry was performed as described in De Cuyper *et al*.[27] As agonists we used 100 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich, St Louis, MO), 10 µg/ml type-I collagen (Horm, Nycomed Arzneimittel GmbH, München, Germany), and 1.5 mg/ml Ristocetin (Biopool, Trinity Biotech Plc, Bray, Co Wincklow, Ireland). Samples were measured by flow cytometry (LSRII + HTS, BD Biosciences) and analyzed using FlowJo software (Tree star Inc., Ashland, USA).

Mass spectrometry analysis - Proteins in the lysate and releasate of the platelets were separated on SDS-PAGE and processed for mass spectrometry analysis as described.[28,29] Peptides were separated using a reverse-phase C18 Acclaim PepMap RSLC (75 µm × 150 mm, 2 µm particles) at a flow rate of 300 nl/min using a one-hour linear gradient from 0.05% acetic acid (v/v) to 0.05% (v/v) acetic acid and 35% (v/v) acetonitrile employing a Dionex Ultimate 3000 RSLC. Once separated, the peptides were directly sprayed into the LTQ Orbitrap XL mass spectrometer. Peptides were identified using the Sequest search algorithm using proteome discoverer 1.2 as described.[29] A maximum false discovery rate of 5% was allowed. The mass spectrometer, the nanoelectrospray source and emitters, the C18 column, nanoLC system, and analysis software were from Thermo Fisher Scientific (Bremen, Germany). Normalized spectral count of the proteins was obtained employing Scaffold (version Scaffold_4.0.4, Proteome Software Inc., Portland, OR).

Electron microscopy analysis of platelets - Platelets were fixed in Karnovsky's fixative. Postfixation was done with 1% Osmiumtetroxide in 0,1M cacodylate buffer, after washing the pellets were stained and blocked with Ultrastain 1 (Leica, Vienna, Austria), followed by ethanol dehydration series. Finally, the platelets were embedded in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, USA), sectioned and stained with Ultrastain 2 and analyzed with a Philips CM10 electron microscope (FEI company, Eindhoven, the Netherlands). For immunoelectron microscopy analysis, platelets were fixed in 2% paraformaldehyde and 0,2% glutaraldehyde in 0.1M PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM MgCl2, 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described[30]. Briefly, 50-nm cryosections were cut at -115º C using diamond knives in a cryoultramicrotome (Leica) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids. The grids were placed on 35-mm petri dishes containing 2% gelatine. Ultrathin frozen sections were incubated at room temperature with anti-Von Willebrand Factor (DAKO, Glostrup, Denmark) followed by a rabbit anti-mouse bridging antibody, and then incubated with 10-nm protein A-conjugated colloidal gold (EM Lab, Utrecht University, Netherlands). After immunolabeling, the sections were embedded in a mixture of methylcellulose and uranyl acetate and examined with the CM10 electron microscope.

RESULTS

Mass spectrometry analysis of the platelet secretome of FHL-5 patients carrying the Munc18-2 splice-site mutation. The two FHL-5 patients that are homozygous for the Munc18-2 splice-site variant are referred to as $P1^{splice}$ and $P2^{splice}$ and the heterozygous patient as $P3^{splice/G541S}$. To assess whether or not the splice-site variant supports α-granule release, the platelets of the patients were activated via protease-activating-receptor-1 (PAR1) with a PAR1-activating peptide. The proteins in the supernatant of stimulated and non-stimulated platelets were identified by mass spectrometry. The normalized spectral count of the identified proteins was utilized to estimate their relative abundance in the platelet supernatants. Figure 1A shows the fold increase in spectral counts of a subset of identified typical α-granule proteins in the supernatant of stimulated platelets with respect to their own non-stimulated platelets. The result showed that there is an increase in the presence of α-granule proteins in the supernatant of the stimulated FHL-5 platelets. Figure 1B shows the average spectral count of proteins released from the combined non-stimulated FHL-5

platelets versus the combined stimulated FHL-5 platelets. Data points that are above the indicated diagonal represent proteins that are enriched in the supernatant of the stimulated platelets. These enriched proteins appear to be typical α-granule proteins, including e.g. von Willebrand factor, fibrinogen and platelet factor 4 (see table in Figure 1B). The results together imply that the Munc18-2 variants in homozygous patients $P1^{splice}$ and $P2^{splice}$ and the heterozygous patient $P3^{splice/G541S}$ support PAR1-mediated degranulation of α-granules from the platelets.

Relative abundance of Munc18-2 and identification of the protein product of Munc18-2 splice-site variant. The lysates of the platelets were analyzed by mass spectrometry for the presence of Munc18-2. Figure 2A shows that the normalized spectral count of Munc18-2 derived from patient platelets $P1^{splice}$ and $P2^{splice}$ was about 40% of control value. The spectral count dropped to less than 3% for patient $P3^{splice/G541S}$. This implies that the expression of the Munc18-2 variant in the platelets of $P1^{splice}$ and $P2^{splice}$ and especially in $P3^{splice/G541S}$ is markedly reduced. Compatible with earlier reports, the level of STX11 was also reduced in the platelets of the patients (Fig. 2A). Surprisingly, irrespective of the protein level of Munc18-2 in the platelets, the same residual level of STX11 was found. No significant change was observed in the spectral count of other members of the SNARE complex in the platelet lysates (data not shown). The identified peptides of Munc18-2 covered about 41% of the primary sequence of the protein in control platelets and about 32% in the platelets of $P1^{splice}$ and $P2^{splice}$ (Fig. 2B). Strikingly, from the lysate of $P2^{splice}$, a MS/MS fragmentation spectrum was obtained that could be reliably matched to peptide sequence NATPLDPGTLLHWLGDSSTEAHSSLIR (Fig. 2C). Part of this peptide can only be derived from intron 14[12] (Fig. 2B,C). This finding demonstrates that the splice-site variant results in a protein product in which 17 amino acids from exon 15 are swapped by 19 new amino acids derived from intron 14. No MS/MS spectra were identified that could be matched to peptides derived from the other proposed protein products. Even though the presence of minor products cannot be excluded, this result indicates that the splice-site mutation leads to the transcription of a protein comprising 19 amino acids derived from intron 14.

The Munc18-2 variants exhibit a modest reduction in degranulation efficiency. Flow cytometry was employed to follow the appearance of the degranulation marker P-selectin (CD62P) on the surface of platelets of the patients upon PAR1 stimulation. We observed that platelets from all three patients showed surface exposure of the α-granule protein CD62P in response to saturating concentrations of the PAR1-activating peptide. The surface exposure was, however, reduced in the platelets of especially $P3^{splice/G541S}$ compared to the activated platelets of healthy individuals (Fig. 3A). As the platelets of $P3^{splice/G541S}$ also exhibit the strongest decrease in the protein level of Munc18-2 (Fig. 2A), we further assessed α-granule release from these platelets employing increasing concentrations of the PAR1-activating peptide. The result showed a reduced, but dose-dependent, appearance of the α-granule degranulation marker at the surface of the platelets of the patient (Fig. 3B). This suggests that the platelets are still able to release α-granules even in almost complete absence of Munc18-2. The results together confirm the findings obtained with mass spectrometry and show that the platelets of the FHL-5 patients are not incapacitated to degranulate.

The ability of the platelets of $P1^{splice}$, $P2^{splice}$ and $P3^{splice/G541S}$ to aggregate in response to stimuli was also assessed. Flow cytometry analysis revealed no change in the expression of GPIIbIIIa (CD41/61,

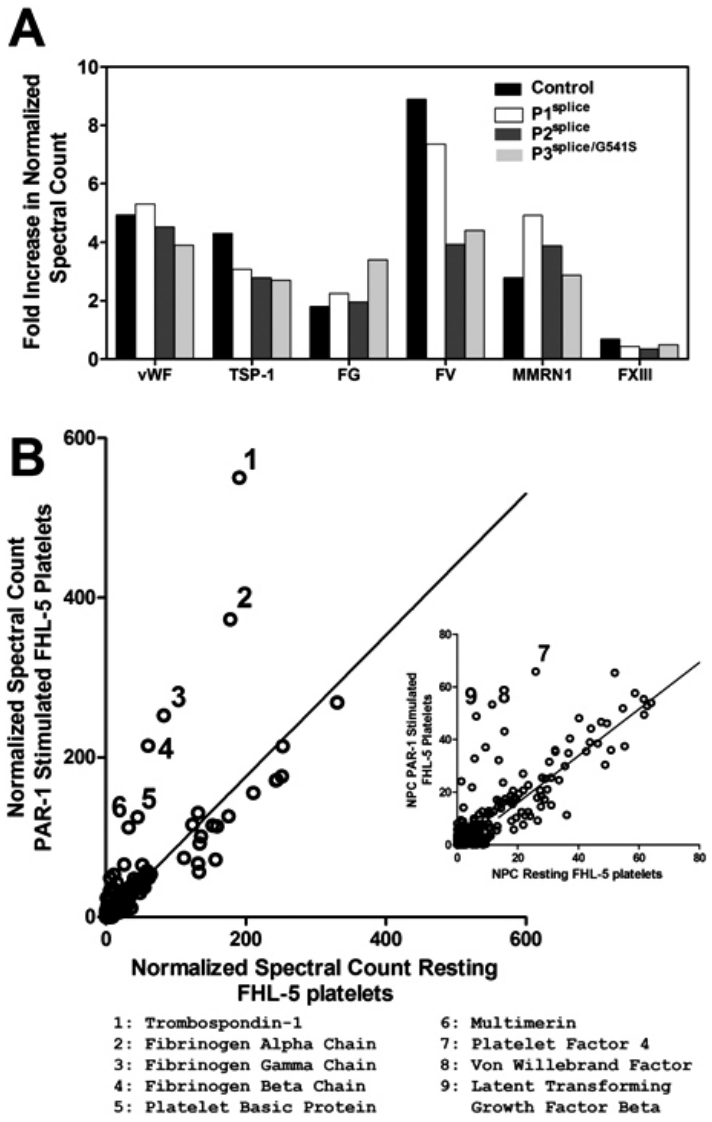

Figure 1. Semi-quantitative analysis of α-granule protein secretion in platelets of healthy individuals and FHL-5 patients. (A) The proteins in the supernatant of non-stimulated and PAR1-stimulated platelets of controls and the patients $P1^{splice}$, $P2^{splice}$ and $P3^{splice/G541S}$ were analyzed by mass spectrometry. To obtain the fold-increase in released proteins, normalized spectral counts of the proteins in the supernatant of the PAR1-stimulated platelets were divided with that obtained from the supernatant of their own non-stimulated platelets. A selection of identified α-granule proteins is displayed, including VWF, thrombospondin-1 (TSP-1), and fibrinogen (FG), coagulation factors (FV, FXIII) and Multimerin-1 (MMRN1). (B) The average normalized spectral count from the proteins of the supernatant of the non-stimulated platelets of the three FHL-5 patients was plotted as a function of that of the stimulated FHL-5 platelets. Data points that are close to the displayed diagonal represent proteins that are about equally present in the supernatant of the resting and stimulated platelets. The proteins that belong to the numbered data points are indicated in panel B. The inset is a zoomed-in section of the figure.

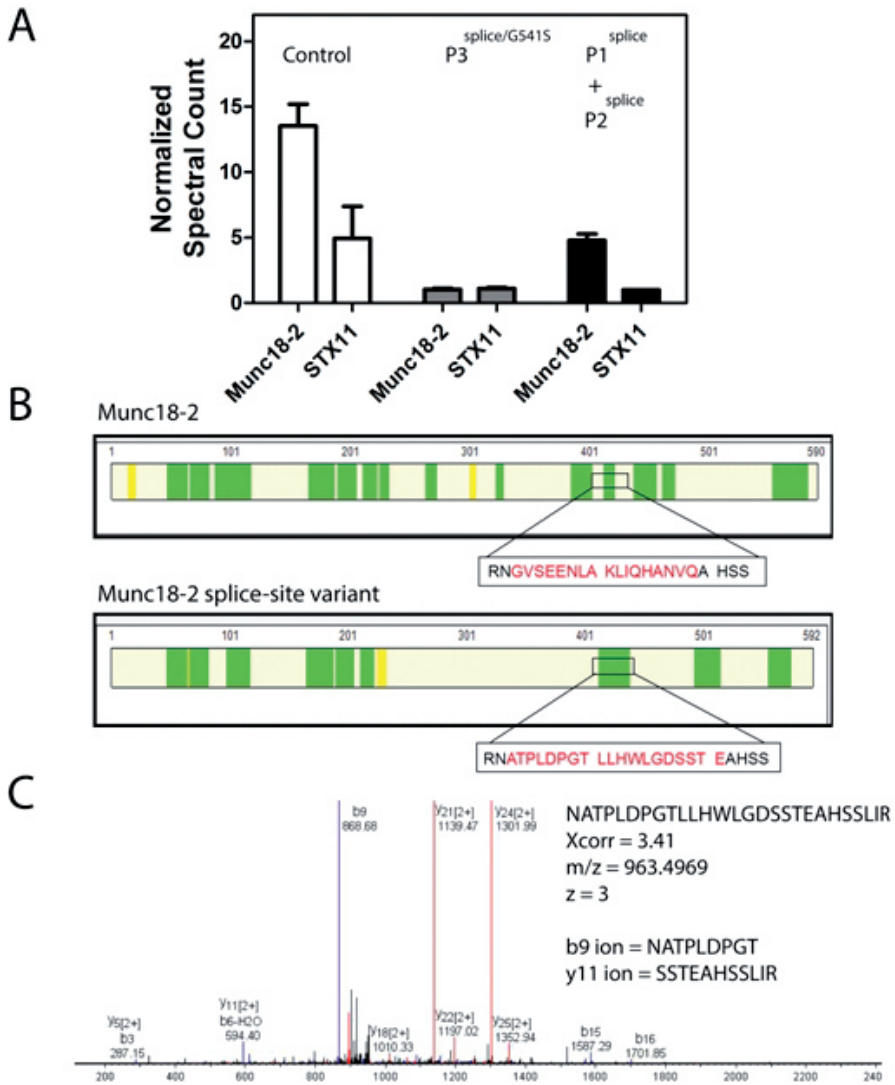

Figure 2. Relative abundance of Munc18-2 and STX11 and identification of the protein product of the splice-site variant. (A) The average of the normalized spectral counts of the proteins in the platelet lysate of healthy controls, P3$^{splice/G541S}$, and the platelets of the patients that are homozygous for the Munc18-2 splice-site mutation (P1^{splice} and P2^{splice}) are displayed. Error bars represent standard deviation of the data obtained from the platelets of healthy controls (5 platelets donors), and P3$^{splice/G541S}$ (three independent MS analysis). Error bar on the data obtained for P1^{splice} and P2^{splice} represent the spread around the mean. (B) Schematic representation of the primary amino acid sequence of WT Munc18-2 and the identified Munc18-2 splice-site variant. Areas in the sequence that are indicated in green are identified with high confidence (false discovery rate of 1%). Areas in yellow are identified with medium confidence (false discovery rate of 5%). The difference in amino acid sequence between WT Munc18-2 and the splice-site variant are indicated. (C) MS/MS spectrum of the identified peptide (in patient P2^{splice}) that includes 19 amino acids derived from intron 14. Xcorrelation score (Xcor), mass over charge ratio (m/z) and the charge (z) of the peptide-ion are displayed. The most intense y- and b-ions are indicated as well as the peptide fragments that belong to the b9 and y11-ions.

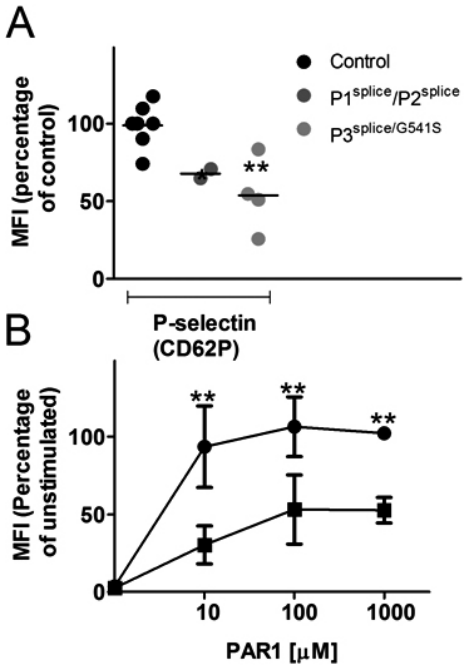

Figure 3. Analysis of platelet degranulation by flow cytometry. (A) Platelets of the patients and healthy individuals were stimulated for 5 minutes employing 100 μM PAR1-activating peptide. The average increase in mean fluorescence intensity (MFI) of P-selectin (CD62P) of the stimulated control platelets was set to 100%. The increase in MFI of the platelets of the patients is shown as a percentage of control. Black dashes indicate the mean value of all individual measurements, which are represented as dots. (B) P-selectin (CD62P) at the platelet surface of $P3^{splice/G541S}$ (squares) and control (circles) platelets was assessed as a function of the concentration of the PAR1-activating peptide. Results are expressed as the percentage of mean fluorescence intensity (MFI) in respective un-stimulated platelet samples. Data are expressed as mean value ± standard deviation of at least three measurements; * $p<0.005$; ** $p<0.001$.

i.e. the fibrinogen receptor), GPIb (CD42b, i.e. the subunit of the VWF receptor) and PECAM-1 (CD31) in non-stimulated platelets of the patients compared to that of control platelets (Fig. 4A). Platelet aggregation assessed by flow cytometry demonstrated that the platelets of the FHL-5 patients effectively aggregate in the presence of phorbol 12-myristate 13-acetate (PMA) (via GPIIbIIIa), ristocetin (via VWF receptor), and collagen (via GPIaIIa) (Fig. 4B). The findings show that $P1^{splice}$, $P2^{splice}$ and $P3^{splice/G541S}$ exhibit functional platelets that aggregate and degranulate upon stimulation.

EM study reveals limited effect of the Munc18-2 variants on platelet degranulation. EM studies were employed as a third independent experimental approach to assess the putative release of granules from the platelets of the FHL-5 patient $P3^{splice/G541S}$. Figure 5A displays images of resting control platelets and of the platelets of the patient. The α-granule protein VWF was immunostained with gold particles in this image. The results show that the platelets from $P3^{splice/G541S}$ comprise morphologically intact α-granules. We next examined by EM the platelet aggregates after PAR1 stimulation. EM images were taken of the edge of the aggregate

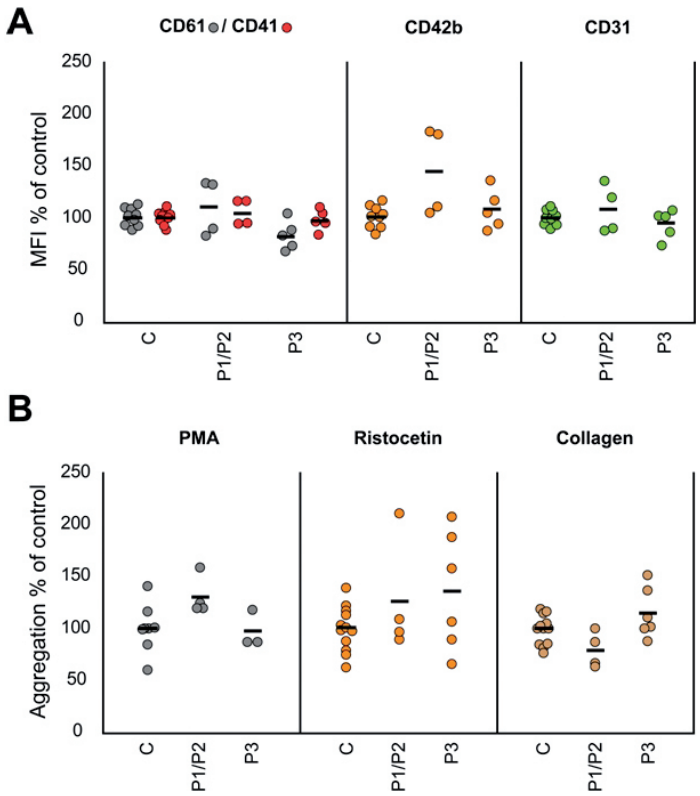

Figure 4. FHL-5 platelets do not present aggregation defects. (A) Surface marker expression (CD61, CD41, CD42b and CD31) is shown in un-stimulated control, $P1^{splice}$/$P2^{splice}$ and $P3^{splice/G541S}$ platelets. (B). Platelet aggregation in response to agonists PMA, ristocetin and collagen as measured by flow cytometry-based aggregation assay. Black dashes indicate the mean value of all individual measurements, which are represented as dots.

as individual platelets cannot be recognized in the center of the aggregate. As shown in figure 5B, platelets from patient $P3^{splice/G541S}$ and healthy controls completely lacked granules after stimulation. EM image analysis of multiple platelets from $P1^{splice}$, $P2^{splice}$ and $P3^{splice/G541S}$ demonstrated a reduction in the number of granules in resting platelets of $P2^{splice}$ and especially of $P3^{splice/G541S}$ (Fig. 6). The finding may provide an explanation for the reduced surface expression of P-selectin after stimulation (Fig. 4). Collectively, this study shows that platelets with limited expression of the Munc18-2 variants retain their ability to degranulate.

DISCUSSION

Late onset and/or atypical clinical presentation of FHL-5 has been associated with the splice-site variant affecting exon 15, which is the most common and least understood Munc18-2 variant.[13,15,16,18] In the present study, a MS approach, flow cytometry and EM studies were employed to assess the role of the splice-site variant on platelet degranulation. The analysis showed that

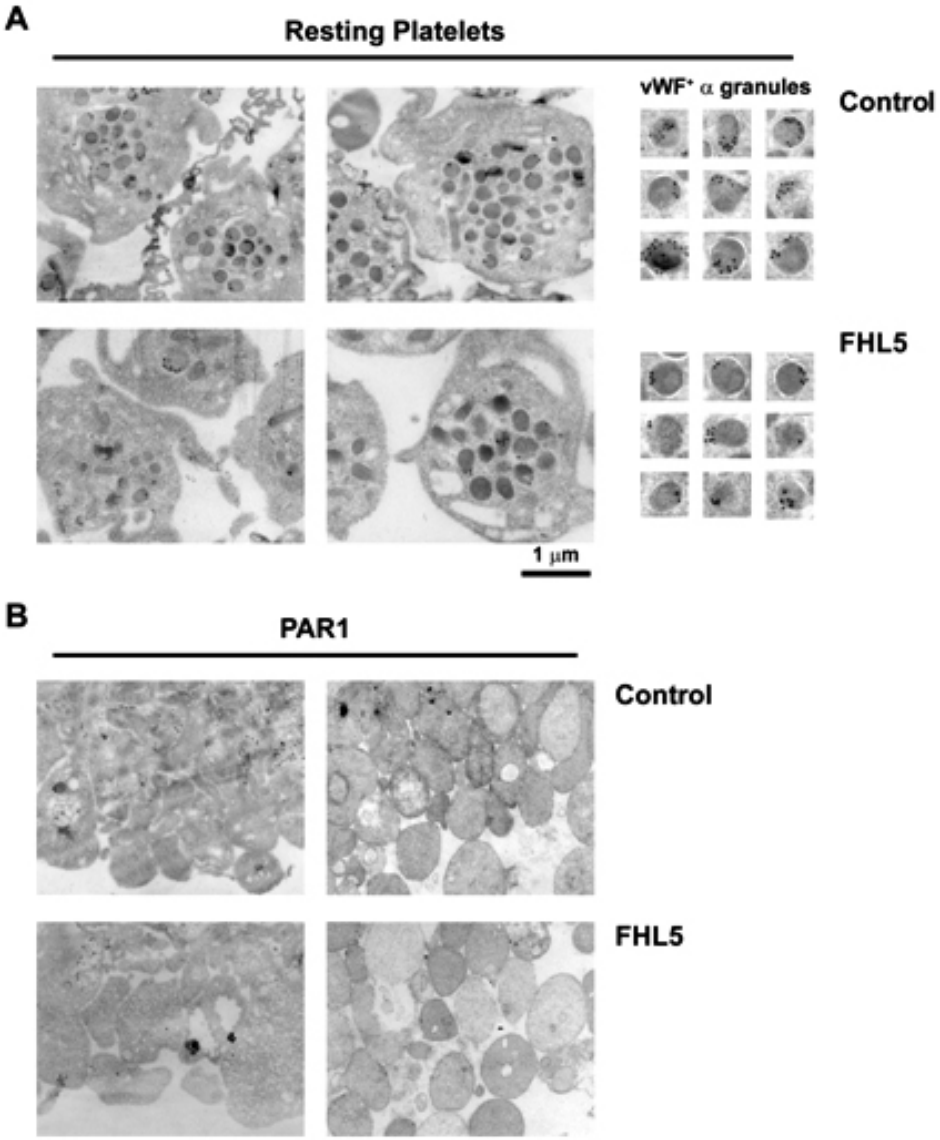

Figure 5. EM image analysis reveals complete degranulation of the platelets of P3$^{splice/G541S}$. (A) Two representative EM images of healthy control platelets (upper panel) and platelets obtained from patient P3$^{splice/G541S}$ (lower panel). VWF is immunostained with gold beads, which is displayed as black dots in the images. On the right, EM images of individual α-granules are shown. (B) EM images of control platelets (upper panel) and the platelets of P3$^{splice/G541S}$ (lower panel) that were stimulated for 5 minutes with PAR1-activating peptide. The images display the edge of the platelet aggregate. In the left two images, VWF is immunostained with gold beads.

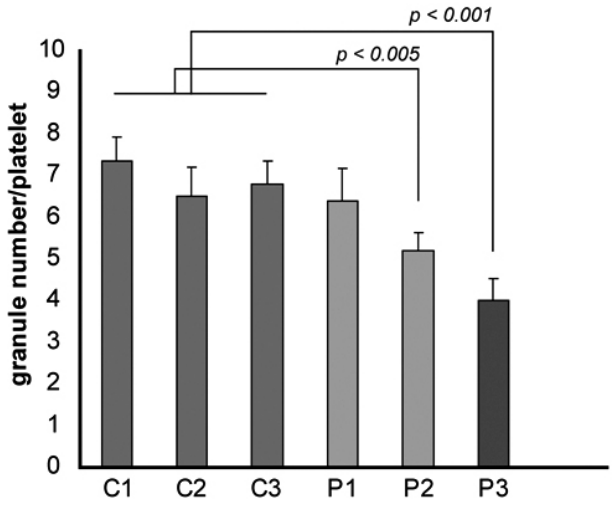

Figure 6. FHL-5 P3$^{splice/G541S}$ platelets have a significant decrease in the granule number. The granules identified in EM imaging of the platelets of healthy controls, P1^{splice}, P2^{splice}, and P3$^{splice/G541S}$ were quantified. Data represent the average ± standard deviation of the granule count of at least 20 platelets.

the splice-site variant comprises 19 amino acids derived from intron 14 instead of 17 amino acids of exon 15 (Fig. 2B-C). In addition, next to a decrease in the protein level of Munc18-2, the analysis demonstrated a reduced expression of STX11 (Fig. 2A). This agrees with the previous suggestion that complex formation between these proteins is required for a stable expression of STX11.[12,15,17] In spite of these findings, no defect is observed in aggregation and degranulation of the platelets of the FHL-5 patients (Figs 1, 3-5). This observation correlates with the clinical features of these patients, which showed no bleeding phenotype, not even during interventions.

Primary sequence homology between Munc18-2 and Munc18-1, of which the crystal structure has been solved, reveals that Munc18-2 comprises three domains. The third domain can be further subdivided into a 3a and a 3b domain. It has been proposed that the 3a domain and the 1 domain contribute to STX11 binding.[31,32] The newly introduced 19 amino acids of the splice-site variant are, however, located in the 3b domain of Munc18-2. This suggests that the reduced level of STX11 in the platelets of the patients is not caused by a direct STX11 binding defect of the splice-site variant. Apparently, the expression level of the Munc18-2 variant is too low to effectively stabilize the level of STX11 in these platelets. Strikingly, although the expression of Munc18-2 is even further reduced in the platelets of P3$^{splice/G541S}$, these platelets show the same decrease in the level of STX11 as found for P1^{splice} and P2^{splice} (Fig. 2A). This implies that other proteins may contribute to the stabilization of STX11 in platelets as well. This may involve other Munc18 family members, which are expressed in platelets, i.e. Munc18-1, Munc18-3 and Munc18-5.[23-25,33,34]

A low expression of the Munc18-2 G541S variant can be understood taking into account that the glycine at position 541 (Gly541) is conserved in all Munc18 family members.[9] As such, this glycine may be of critical importance for a proper folding of the protein. Its replacement may therefore affect protein expression and/or stability. For the splice-site variant, however, it has

previously been proposed that the 19 new amino acid residues may have a limited structural impact on the protein as it may retain the α-helical nature of the 17 amino acids of the wild type protein.[12] Interestingly, comparative homology modeling reveals that part of the 19 new amino acid residues of the splice-site variant may indeed adopt an α-helical structure (Fig. 7). However, the model of WT Munc18-2 shows that the replaced 17 amino acids also include a non-helical spacer region of which valine 417 (Val417) exhibits a hydrophobic interaction with a α-helix within the 3b domain. This suggests that the spacer region may be important for the local structure of the 3b domain. This view is strengthened by the observation that mutation of the neighboring conserved Gly416 in homologous Sec1p of yeast indeed leads to secretion defects.[35] Within the splice-site variant, Val417 has been replaced by the polar threonine, which precludes the stabilizing hydrophobic interaction with the α-helix. In addition, the spacer region and the start of the putative α-helix comprise two conformational rigid prolines that are absent in WT Munc18-2. It seems therefore likely that the splice-site variant will exhibit a folding defect in the 3b domain. This may explain the reduced expression/stability of the splice-site variant compared to WT Munc18-2 (Fig. 2A).

Flow cytometry analysis revealed a reduced expression of the α-granule degranulation marker CD62P at the surface of the platelets of patient P3$^{splice/G541S}$ upon PAR1 stimulation (Fig. 3).

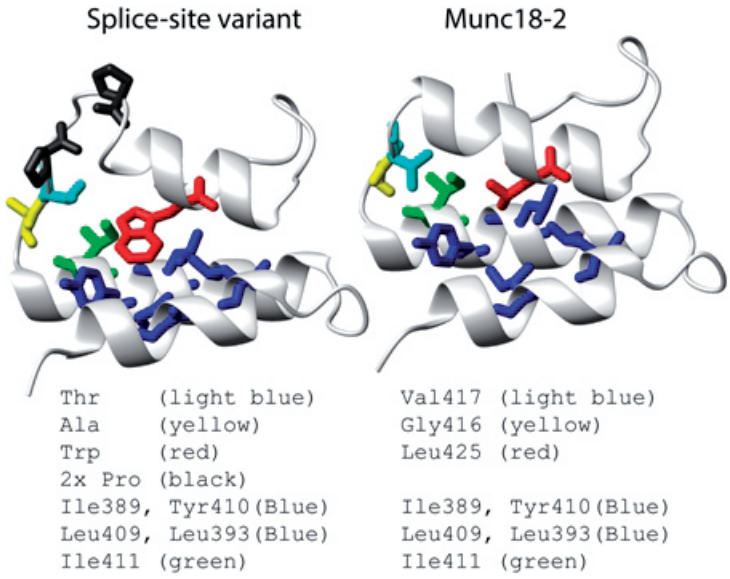

Figure 7. Model of part of the 3b domain of Munc18-2 and the splice-variant. Comparative homology modeling with modeller9V7[36] was utilized to obtain part of the 3b domain of Munc18-2 and the splice-site variant. The crystal structure of Munc18-1 was used as a template (3PUJ.pdb).[32] In the WT model (shown on the right) Val417 displays hydrophobic contact with Ile411. Replacement of Gly416 leads to secretion defects in the homologous S. cerevisiae Sec1p.[35] In the splice-site variant (shown on the left) these residues are replaced by Thr and Ala. The prolines in the spacer region of the splice-site variant are displayed. The WT model further shows hydrophobic interactions between Leu425 and Ile389, Tyr410, Leu409, and Leu393 on the opposite helices. In the splice-site variant Leu425 has been replaced by a tryptophan, which may preserve the hydrophobic interaction.

On first thought, this observation suggests that the almost complete absence of Munc18-2 does affect platelet degranulation. However, EM image analysis revealed a complete absence of granules in the stimulated platelets of patient P3$^{splice/G541S}$ (Fig. 5B). EM analysis also showed that the total granule count per platelet was reduced for patient P3$^{splice/G541S}$ (Fig. 6). This reduction may explain the reduced surface expression of CD62P upon stimulation of the platelets. Apparently, care should be taken when using only CD62P surface expression as readout for degranulation efficiency. A reduced expression can be also attributed to a decreased content of CD62P in resting platelets. Although it is highly speculative, our observations may even imply that there is a role for Munc18-2 in the biogenesis of α-granules.

Our findings may seem in apparent contrast with the studies by Al Hawas *et al*. and Sandrock *et al*. who showed that Munc18-2 is critical for platelet degranulation.[25,26] However, we cannot exclude the possibility that the Munc18-2 splice-site variant is still functional and that only few copies of the protein are sufficient to drive platelet degranulation. Yet, the presence of the other Munc18 family members, which may assist in the stabilization of STX11, may also explain why the platelets of the investigated FHL-5 patients are still able to degranulate (Figs. 1,3,5). Possibly, Munc18-1, Munc18-3 and/or Munc18-5 may contribute to platelet degranulation as well. This possibility is supported by the observation that intracellular peptide antagonists of Munc18-1 and Munc18-3 impair platelet degranulation.[24] The relative importance of the involved Munc18 proteins for the degranulation remains therefore a topic for further investigation.

Based on our results, we propose that defects in degranulation in the platelets of patients carrying the splice-site and G541S variants have a limited contribution, if any at all, to clinical symptoms that are associated with FHL-5.

ACKNOWLEDGEMENTS

This work was supported by Sanquin Blood Supply, grant number PPOC-08-004 (I.M.D.C.) and PPOC-10-012 (A.B.M). This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl), project INCOAG (grant 01C-201), and supported by the Dutch Heart Foundation. The Netherlands Organization for Scientific Research (863.09.012) (L.G.).

AUTHORSHIP AND CONFLICT OF INTEREST STATEMENTS

C.Z. and I.M.D.C. performed experiments, analyzed the data and wrote the paper; H.J. performed experiments; J.vdW.-T.B. and I.K. provided material and participated in discussions; T.vdB., T.W.K. and K.M. participated in discussions; L.G. and A.B.M. designed and performed experiments, analyzed the data and wrote the paper. We disclose that there is no conflict of interest.

REFERENCES

1 Hata Y, Slaughter CA, Sudhof TC. Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. *Nature*. 1993;366(6453):347-351.

2 Sudhof TC, Rothman JE. Membrane fusion: grappling with SNARE and SM proteins. *Science*. 2009;323(5913):474-477.

3 Rizo J, Sudhof TC. The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplices--guilty as charged? *AnnuRevCell DevBiol*. 2012;28:279-308.

4 Yu H, Rathore SS, Lopez JA, et al. Comparative studies of Munc18c and Munc18-1 reveal conserved and divergent mechanisms of Sec1/Munc18 proteins. *Proc Natl Acad Sci USA*. 2013;110(35):E3271-80

5 Hata Y, Sudhof TC. A novel ubiquitous form of Munc-18 interacts with multiple syntaxins. Use of the yeast two-hybrid system to study interactions between proteins involved in membrane traffic. *J Biol Chem*. 1995;270(22):13022-13028.

6 Katagiri H, Terasaki J, Murata T, et al. A novel isoform of syntaxin-binding protein homologous to yeast Sec1 expressed ubiquitously in mammalian cells. *J Biol Chem*. 1995;270(10):4963-4966.

7 Tellam JT, McIntosh S, James DE. Molecular identification of two novel Munc-18 isoforms expressed in non-neuronal tissues. *J Biol Chem*. 1995;270(11):5857-5863.

8 Riento K, Galli T, Jansson S, Ehnholm C, Lehtonen E, Olkkonen VM. Interaction of Munc-18-2 with syntaxin 3 controls the association of apical SNAREs in epithelial cells. *J Cell Sci*. 1998;111 (Pt 17):2681-2688.

9 Riento K, Kauppi M, Keranen S, Olkkonen VM. Munc18-2, a functional partner of syntaxin 3, controls apical membrane trafficking in epithelial cells. *J Biol Chem*. 2000;275(18):13476-13483.

10 Martin-Verdeaux S, Pombo I, Iannascoli B, et al. Evidence of a role for Munc18-2 and microtubules in mast cell granule exocytosis. *J Cell Sci*. 2003;116(Pt 2):325-334.

11 Cetica V, Santoro A, Gilmour KC, et al. STXBP2 mutations in children with familial haemophagocytic lymphohistiocytosis type 5. *J Med Genet*. 2010;47(9):595-600.

12 Cote M, Menager MM, Burgess A, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J Clin Invest*. 2009;119(12):3765-3773.

13 Meeths M, Entesarian M, Al-Herz W, et al. Spectrum of clinical presentations in familial hemophagocytic lymphohistiocytosis type 5 patients with mutations in STXBP2. *Blood*. 2010;116(15):2635-2643.

14 Nagai K, Yamamoto K, Fujiwara H, et al. Subtypes of familial hemophagocytic lymphohistiocytosis in Japan based on genetic and functional analyses of cytotoxic T lymphocytes. *PLoS One*. 2010;5(11):e14173.

15 Pagel J, Beutel K, Lehmberg K, et al. Distinct mutations in STXBP2 are associated with variable clinical presentations in patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL5). *Blood*. 2012;119(25):6016-6024.

16 Zhang K, Jordan MB, Marsh RA, et al. Hypomorphic mutations in PRF1, MUNC13-4, and STXBP2 are associated with adult-onset familial HLH. *Blood*. 2011;118(22):5794-5798.

17 zur Stadt U, Rohr J, Seifert W, et al. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. *Am J Hum Genet*. 2009;85(4):482-492.

18 Janka GE. Familial and acquired hemophagocytic lymphohistiocytosis. *Annu Rev Med*. 2012;63:233-246.

19 Machaczka M, Klimkowska M, Chiang SC, et al. Development of classical Hodgkin's lymphoma in an adult with biallelic STXBP2 mutations. *Haematologica*. 2013;98(5):760-764.

20 Rohr J, Beutel K, Maul-Pavicic A, et al. Atypical familial hemophagocytic lymphohistiocytosis due to mutations in UNC13D and STXBP2 overlaps with primary immunodeficiency diseases. *Haematologica*. 2010;95(12):2080-2087.

21 Sieni E, Cetica V, Mastrodicasa E, et al. Familial hemophagocytic lymphohistiocytosis: a model for understanding the human machinery of cellular cytotoxicity. *Cell Mol Life Sci*. 2012;69(1):29-40.

22 Zhao XW, Gazendam RP, Drewniak A, et al. Defects in neutrophil granule mobilization and bactericidal activity in familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) syndrome caused by STXBP2/Munc18-2 mutations. *Blood*. 2013;122(1):109-111.

23 Houng A, Polgar J, Reed GL. Munc18-syntaxin complexes and exocytosis in human platelets. *J Biol Chem*. 2003;278(22):19627-19633.

24 Schraw TD, Lemons PP, Dean WL, Whiteheart SW. A role for Sec1/Munc18 proteins in platelet exocytosis. *Biochem J*. 2003;374(Pt 1):207-217.

25 Al Hawas R, Ren Q, Ye S, Karim ZA, Filipovich AH, Whiteheart SW. Munc18b/STXBP2 is required for platelet secretion. *Blood*. 2012;120(12):2493-2500.

26 Sandrock K, Nakamura L, Vraetz T, Beutel K, Ehl S, Zieger B. Platelet secretion defect in patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL-5). *Blood*. 2010;116(26):6148-6150.

27 De Cuyper IM, Meinders M, van de Vijver E, et al. A novel flow cytometry-based platelet aggregation assay. *Blood*. 2013;121(10):e70-e80.

28 Piersma SR, Broxterman HJ, Kapci M, et al. Proteomics of the TRAP-induced platelet releasate. *J Proteomics*. 2009;72(1):91-109.

29 van Breevoord, van Agtmaal EL, Dragt BS, et al. Proteomic screen identifies IGFBP7 as a novel component of endothelial cell-specific Weibel-Palade bodies. *J Proteome Res*. 2012;11(5):2925-2936.

30 Calafat J, Janssen H, Stahle-Backdahl M, Zuurbier AE, Knol EF, Egesten A. Human monocytes and neutrophils store transforming growth factor-alpha in a subpopulation of cytoplasmic granules. *Blood*. 1997;90(3):1255-1266.

31 Misura KM, Scheller RH, Weis WI. Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature*. 2000;404(6776):355-362.

32 Hu SH, Christie MP, Saez NJ, et al. Possible roles for Munc18-1 domain 3a and Syntaxin1 N-peptide and C-terminal anchor in SNARE complex formation. *Proc Natl Acad Sci USA*. 2011;108(3):1040-1045.

33 Ye S, Karim ZA, Al HR, Pessin JE, Filipovich AH, Whiteheart SW. Syntaxin-11, but not syntaxin-2 or syntaxin-4, is required for platelet secretion. *Blood*. 2012;120(12):2484-2492.

34 Burkhardt P, Hattendorf DA, Weis WI, Fasshauer D. Munc18a controls SNARE assembly through its interaction with the syntaxin N-peptide. *EMBO J*. 2008;27(7):923-933.

35 Brummer MH, Kivinen KJ, Jantti J, Toikkanen J, Soderlund H, Keranen S. Characterization of the sec1-1 and sec1-11 mutations. *Yeast*. 2001;18(16):1525-1536.

36 Eswar N, Webb B, Marti-Renom MA, et al. Comparative protein structure modeling using MODELLER. *Curr Protoc Protein Sci*. 2007 Chapter 2: Unit 2.9.

CHAPTER 5

Galectin-8: a potential role as bridging molecule between Factor V and Glycoprotein V

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Manuscript in preparation

ABSTRACT

Galectin-8 (Gal8) is a tandem-repeat β-galactoside-binding lectin comprising two carbohydrate recognition domains (CRD) that can cross-link glycoproteins in the intracellular and extracellular space. Recently, we have obtained evidence that Gal8 mediates endocytosis of Factor V (FV) by megakaryocytes ultimately leading to storage of plasma-derived FV in the α-granules of platelets. Whereas one CRD of Gal8 binds FV, the identity of the counter-receptor on the platelet surface remains unclear. It has been proposed that Gal8 can interact with glycoprotein αIIbβ3, the major platelet intergrin, which mediates the endocytic uptake of fibrinogen by megakaryocytes. In the present study, mass spectrometry analysis of platelets from Glanzmann Thrombasthenia patients, who lack expression of αIIbβ3on their platelets, revealed normal levels of FV and markedly reduced levels of fibrinogen. This suggests that αIIbβ3 does not contribute to FV uptake and storage by megakaryocytes. However, proteomic analysis of platelets from LAD-IIII patients, which exhibit an impaired integrin activation machinery, indicated a correlation between the levels of FV and glycoprotein V (GPV), which is a component of the GPIb-IX-V complex. Intriguingly, GPV has been previously identified as a putative binding partner for Gal8. Employing surface plasmon resonance analysis and solid phase binding studies, we established that Gal8 can acts as a cross-linker between recombinant GPV and FV. This indirect interaction between GPV and FV is specifically mediated by Gal8 in a concentration and carbohydrate-dependent manner. In view of our previous findings on the role of Gal8 for mediating FV internalization in megakaryocytes, the results of the present study suggest a role for GPV as Gal8 counter-receptor on the cell surface to mediate the uptake of FV.

INTRODUCTION

Galectins are a family of lectins involved in several physiological and pathological processes including differentiation, apoptosis, and adhesion[1–3]. Galectins specifically recognize galactose-containing glycans (Galβ1-4GlcNAc) *via* their carbohydrate-recognition domains (CRD)[4]. Although the CRD domains of galectins show a high primary sequence homology, each individual member of the galectin family recognizes unique glycan structures[5–7]. Galectin-8 (Gal8) comprises two CRDs connected by a linker peptide[8–10] and, like other galectins, has been identified in the cytosol, the nucleus or in the extracellular space[9]. It has been suggested that the two CRDs in Gal8 exhibit a different glycan-binding specificity. Gal8 has therefore been proposed to facilitate cross-linking of specific glycoproteins[4,9,11].

We have previously provided evidence that cell-surface bound Gal8 mediates Factor V (FV) endocytosis by the platelet precursor cells megakaryocytes[12]. This process ultimately leads to the storage of FV in platelet α-granules. Whereas one of the CDR domains of Gal8 binds FV, the identity of the cell surface receptor that interacts with the second CRD domain remains to be established. It has been proposed that LRP-1 contributes to the endocytosis of FV as well[13,14]. However, whereas LRP-1-mediated endocytosis usually takes place on the minute time scale, we and Bouchard *et al*. found that the endocytosis of FV requires one or more hours of incubation of FV with the megakaryocytes[12-14]. This observation suggests that the mechanism underlying FV endocytosis is still unclear and possibly requires additional unknown key players[12,13].

It has previously been demonstrated that galectins can interact with platelet integrins[15,16]. Gal1 binding to αIIbβ3 on platelets has been shown to trigger outside-in signaling leading to platelet aggregation[17]. Employing platelets from Bernard-Soulier syndrome (BSS) patients, with low or absent expression of the glycoprotein (GP)Ib-IX-V complex, it has further been proposed that Gal8 can activate platelets *via* this complex[18]. The GPIb-IX-V complex is a specific marker of platelets and is essential for platelet adhesion and aggregation by mediating the binding to VWF[19]. Pull-down experiments revealed, however, that Gal8 may not only interact with GPIb and GPV but also with αIIbβ3[18]. Intriguingly, the latter platelet receptor has been shown to be responsible of the uptake and storage of plasma-derived fibrinogen in the α-granules of platelets[20]. The observation that Gal8 interacts with FV and with αIIbβ3 raises the question whether the integrin may contribute to the uptake of FV as well.

The role of αIIbβ3 for endocytosis of fibrinogen by megakaryocytes has been shown employing studies on the platelets of Glanzmann Thrombastenia (GT) Type I patients[21]. These patients completely lack αIIbβ3 in their platelets and show absence of plasma-derived fibrinogen in their α-granules. By means of specific antagonists, other studies also concluded that αIIbβ3 is the receptor responsible for fibrinogen endocytosis[22].

In the present study, we assessed whether there was a correlation between the levels of FV and αIIbβ3 in the platelets of GT patients and of healthy individuals. We further analyzed the platelets of LAD-III patients, which exhibit an activation defect of β integrins due to Kindlin-3 protein deficiency[23]. We have previously shown that LAD-III platelets display a specific reduction in the levels of FV[24]. Mass spectrometry analysis and direct binding studies surprising revealed that GPV and not αIIbβ3 may be the counter-receptor for Gal8 that contributes to the internalization and storage of FV in the α-granules of platelets.

MATERIALS AND METHODS

Materials, antibodies and proteins – Recombinant human full-length GPV, recombinant human Gal8 were purchased from R&D (Abingdon, United Kingdom). D-Galactopyranosyl-D-thio-galactopyranoside (TDG) was from Carbosynth (Compton, Berkshire, UK). Polyclonal FV-HRP sheep anti-human FV was from Kordia (Kordia Life Sciences, Leiden, the Netherlands). Unless specified, all other chemicals were from Sigma-Aldrich (Sigma Aldrich, Zwijndrecht, the Netherlands). Human plasma-derived FV was obtained essentially as described[12,25].

Surface Plasmon Resonance analysis – SPR analysis was performed employing a BIAcore™ 3000 biosensor system (Biacore AB, Uppsala, Sweden) as previously described[12]. Briefly, recombinant GPV (1000 RU) or plasma FV (1000 RU) was covalently coupled to the activated surface of a CM5-sensor chip (GE Healthcare Bio Sciences, Uppsala, Sweden) *via* primary amino-groups using an amine coupling kit (Biacore) as prescribed by the supplier. One control flow channel was routinely activated and blocked in the absence of protein. Increasing concentrations of Gal8 (0.78-100 nM) were subsequently perfused over chip-immobilized GPV. Association of ligand was assessed in 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$ and 0.005% Tween 20 for 120 sec at a flow rate of 20 µl/min. Dissociation was initiated upon replacement of the ligand solution with the buffer alone 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 chip-immobilized GPV (1000 RU) in the presence of increasing concentrations of lactose or sucrose.

In vitro binding of GPV to Gal8 and FV: solid-phase binding analysis – Binding of soluble recombinant Gal8, Gal1 and plasma FV to GPV was monitored as follows. Recombinant GPV (5 µg/ml) was adsorbed onto microtiter wells MaxiSorp plate (Nunc) in a volume of 50 µl in 50 mM $NaHCO_3$ (pH 9.5) overnight at 4°C. Wells were washed with 150 mM NaCl, 5 mM $CaCl_2$, 0,1% Tween-20, 50 mM Tris (pH 7.4) and subsequently blocked for 1 h in TSM buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$) containing 2% BSA (200 µl/well). FV (5 µg/ml) was pre-incubated with increasing concentrations of Gal8 or Gal1 for 30 min at 37 °C or buffer alone and then added to GPV-coated plates. After 1h incubation, unbound fragments were washed away and FV binding to GPV was determined by incubation with the HRP-labeled anti–human FV antibody. Binding specificity was verified by pre-incubating the galectin-FV complex with increasing concentrations of lactose or sucrose before the addition to GPV-coated plates.

Platelet isolation from healthy donors and patients – The study was conducted on platelets of four healthy donors, two GT Type I, one GT Type III, and three LAD-III patients. The study was approved by the Amsterdam Medical Center Institutional Medical Ethics Committee in accordance with the Declaration of Helsinki. Venous blood was collected from healthy donors and from GT and LAD-III patients after obtaining informed consent. The blood was anticoagulated with heparin. Washed platelets were obtained as previously described[26]. The collected platelets contained less than 1 leukocyte/10^6 platelets and less than 1 erythrocyte/10^4 platelets.

Mass spectrometry analysis – Platelets were lysed as previously described[24]. Equal amounts of platelet lysates were employed for SDS-PAGE (4-12% acrylamide) gel electrophoresis to

separate the proteins. The proteins in the gel bands were processed for mass spectrometry analysis and the data was analyzed as described previously[24].

RESULTS

Putative receptor involved in FV endocytosis: ex-vivo studies

GT-derived platelets were analyzed by mass spectrometry for the presence of FV and fibrinogen. GT patients included two GT Type I and one GT Type III patients, the latter displaying normal or near-normal levels of not functional αIIbβ3[24]. Figure 1 shows the normalized spectral counts for αIIb, β3, fibrinogen chains, and FV in these patients. The results reveal that the functionally defective αIIbβ3 of the platelets of the GT Type III patient does not lead to a reduction in the spectral count of neither fibrinogen nor FV in the platelets. Moreover, the reduction of spectral counts of FV is also not observed in the almost complete absence of αIIbβ3 in the platelets of the GT Type I patients. Yet, the latter platelets do reveal an almost complete absence of fibrinogen. Compatible with previous findings, these results show on one hand that αIIbβ3 contributes to fibrinogen internalization and on the other hand that this integrin is not involved in FV endocytosis at all.

As the platelets of the LAD-III patients did reveal a reduction in the level of FV[24], we reanalyzed these platelets to assess whether there is any correlation between the expression levels of a specific platelet glycoprotein and FV. The LAD-III platelets showed markedly reduced spectral counts of platelet GPV of the VWF receptor complex (Fig. 2). On the other hand, the spectral counts of GPIb α and β chains were not reduced in these platelets. The slight lower mean value of GPIX counts in LAD-III patients might be not reliable because of the fluctuating content of this subunit either in controls and LAD-III patients. LAD-III platelets displayed also reduced spectral counts of β1 integrin subunit and its partners (α2 and α6) and less markedly of β3 and αIIb (Fig. 2). These observations together open the possibility that GPV and/or β1-comprising integrins may participate in the endocytosis of FV.

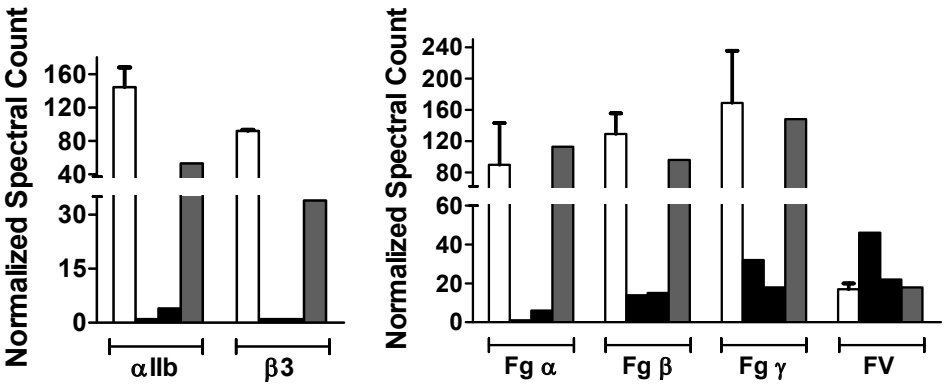

Figure 1. Relative abundance of αIIbβ3 and Factor V in GT patients. Mass spectrometry analysis of lysates of washed platelets of healthy controls (white bars), GT-I patients (black bars) and the GT-III patient (grey bars). Normalized spectral counts of: (left panel) glycoprotein αIIb and β3; (right panel) fibrinogen (Fg) chains (α, β and γ) and Factor V. Data of healthy controls are the mean value ± SD (n = 4). Normalized spectral counts were obtained as described in Materials and methods.

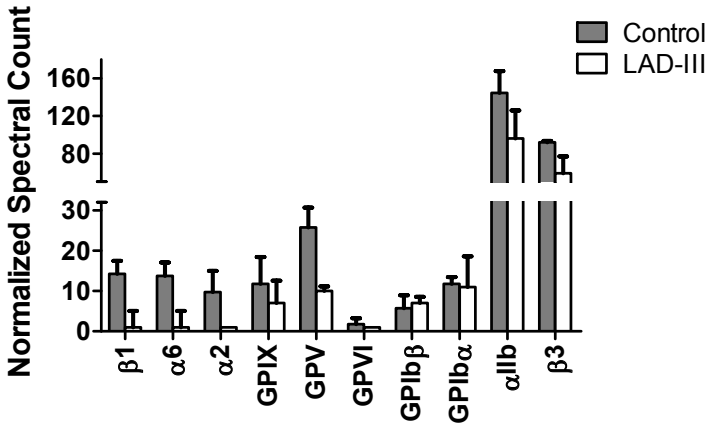

Figure 2. Glycoproteins relative abundance in LAD-III patients. Mass spectrometry analysis of lysates of washed platelets of controls, LAD-III patients (LAD-III). Values from the normalized spectral counts of platelet glycoproteins are shown. Data are the mean value ± SD from 4 independent healthy controls and three LAD-III patients.

Glycoprotein V binding to Galectin-8 and by SPR

As GPV has been identified in a pull-down experiment with Gal8[18], we assessed by SPR analysis the putative binding of Gal8 to GPV. To this end, increasing concentrations of Gal8 were passed over immobilized GPV and response units were measured. Figure 3 shows that Gal8 interacts with GPV in a dose-dependent manner. The binding response at equilibrium for each Gal8 concentration was estimated 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. The apparent Kd for the GPV-Gal8 complex, which is reflected by the concentration of Gal8 at which half-maximum binding is reached, was about 42 nM (inset Fig. 3). We next addressed whether Gal8 binding to GPV was mediated by one of the β-galactoside-binding CRD domains of Gal8. To this end, a fixed concentration of Gal8 was incubated with immobilized GPV in presence of increasing concentrations of the β-galactoside carbohydrate lactose (Fig. 4). As a control, we included sucrose in the experiment. The results showed that Gal8-GPV binding was specifically inhibited in a concentration-dependent manner by lactose. These data indicate that there is a specific interaction between the carbohydrate residues of GPV and the CRD domains of Gal8.

Galectin-8 mediates FV binding to GPV

Based on the observation that Gal8 binds both GPV and FV[12], we assessed whether the presence of Gal8 affects the binding of FV to plate-immobilized GPV in the presence of Gal8. As shown in Figure 5, Gal8 promoted dose-dependently the indirect FV binding to GPV. Conversely, no FV-GPV direct interaction was detectable in the absence of Gal8. SPR analysis confirmed the absence of a direct interaction between FV and GPV (data not shown). Competition experiments with β-galactoside sugars were performed to evaluate the requirement of Gal8 CRDs for FV-GPV interaction (Fig. 5B). Increasing concentrations of lactose and TDG, but not of sucrose, were effective in reducing

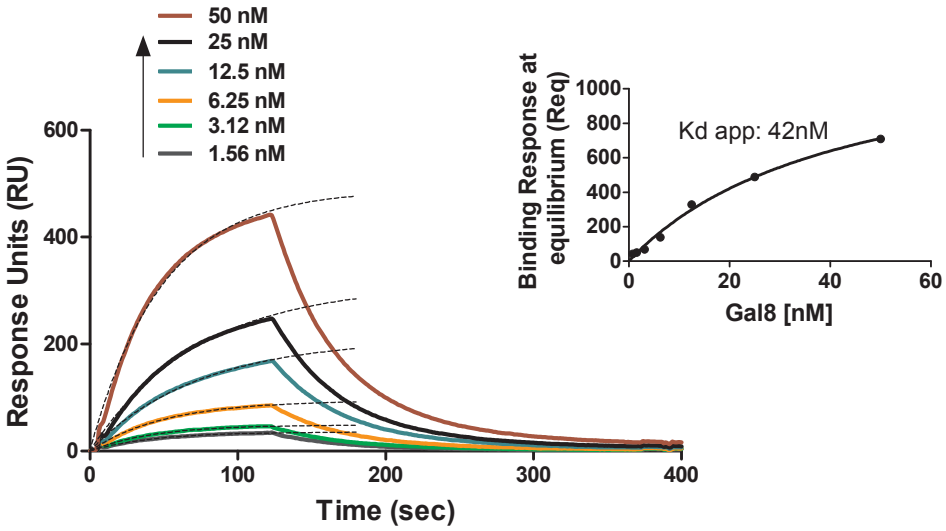

Figure 3. Gal8 binds GPV in a concentration-dependent manner. 1000 Response units (RU) of recombinant GPV were immobilized on the surface of a CM5 sensor chip. The indicated concentrations of Gal8 were subsequently perfused over the immobilized GPV. Association and dissociation were followed as described in the Material and Methods section. Inset shows the apparent equilibrium dissociation constant (Kd-app), which was estimated by plotting the maximum response at equilibrium (Req) for a range of ligand concentrations as a function of those concentrations.

FV binding to GPV. Taken together these observations suggest that Gal8 acts as a cross-linker between FV and GPV and that the interaction is actually mediated *via* Gal8 CRD domains.

DISCUSSION

It has been proposed that the protein composition of the α-granule membrane mirrors that of the platelet membrane[27]. This may be the consequence of active recycling of the plasma membrane receptors between the α-granules and the platelet surface. Via this mechanism, it has been suggested that αIIbβ3 mediated the loading of fibrinogen into the α-granules of platelets and megakaryocytes[20,21]. As Gal8 binds both FV and αIIbβ3[12,18], it would seem feasible that αIIbβ3 contributes to the uptake of FV as well. However, whereas complete absence of αIIbβ3 in the platelets of the GT patients did affect the level of fibrinogen, the platelet content of FV was not altered (Fig. 1). This result excludes therefore a role of αIIbβ3 as a counter receptor for the Gal8 during the uptake of FV.

The results of our study do show that a complete integrin activation defect in LAD-III platelets does affect the level of FV. Proteomic analysis of these platelets also revealed a reduced level of GPV and the α2β1 and α6β1 integrins (Fig. 2). A defect in the expression of the β subunit has previously been shown to results in the rapid degradation of the α subunit[28]. A selective impaired expression or enhanced degradation of the β1 subunit in the LAD-III platelets may therefore explain the reduced levels of the α subunit partners. Conversely, the observed decrease of GPV

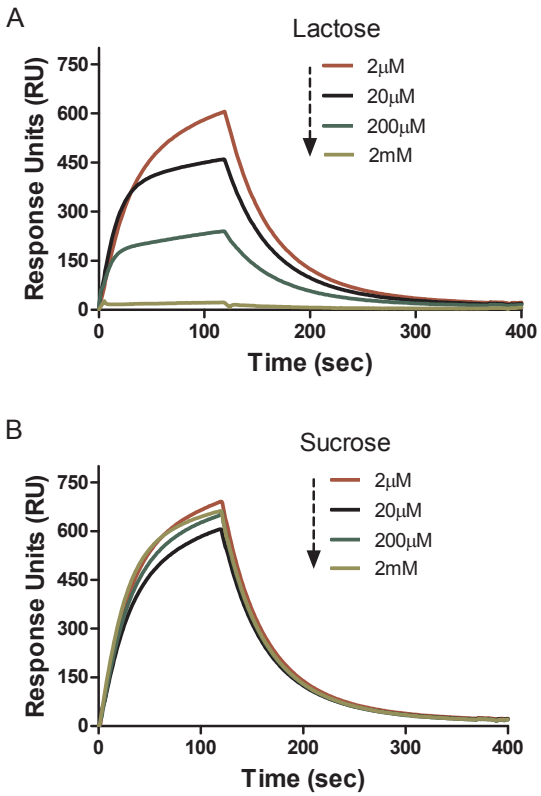

Figure 4. Effect of lactose and sucrose on Gal8-GPV binding. A fixed amount of Gal8 (50 nM) was associated to immobilized GPV (1000 RU) in the presence or absence of the indicated doses of (A) lactose or (B) sucrose. Association and dissociation were followed as described in Material and Methods section. After 120 sec of association, the buffer comprising the carbohydrates and Gal8 was replaced with the buffer alone.

content was not accommodated with a reduction of other components of the VWF receptor complex, i.e. GPIX and GPIb (Fig. 2). On first thought, it may therefore seem remarkable that GPV expression was reduced independently of the other members of the GP Ib-IX-V complex. However, it has been suggested that GPV does not form a tight complex with the GPIb-GPIX complex[29,30]. Recently, it has also been proposed that a fraction of GPV is present as a monomer on the platelet surface[31]. It has further been shown that GPV deletion in mice has little impact on the surface expression of GPIb-IX complex[32]. These observations imply that the expression of GPV at the platelet surface may be independent of the presence of GPIb-GPIX. If so, an impaired expression of GPV does not necessary lead to an altered level of GPIb-GPIX as well.

The reason behind the decrease of the β1 integrins and GPV remains to be elucidated. Compatible with our finding, also the platelets from FERMT3-deficient mice were found to display reduced levels of GPV and β1-comprising integrins[33]. Intriguingly, the red blood cells of these mice also revealed a structural defect of the membrane cytoskeleton[33]. In platelets, the interaction of integrins and GPV with the cytoskeleton is well documented[34,35]. Possibly, the

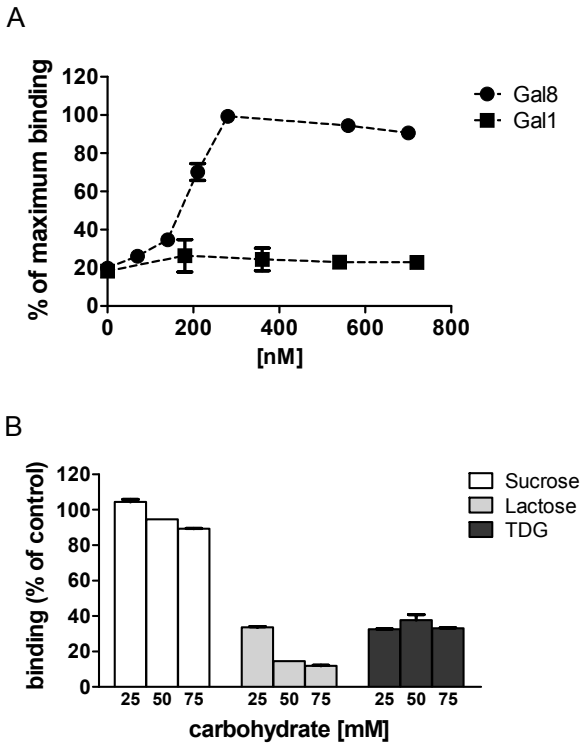

Figure 5. Gal8 mediates indirect GPV-FV interaction. (A) Plasma FV (5 µg/ml) was pre-incubated in the presence of the indicated concentrations of Gal8 or Gal1 for 30 min at 37 °C or buffer alone. After incubations the complexes were added to GPV–coated plates. The amount of FV bound to plates was revealed using the HRP-labeled anti–FV antibody. Interaction is expressed as % of maximum binding. (B) Gal8 (280 nM) and FV (5 µg/ml) were mixed in the absence and in the presence of sucrose, lactose or TDG and then added to GPV-coated plates. FV binding was revealed as in (A). Values are expressed as % of binding in the absence of carbohydrates (control).

absence of FERMT3 in LAD-III patients might affect the platelet cytoskeleton as well, thereby indirectly contributing to the altered expression of GPV and the α2β1 and α6β1 integrins. If so, and why other integrins in the LADIII platelets are not also affected, remains to be established.

The notion that integrins can actively recycle between the cell surface and intracellular organelles[27] opens the possibility that one of the β1 integrins contribute to the endocytosis of the Gal8-FV complex. The reduced expression of FV in the LAD-III platelets can then be explained by the reduced expression of the β1 comprising integrins. A selective knock down of the β1 subunit in the megakaryocytes may provide insight in the specific role of this integrin subunit for FV uptake. Yet, the pull-down study by Romaniuk *et al*.[18] has provided evidence that there may be an interaction between GPV and Gal8. Employing SPR analysis and immunoabsorbent assays, we now show that Gal8 and GPV interact in a carbohydrate-dependent manner (Fig. 4). In addition, we found that FV binds GPV only in the presence of Gal8 (Fig. 5). These findings suggest that Gal8 may act as a cross-linker between GPV and FV. Possibly, GPV may acts as a

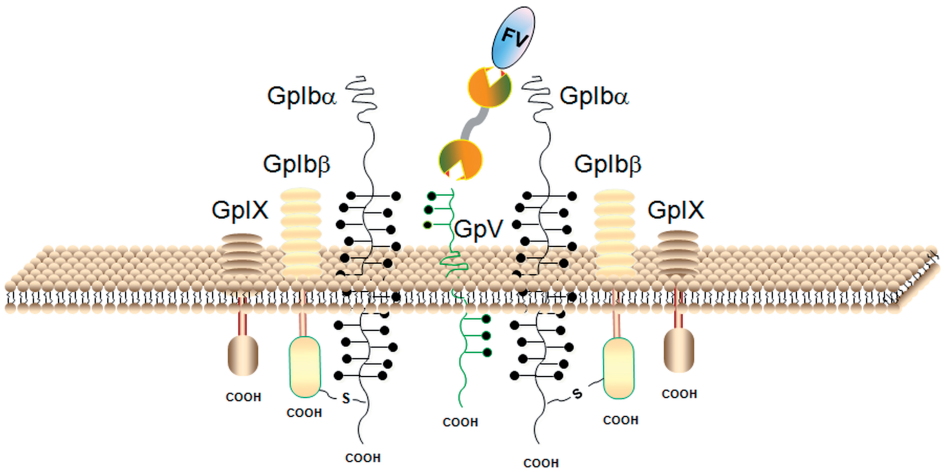

Figure 6. Proposed mechanism for Gal8 mediated GPV-FV binding. A model of the hypothesized interaction between FV, Gal8 and GPIb-IX-V complex, is shown. The multivalent Gal8 could bind GPV *via* one CRD domain and FV *via* the other CRD. The model proposes that GPV is a Gal8 counter-receptor on the megakaryocytes and that the formed complex is internalized allowing FV accumulation in α-granules.

transport receptor for the Gal8-FV complex. This may then also explain the observation of our study that reduction of GPV in LAD-III platelets is accommodated by the decrease of FV.

A model that could explain the uptake mechanisms of FV is show in figure 6. The tandem-repeat Gal8 binds GPV via one of its CRD domain to one or more of the 8 potential glycosylation sites present in GPV extracellular domain[36]. The second free CRD domain acts as a bait for plasma FV. GPV might then drive the internalization of the complex allowing FV accumulation in the α-granules. In the final step, free GPV may be recycled back on the cell surface. Alternatively, the GPV-Gal8 complex might function as docking site promoting pre-concentration of FV on the cell surface and binding to the endocytic receptor. It has been suggested that LRP-1 acts as endocytic receptor for FV in the two-receptor system[13]. The authors concluded that only upon binding to the first, yet unknown receptor, FV could be transferred to LRP-1[13]. In this view, a role for the GPV-Gal8 complex as first player in FV internalization is conceivable and might be object of future studies. In contrast to megakaryocytes, circulating platelets do not internalize plasma FV[37]. Their surface is loaded with copies of the GPIb-IX-V complex but lacks extracellular Gal8[12,18]. Only upon thrombin stimulation intracellular Gal8 is secreted and retained on the platelet surface[18]. In the absence of Gal8 secretion by platelets, the GPV-Gal8 complex cannot be assembled. This might explain why resting platelet fail to internalize FV.

In conclusion our study provides a new perspective about the role of GPV in platelets as key player in plasma FV internalization by megakaryocytes. Future studies might take advantage of BSS patients which often display reduced expression of GPV[38,39]. Mass spectrometry analysis of these platelets could reveal whether there is a specific decrease of FV in the α-granules of their platelets.

REFERENCES

1 Hughes RC. Galectins as modulators of cell adhesion. *Biochimie*. 2001;83(7):667–676.

2 Leffler H, Carlsson S, Hedlund M, Qian Y, Poirier F. Introduction to galectins. *Glycoconj. J.* 2004;19(7-9):433–440.

3 Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich GA. Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell. Mol. Life Sci.* 2007;64(13):1679–1700.

4 Rabinovich GA, Toscano MA, Jackson SS, Vasta GR. Functions of cell surface galectin-glycoprotein lattices. *Curr. Opin. Struct. Biol.* 2007;17(5):513–520.

5 Leppänen A, Stowell S, Blixt O, Cummings RD. Dimeric Galectin-1 Binds with High Affinity to α2,3-Sialylated and Non-sialylated Terminal N-Acetyllactosamine Units on Surface-bound Extended Glycans. *J. Biol. Chem.* 2005;280(7):5549–5562.

6 Fry SA, Van den Steen PE, Royle L, et al. Cancer-associated glycoforms of gelatinase B exhibit a decreased level of binding to galectin-3. *Biochemistry*. 2006;45(51):15249–15258.

7 Cederfur C, Salomonsson E, Nilsson J, et al. Different affinity of galectins for human serum glycoproteins: galectin-3 binds many protease inhibitors and acute phase proteins. *Glycobiology*. 2008;18(5):384–394.

8 Ideo H, Seko A, Ishizuka I, Yamashita K. The N-terminal carbohydrate recognition domain of galectin-8 recognizes specific glycosphingolipids with high affinity. *Glycobiology*. 2003;13(10):713–723.

9 Carlsson S, Oberg CT, Carlsson MC, et al. Affinity of galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. *Glycobiology*. 2007;17(6):663–676.

10 Ideo H, Matsuzaka T, Nonaka T, Seko A, Yamashita K. Galectin-8-N-domain recognition mechanism for sialylated and sulfated glycans. *J. Biol. Chem.* 2011;286(13):11346–11355.

11 Thijssen VL, Hulsmans S, Griffioen AW. The Galectin Profile of the Endothelium. *Am. J. Pathol.* 2008;172(2):545–553.

12 Zappelli C, van der Zwaan C, Thijssen-Timmer DC, Mertens K, Meijer AB. Novel role for galectin-8 protein as mediator of coagulation factor V endocytosis by megakaryocytes. *J. Biol. Chem.* 2012;287(11):8327–8335.

13 Bouchard BA, Meisler NT, Nesheim ME, et al. A unique function for LRP-1: a component of a two-receptor system mediating specific endocytosis of plasma-derived factor V by megakaryocytes. *J. Thromb. Haemost*. 2008;6(4):638–644.

14 Bouchard BA, Abdalla S, Tracy PB. The Factor V Light Chain Mediates the Binding and Endocytosis of Plasma-derived Factor V by Megakaryocytes. *J. Thromb. Haemost*. 2013;11(12):2181-2183.

15 Hadari YR, Arbel-Goren R, Levy Y, et al. Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis. *J. Cell Sci.* 2000;113 (Pt 13):2385–2397.

16 Levy Y, Arbel-Goren R, Hadari YR, et al. Galectin-8 Functions as a Matricellular Modulator of Cell Adhesion. *J. Biol. Chem*. 2001;276(33):31285–31295.

17 Romaniuk MA, Croci DO, Lapponi MJ, et al. Binding of galectin-1 to $\alpha IIb\beta_3$ integrin triggers "outside-in" signals, stimulates platelet activation, and controls primary hemostasis. *FASEB*. 2012;26(7):2788–2798.

18 Romaniuk MA, Tribulatti MV, Cattaneo V, et al. Human platelets express and are activated by galectin-8. *Biochem. J.* 2010;432(3):535–547.

19 Clemetson KJ, Clemetson JM. Platelet GPIb-V-IX complex. Structure, function, physiology, and pathology. *Semin. Thromb. Hemost*. 1995;21(2):130–136.

20 Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa). *Blood*. 1993;82(1):135–138.

21 Coller BS, Seligsohn U, West SM, Scudder LE, Norton KJ. Platelet fibrinogen and vitronectin in Glanzmann thrombasthenia: evidence consistent with specific roles for glycoprotein IIb/IIIA and alpha v beta 3 integrins in platelet protein trafficking. *Blood*. 1991;78(10):2603–2610.

22 Handagama P, Bainton DF, Jacques Y, et al. Kistrin, an integrin antagonist, blocks endocytosis of fibrinogen into guinea pig megakaryocyte and platelet alpha-granules. *J. Clin. Invest*. 1993;91(1):193–200.

23 Kuijpers TW, van de Vijver E, Weterman MAJ, et al. LAD-1/variant syndrome is caused by mutations in FERMT3. *Blood*. 2009;113(19):4740–4746.

24 Zappelli C, Zwaan van der C, De Cuyper I, et al. The effect on platelet protein expression of single $\alpha IIb\beta 3$ deficiency or a complete integrin activation defect in rare platelet disorders explored by mass spectrometry. *Chapter III of this thesis*.

25 Bos MHA, Meijerman DWE, van der Zwaan C, Mertens K. Does activated protein C-resistant factor V contribute to thrombin generation in hemophilic plasma? *J. Thromb. Haemost*. 2005;3(3):522–530.

26 De Cuyper IM, Meinders M, van de Vijver E, et al. A novel flow cytometry-based platelet aggregation assay. *Blood*. 2013;121(10):e70–80.

27 Berger G, Massé JM, Cramer EM. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood*. 1996;87(4):1385–1395.

28 Hynes RO. IntegrinsBidirectional, Allosteric Signaling Machines. *Cell*. 2002;110(6):673–687.

29 Modderman PW, Admiraal LG, Sonnenberg A, von dem Borne AE. Glycoproteins V and Ib-IX form a noncovalent complex in the platelet membrane. *J. Biol. Chem*. 1992;267(1):364–369.

30 Mo X, Liu L, López JA, Li R. Transmembrane domains are critical to the interaction between platelet glycoprotein V and glycoprotein Ib-IX complex. *J. Thromb. Haemost*. 2012;10(9):1875–1886.

31 Li R, Emsley J. The organizing principle of the platelet glycoprotein Ib-IX-V complex. *J. Thromb. Haemost*. 2013;11(4):605–614.

32 Nonne C, Hechler B, Cazenave J-P, Gachet C, Lanza F. Reassessment of in vivo thrombus formation in glycoprotein V deficient mice backcrossed on a C57Bl/6 strain. *J. Thromb. Haemost*. 2008;6(1):210–212.

33 Krüger M, Moser M, Ussar S, et al. SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell*. 2008;134(2):353–364.

34 Mistry N, Cranmer SL, Yuan Y, et al. Cytoskeletal regulation of the platelet glycoprotein Ib/V/IX-von willebrand factor interaction. *Blood*. 2000;96(10):3480–3489.

35 Lad Y, Harburger DS, Calderwood DA. Integrin cytoskeletal interactions. *Methods Enzymol*. 2007;426:69–84.

36 Strassel C, Moog S, Baas M-J, Cazenave J-P, Lanza F. Biosynthesis of platelet glycoprotein V expressed as a single subunit or in association with GPIb-IX. *Eur. J. Biochem*. 2004;271(18):3671–3677.

37 Camire RM, Pollak ES, Kaushansky K, Tracy PB. Secretable human platelet-derived factor V originates from the plasma pool. *Blood*. 1998;92(9):3035–3041.

38 López JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard-Soulier syndrome. *Blood*. 1998;91(12):4397–4418.

39 Lanza F. Bernard-Soulier syndrome (Hemorrhagiparous thrombocytic dystrophy). *Orphanet J. Rare Dis*. 2006;1(1):46.

CHAPTER 6

General Discussion

GENERAL DISCUSSION

Research over the last decade proved that platelet biology is not as simple and straightforward as originally anticipated. Next to their established role in wound repair, platelets show unsuspected key functions in a number of fundamental biological processes including inflammation, immunity and cancer[1,2]. To effectively perform their function, platelets store hundreds of critical proteins[3] in secretory α-granules that are released at sites of injury upon platelet activation. Megakaryocyte (MK) biosynthesis is one source for the proteins that reside in the α-granules of platelets. MKs and also platelets further have the unique capacity to take up plasma-derived proteins that end up in the α-granules. The present thesis focuses on the mechanism behind endocytosis of plasma proteins, and the role of platelet integrins and glycoproteins therein. We further address the mechanism of the release of α-granules. The findings and implications of this study are discussed below.

FACTOR V ENDOCYTOSIS BY MEGAKARYOCYTES

The contribution of galectin-8 in the endocytosis of factor V

The origin of platelet α-granule factor V (FV) has been debated for several years [4–8]. It has now been established that platelet FV is not synthesized by the megakaryocytes[6–8]. Instead, it is taken up from plasma via a poorly understood, receptor-mediated mechanism[8]. In Chapter II, we show that FV endocytosis by megakaryocyte-like cells occurs through recognition of its sugar residues, which account for approximately 13% to 25% of the mass of human FV[9–11]. Cellular uptake analysis and direct protein binding studies revealed that the interaction between FV and MKs requires the presence of the lectin Galectin-8 (Gal8) at the cell surface. Gal8 comprises two carbohydrate recognition domains (CRD) of which one binds FV and the second a yet to be identified cellular component that facilitates endocytosis. The observation that Gal8 interacts with FV is supported by data obtained by Romaniuk *et al*.[12], which identified FV as one of the binding partners of Gal8 in platelets.

To gain additional insight into the role of Gal8 for FV endocytosis, we assessed the location of Gal8 in $CD34^+$ mobilized peripheral blood cells that are differentiated towards MKs. Confocal microscopy revealed that Gal8 partially co-localizes with endogenously synthesized α-granule protein VWF (Fig. 1). Flow cytometry analysis further revealed that Gal8 is at the surface of the differentiating MKs (Fig. 2). This shows that Gal8 is present at the cell surface as well as in the α-granules. This opens the possibility that Gal8 may actively recycle between the MK cells surface and the α-granules. This is in agreement with the observation that members of galectin family can recycle between membrane compartments[13–15]. If so, the cell surface bound Gal8-FV complex may be internalized and fuse with the maturating α-granules in the MKs. Dissociation of the FV-Gal8 complex inside the maturating α-granules could then be driving force for FV storage in the platelet organelles. However, as mentioned above, the identity of the cell surface component that facilitates Gal8 recycling remains to be identified. It further seems remarkable that Gal8 would contribute to the uptake of FV only. Future studies should assess whether other α-granule proteins are taken up via Gal8 as well.

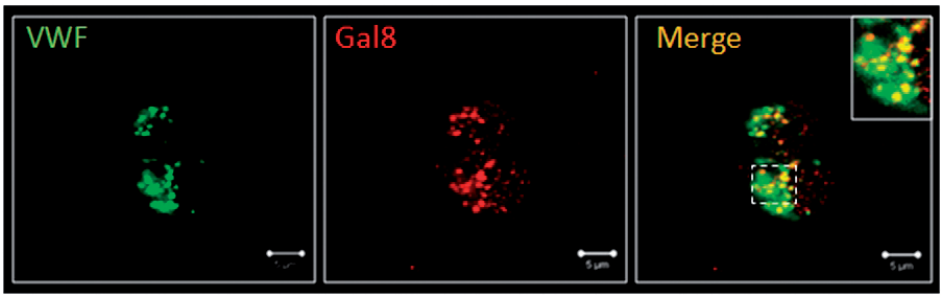

Figure 1. Galectin-8 is expressed in primary megakaryocytes and partially co-localizes with VWF. Shown is a confocal immunodetection performed as described in Chapter II. Mobilized peripheral blood CD34+ cells were maturated for 10 days in Cellgro medium supplemented with TPO (100 ng/ml) and IL-1β (10 ng/ml), and were stained for VWF (green) and Gal8 (red). Inset shows a higher magnification of the boxed region. The white scale bars represent 5 µm.

The contribution of LRP-1 for FV endocytosis

A function for low density lipoprotein-receptor related protein-1 (LRP-1) in FV endocytosis by megakaryocytes has been suggested by Bouchard *et al*.[16,17]. It has previously been shown that LRP-1 is transiently expressed by differentiating MKs[18]. The putative role of LRP-1 for the uptake of FV is therefore compatible with the observation that MKs[16,17] and not platelets[19] internalize and store FV in the α-granules. Bouchard *et al*. based their findings on the observation that an anti-LRP-1 antibody and an excess of the LRP-1 antagonist Receptor-Associated-Proteins (RAP) decrease FV endocytosis by the megakaryocyte-like CMK cells[16,17]. Only a fraction of the CMK cells was, however, capable to support FV endocytosis. Flow cytometry analysis of the cells revealed that CMK cells became positive for both RAP and FV upon incubation of the cells with these proteins. The authors proposed that the uptake mechanism for FV by megakaryocytes involves two steps. A specific, yet unknown, receptor assists in the binding of FV light chain to the cells surface after which FV is transferred to LRP-1 for endocytosis[16,17]. Bouchard *et al*. further provided evidence that the initial cell binding step of FV occurs in a Ca^{2+}-independent manner[8].

It has been previously demonstrated that LRP-1 also serves as endocytic receptor involved in the clearance of factor VIII (FVIII)[20–23], a FV homologous coagulation cofactor. Also for FVIII, a two-step mechanism behind the LRP-1 dependent endocytosis has been proposed[24–26]. FVIII endocytosis requires a pre-concentration step at the cell surface via an unknown receptor prior to the transfer to LRP for effective endocytosis[23,26]. However, unlike FV, FVIII has not been identified in platelets[27]. This implies that FV and FVIII may require distinct cell surface binding receptors to assist LRP-1 dependent uptake of the proteins.

The intrinsic ability of Gal8 to bind distinct glycans with its two independent carbohydrate-recognition-domains (CDR) in combination with the observation that Gal8 binds ligands in a Ca^{2+}-independent manner[28,29] makes Gal8 a suitable candidate to serve as the initial cell surface binding receptor for FV. However, the notion that Gal8 itself is also inside the α-granules (Fig. 1) argues against the proposed mechanism that FV would be transferred to LRP-1. Instead, LRP-1 may contribute to the uptake of the Gal8-FV complex. To verify whether LRP-1 may indeed

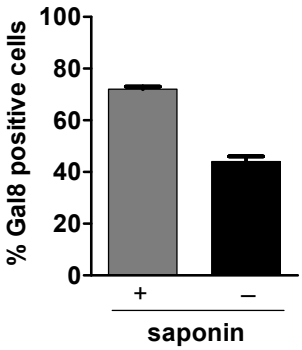

Figure 2. Surface and intracellular galectin 8 expression in primary megakaryocytes. Mobilized peripheral blood-derived CD34+ cells were maturated for 10 days in Cellgro medium supplemented with TPO 100ng/ml and IL-1β 10ng/ml. Staining of galectin 8 (Gal8) in permeabilized (+ saponin) and non-permeabilized (- saponin) cells was performed by flow cytometry as described in Chapter II. Data are the mean values of two independent experiments and are expressed as the percentage of Gal8 positive cells. The error bars represent the spread around the mean.

act as the second receptor contributing to FV endocytosis, we reduced expression of LRP-1 in the megakaryocyte-like DAMI cells using RNAi technology. FV endocytosis was evaluated by incubating the cells with 40 nM FITC FV for 3 hours at 37°C. As shown in Figure 3, the FV uptake capacity of the DAMI cells was unaltered after silencing of LRP-1. This suggests a minor, if any, contribution of LRP-1 to the uptake of FV. We could therefore not confirm the LRP-1 dependent uptake mechanism suggested by Bouchard *et al*.[16,17]. The reason for the different observations is at present unclear but may be related to the employed cellular systems. A targeted knock-down of LRP-1 in the CMK cells should, however, be performed to confirm a role for LRP-1 in these cells. Our findings suggest that in megakaryocytes other receptors than LRP-1 assist Gal8 in the uptake of FV by megakaryocytes.

A role of Gal8 as a cross-linker between FV and platelet glycoproteins

Next to FV, Romaniuk *et al*.[12] identified VWF, multimerin-1 (MNRN-1), integrin GPIIbIIIa and GPV among the proteins that were immunoprecipitated from platelet lysates via Gal8. The identification MNRN-1 may not come as a surprise as FV and MNRN-1 are in a covalent complex in the α-granules[30]. Notably, MNRN-1 has been demonstrated to interact with GPIIbIIIa as well[31]. Intriguingly, GPIIbIIIa is the established receptor that mediates endocytosis and storage of fibrinogen in the α-granules[32]. These observations prompted us investigate the possibility that GPIIbIIIa may act as the receptor that facilitates endocytosis of the FV. If so, extracellular Gal8 may then cross-link FV and GPIIbIIIa prior to endocytosis. Yet, the results from Chapter III show that Glanzmann thrombasthenia (GT) patients, who completely lack GPIIbIIIa, comprise normal levels of FV in their platelets (Chapter III, Fig. 1; Chapter V, Fig. 1). This observation excludes a critical role of GPIIbIIIa for FV endocytosis.

In Chapter V we also established by SPR analysis that Gal8 can directly interact with immobilized GPV. This glycoprotein is found in complex with GPIb-IX on platelets. However, the precise role of GPV for platelet and megakaryocyte biology is yet unclear. It has been reported that GPV is only loosely associated with GPIb-GPIX and its expression is not required for the complex to perform its function as a VWF-binding receptor[33]. We further show in Chapter V that Gal8 may serve as a cross-linker between FV and GPV, and that this "bridge-like" binding interaction can be specifically inhibited by the Gal8-antogonists lactose and TDG (Chapter V,

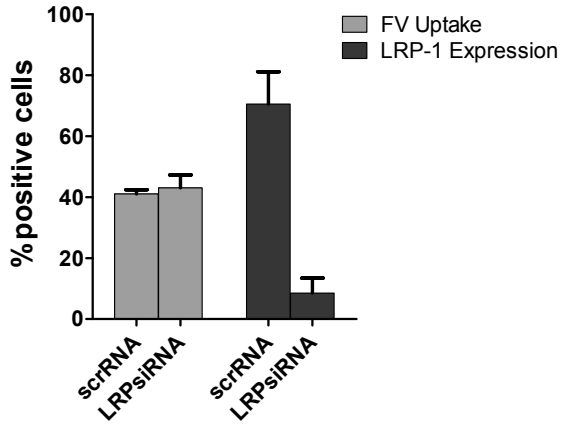

Figure 3. Gene silencing of LRP-1 does not affect uptake of FV by DAMI cells. DAMI cells were transfected with scramble RNA (scrRNA) or targeting LRP-1 siRNA (LRPsiRNA) as described in Chapter II. At 72 hours following transfection, LRPsiRNA-and scrRNA-transfected cells were incubated with 40 nM FITC-FV as described in Chapter II and the uptake and LRP-1 expression were monitored by flow cytometry. Results are expressed as percentage of positive cells. Graphs represent data from two independent experiments and are expressed as means. The errors bars represent the spread around the mean.

Fig. 5). Like Gal8, GPV has been identified on the MK surface as well as in the α-granules[34]. These findings together imply that GPV may facilitate the uptake of the Gal8-FV complex by megakaryocytes. Compatible with this suggestion is our finding that LAD-III patients show a reduction of FV and GPV in their platelets (Chapter III, Table 2; Chapter V, Fig. 2). However, additional experiments with cells where GPV expression is specifically reduced could provide a suitable model to assess the role for GPV in the uptake mechanism.

FIBRINOGEN ENDOCYTOSIS BY MEGAKARYOCYTES

The role of GPIIbIIIa

Fibrinogen constitutes 10% of the protein content of the α-granules[27]. Whereas FV endocytosis occurs exclusively by MKs[8], fibrinogen can be internalized and stored in the α-granules by both platelets and MKs[35]. It has been established that GPIIbIIIa mediates endocytosis of fibrinogen by MKs and platelets[32], but the mechanism thereof remains incompletely understood. The integrin requires activation for high affinity binding to fibrinogen[36]. However, activation of GPIIbIIIa will also lead to activation of the platelets and release of the stored proteins from the α-granules. This implies that the mechanism of fibrinogen endocytosis by GPIIbIIIa in resting platelets cannot involve high affinity interaction between an activated integrin and fibrinogen. This agrees with the findings of this study and of others showing that expression of a functionally defective integrin is sufficient to drive the uptake and storage of the protein (Chapter III, Fig. 3). In addition, the platelets of the LAD-III patients, which exhibit a general integrin activation defect, also revealed normal levels of fibrinogen. Only complete absence of GPIIbIIIa as found in GT type I patients appears to affect fibrinogen storage in α-granules (Chapter III, Fig. 3).

Mass spectrometry analysis of the previously uncharacterized GT patient has shed light on the actual mechanisms of fibrinogen endocytosis. The analysis demonstrated that the GT patient carried the Arg214Trp (R214W) mutation in the region adjacent to the Metal Ion-dependent Adhesion Site (ADMIDAS), the Ca^{2+}-binding domain of GPIIIa[37,38]. The R214W variant of GPIIbIIIa has been shown to have an impaired high affinity binding to fibrinogen. This mutation has previously been associated with GT type III[39,40], which is characterized by normal levels of fibrinogen in the platelets and more than 40% expression of a functionally defective GPIIbIIIa. Our proteomics data obtained from the previously uncharacterized GT patient are in full agreement with this classification (Chapter III).

ADMIDAS has been recently shown to be the binding site for the amino acid sequence KQAGDV of the C-terminus of the fibrinogen γ-chain[41,42]. This sequence is exclusively recognized by activated GPIIbIIIa and mediates the high affinity binding of soluble fibrinogen to the integrin upon agonist-induced activation. Lanza *et al*.[39] reported, however, that the R214W variant of GPIIbIIIa is still able to bind RGD-containing peptides. This may imply that the RGD motifs in fibrinogen may also still bind the GPIIbIIIa R214W variant. It is therefore tempting to speculate that the uptake of fibrinogen is mediated by one of the RGD sequences in fibrinogen. This would then explain why activation of the integrin is not required for fibrinogen endocytosis by platelets and megakaryocytes. Site-directed mutagenesis studies on fibrinogen may provide the answer whether or not the RGD sequences are critical for fibrinogen storage in the α-granules of platelets.

A role of galectins in fibrinogen endocytosis

The observation that Gal8 facilitates endocytosis of FV opens the possibility that uptake of fibrinogen may also involve a galectin family member. However, Gal8 is an unlikely candidate to contribute to fibrinogen uptake since resting platelets, which still support fibrinogen endocytosis, do not express this galectin on their membrane surface[12,43]. Galectin-1 (Gal1) has, however, been identified on the surface of resting platelets[44]. Although Gal1 comprised only a single CRD domain, the protein can exist in a homodimeric form[45]. Like Gal8, it may therefore also act as a cross-linker between two glycoproteins. Intriguingly, Romaniuk *et al*. showed that Gal1 can indeed interact with GPIIbIIIa[46]. If Gal1 can also bind fibrinogen, it would provide an alternative mechanism behind the integrin activation-independent uptake of fibrinogen by platelets and MKs. Gal1 could then serve its role as a cross-linker between fibrinogen and GPIIbIIIa, which in turn mediates internalization of the Gal1-fibrinogen complex.

Unraveling the mechanisms controlling fibrinogen uptake via GPIIbIIIa, including the identification of residues involved in this mechanism will provide significant progress in our understanding of platelet biology.

THE MUNC18-2 SPLICE-SITE VARIANT DOES NOT AFFECT α-GRANULE RELEASE

FHL-5 is caused by defects in Munc18-2 and is characterized by a disturbance of the highly regulated process that controls the secretion of granules from neutrophils[47]. Munc18-2 is also abundantly expressed in platelets and its functional absence has previously been demonstrated to affect

α-granule secretion[48]. Yet, bleeding episodes are inconsistently observed in patients that carry a Munc18-2 (c.1247-1G>C) splice-site mutation,the most frequently observed Munc18-2 variant in FHL-5[49,50]. The role of Munc18-2 for the release of granules from platelets remains, therefore, unclear.

In Chapter IV of this thesis, mass spectrometry analysis of the platelet lysates from FHL-5 patients identified the previously unresolved protein product of the Munc18-2 splice-site variant (Chapter IV, Fig. 2). We also found that the platelets of patients, who are homozygous for this splice-site variant, did not show a defect in platelet degranulation at all. Yet, the neutrophils of these patients did reveal a markedly impaired granule secretion[51].

Two alternative explanations can be given to understand the distinct phenotype of the Munc18-2 splice-site variant in platelets and in neutrophils. This variant leads to a protein product in platelets in which the first 17 amino acids of exon 15 are replaced by 19 residues from intron 14 (Chapter IV, Fig. 7). As it has also been proposed by Cote *et al*.[52], it cannot be excluded that the protein fully retains its functionality in spite of this replacement. Although a decreased expression of the splice-site variant was observed in the platelets of the patients, the residual amount of protein may then be still sufficient to facilitate α-granule release. In neutrophils, the expression level of the splice-site variant might have dropped under the threshold level required for granule release. This would explain why the patients have functional platelets but dysfunctional neutrophils. An alternative explanation is that other Munc18 family members can compensate for functional absence of Munc18-2 in platelets but not in neutrophils. Platelets have been demonstrated to express also Munc18-1, Munc18-3 and Munc18-5[53], each of which may take over the role of Munc18-2. Interestingly, a genome wide association study has shown that there is an association between genetic variations in Munc18-5 and the plasma level of VWF[54]. Although this can be attributed to a differential release of VWF from endothelial cells, an altered release of VWF from the α-granule platelets may contribute to the change in the VWF plasma level as well. If so, this suggests that Munc18-5 may be involved in the α-granule release from platelets. Evidence of a putative role of other Munc18 family members for α-granule release has also been previously provided by Schraw *et al*.[53]. Those authors demonstrated that antagonists of Munc18-1 and Munc18-3 inhibit α-granule release by activated platelets. The contribution of the individual Munc18 family members for α-granule release remains therefore to be established.

A systematic analysis of the change in protein expression in the presence and absence of Munc18-2 in platelets may provide insight into the compensatory mechanism of other family members. In addition, a targeted known-down of Munc18-1, Munc18-3 and Munc18-5 in MKs may unravel the role of these Munc18 family members for platelet functioning.

PLATELET GRANULE SUBSET

It has been reported that platelets comprise two subsets of α-granules that can be differentially released depending on the applied stimulus[55,56]. This hypothesis has been based on two observations. First, pro- and anti-angiogenic factors were detected in distinct non-overlapping compartments in the platelets[55,57]. Second, platelet stimulation via protease-activating-receptor-1 (PAR1) and PAR4 resulted in a differential release of these cargo proteins[58]. The idea of platelets being smart effectors that are unexpectedly able to differentially release a class of appropriate proteins represented a breakthrough in platelet biology.

A recent study from Kamykowski *et al*.[59] did not support the hypothesis that there are α-granule subsets. Employing quantitative immunofluorescence co-localization analysis of functionally distinct proteins they showed no clustering of anti-angiogenic and angiogenic proteins in specialized subsets. Based on their findings, however, it was suggested that there is a major group of large granules with spatial distributions of cargo within the α-granule. Further evidences of spatial segregation of cargo within individual α-granules were provided by Van Nispen and co-workers[60]. However, the authors demonstrated also the presence of morphologically different α-granule subtypes.

In Chapter IV, we stimulated the platelets via PAR1 to assess the release of protein from healthy platelets and FHL-5 platelets. To gain insight into the putative differentially release of α-granule proteins, we now also stimulated platelets from healthy donors via PAR4 and analysed the released proteins by mass spectrometry (Fig. 4). We did not observe, however, any thematic release of a specific class of cargo proteins from the platelets depending on the applied stimulus. Our results argue therefore against the existence of differentially releasable proteins from activated platelets.

A limitation of our study as well as of the study of Italiano *et al*.[55] is that the obtained data derive from a single time point after stimulation and that a single dose of agonist is employed. In addition, the studies did not take into account that α-granule proteins, like VWF, FV and fibrinogen, may be retained at the surface upon platelet stimulation[61,62]. Differential protein adhesiveness to the activated platelet membrane has previously been shown by Watkins *et al*.[63]. They showed with immunoelectron microscopy studies that thrombospondin hardly binds to the platelet surface. In contrast, VWF was abundantly present at the surface of the activated platelets. In conclusion, the differential release observed by other authors might be caused by a differential retention of α-granule proteins at the surface after platelet activation. Further studies are required to fully understand the different observation involving the putative presence of

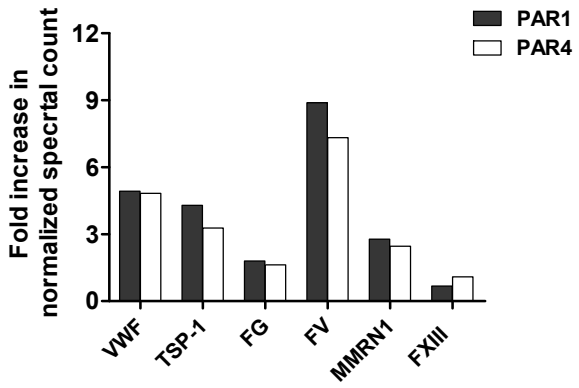

Figure 4. Semi-quantitative analysis of secretable α-granule proteins upon stimulation with PAR1 and PAR4 peptides. Platelets from healthy donors were stimulated via PAR1 (10 μM) and PAR4 (100 μM) as described in Chapter IV. Fold increase of normalized spectral count (average of four healthy controls) were calculated as described in Chapter IV. Figure shows a selection of identified α-granule proteins, including VWF, thrombospondin-1 (TSP-1), fibrinogen (FG), coagulation factors (V and XIII) and Multimerin-1 (MMRN1).

α-granule subsets. The results of our study do not exclude the possibility of spatial segregation of proteins within one α-granule. However, they do imply that there is no differential release of proteins from these granules upon PAR1 or PAR4 stimulation of platelets.

CONCLUSION AND FUTURE PERSPECTIVES

In the present work, we gained more insight a number of events regulating platelet functions. Chapter II, III and V focused on the capacity of MKs to endocytose the plasma proteins FV and fibrinogen. Our findings open a new perspective on the biological functions of Gal8 and other galectin family members during megakaryocytopoiesis. Novel questions that should be addressed in future studies are: (i) At which stage of megakaryocytopoiesis does FV endocytosis occur? (ii) Is Gal8 mediating FV uptake by bridging GPV and FV? (iii) Are there other plasma proteins taken up by the megakaryocytes via Gal8? (iv) Do galectins contribute to a general mechanism of uptake of proteins by megakaryocytes and platelets? Providing an answer to these questions is not merely of scientific interest. As platelets serve as carriers of proteins that are released at sites of injury, these answers might open the perspective of deliberately modifying the protein content of platelets for therapeutic purposes.

The studies described in this thesis further focused on the proteomics of platelets with rare functional defects. Burkhart *et al.*[64] have recently revealed that the difference in platelet proteome of healthy individuals is relatively small. This opens the possibility that proteomic analysis of platelets may assist in the identification of these rare platelet disorders. By analyzing platelet releasates from FHL-5 patients, and the proteome of the platelets of GT, LAD-III and FHL-5 patients, we found that mass spectrometry analysis indeed represents a powerful tool to identify rare platelet disorder (Chapter III, IV). Next to confirming the absence of critical proteins involved in these disorders, differentially expressed proteins were identified that may contribute to the disease as well. An elaborate mass spectrometry-based strategy makes it feasible to establish a protein database that correlates with a specific disorder. This ambitious goal will increase our knowledge of established and previously unidentified platelet disorders and improve their diagnosis.

REFERENCES

1 Leslie M. Beyond Clotting: The Powers of Platelets. *Science*. 2010;328(5978):562–564.

2 Smyth SS, McEver RP, Weyrich AS, et al. Platelet functions beyond hemostasis. *J. Thromb. Haemost*. 2009;7(11):1759–1766.

3 Flaumenhaft R. α-granules: a story in the making. *Blood*. 2012;120(25):4908–4909.

4 Yang TL, Pipe SW, Yang A, Ginsburg D. Biosynthetic origin and functional significance of murine platelet factor V. *Blood*. 2003;102(8):2851–2855.

5 Giampaolo A, Vulcano F, Macioce G, et al. Factor-V expression in platelets from human megakaryocytic culture. *Br. J. Haematol*. 2005;128(1):108–111.

6 Suehiro Y, Veljkovic DK, Fuller N, et al. Endocytosis and storage of plasma factor V by human megakaryocytes. *Thromb. Haemost*. 2005;94(3):585–592.

7 Gould WR, Simioni P, Silveira JR, et al. Megakaryocytes endocytose and subsequently modify human factor V in vivo to form the entire pool of a unique platelet-derived cofactor. *J. Thromb. Haemost*. 2005;3(3):450–456.

8 Bouchard BA, Williams JL, Meisler NT, Long MW, Tracy PB. Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event. *J. Thromb. Haemost*. 2005;3(3):541–551.

9 Kane WH, Majerus PW. Purification and characterization of human coagulation factor V. *J. Biol. Chem*. 1981;256(2):1002–1007.

10 Fernández JA, Hackeng TM, Kojima K, Griffin JH. The carbohydrate moiety of factor V modulates inactivation by activated protein C. *Blood*. 1997;89(12):4348–4354.

11 Preston RJS, Rawley O, Gleeson EM, O'Donnell JS. Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities. *Blood*. 2013;121(19):3801–3810.

12 Romaniuk MA, Tribulatti MV, Cattaneo V, et al. Human platelets express and are activated by galectin-8. *Biochem. J.* 2010;432(3):535–547.

13 Schneider D, Greb C, Koch A, et al. Trafficking of galectin-3 through endosomal organelles of polarized and non-polarized cells. *Eur. J. Cell Biol*. 2010;89(11):788–798.

14 Mishra R, Grzybek M, Niki T, Hirashima M, Simons K. Galectin-9 trafficking regulates apical-basal polarity in Madin–Darby canine kidney epithelial cells. *Proc. Natl. Acad. Sci*. 2010;107(41):17633–17638.

15 Straube T, von Mach T, Hönig E, et al. PH-dependent recycling of galectin-3 at the apical membrane of epithelial cells. *Traffic*. 2013;14(9):1014-1027.

16 Bouchard BA, Meisler NT, Nesheim ME, et al. A unique function for LRP-1: a component of a two-receptor system mediating specific endocytosis of plasma-derived factor V by megakaryocytes. *J. Thromb. Haemost*. 2008;6(4):638–644.

17 Bouchard BA, Abdalla S, Tracy PB. The Factor V Light Chain Mediates the Binding and Endocytosis of Plasma-derived Factor V by Megakaryocytes. *J. Thromb. Haemost*. 2013;doi:10.1111/jth.12417.

18 Lambert MP, Wang Y, Bdeir KH, et al. Platelet factor 4 regulates megakaryopoiesis through low-density lipoprotein receptor-related protein 1 (LRP1) on megakaryocytes. *Blood*. 2009;114(11):2290–2298.

19 Camire RM, Pollak ES, Kaushansky K, Tracy PB. Secretable human platelet-derived factor V originates from the plasma pool. *Blood*. 1998;92(9):3035–3041.

20 Schwarz HP, Lenting PJ, Binder B, et al. Involvement of low-density lipoprotein receptor-related protein (LRP) in the clearance of factor VIII in von Willebrand factor-deficient mice. *Blood*. 2000;95(5):1703–1708.

21 Bovenschen N, van Dijk KW, Havekes LM, Mertens K, van Vlijmen BJM. Clearance of coagulation factor VIII in very low-density lipoprotein receptor knockout mice. *Br. J. Haematol*. 2004;126(5):722–725.

22 Bovenschen N, Mertens K, Hu L, Havekes LM, van Vlijmen BJM. LDL receptor cooperates with LDL receptor-related protein in regulating plasma levels of coagulation factor VIII in vivo. *Blood*. 2005;106(3):906–912.

23 Meems H, van den Biggelaar M, Rondaij M, et al. C1 domain residues Lys 2092 and Phe 2093 are of major importance for the endocytic uptake of coagulation factor VIII. *Int. J. Biochem. Cell Biol*. 2011;43(8):1114–1121.

24 Saenko EL, Yakhyaev AV, Mikhailenko I, Strickland DK, Sarafanov AG. Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. *J. Biol. Chem*. 1999;274(53):37685–37692.

25 Lenting PJ, Neels JG, van den Berg BM, et al. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J. Biol. Chem*. 1999;274(34):23734–23739.

26 Sarafanov AG, Ananyeva NM, Shima M, Saenko EL. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. *J. Biol. Chem*. 2001;276(15):11970–11979.

27 Maynard DM, Heijnen HFG, Horne MK, White JG, Gahl WA. Proteomic analysis of platelet alpha-granules using mass spectrometry. *J. Thromb. Haemost*. 2007;5(9):1945–1955.

28 Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich GA. Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell. Mol. Life Sci.* 2007;64(13):1679–1700.

29 Norling LV, Perretti M, Cooper D. Endogenous galectins and the control of the host inflammatory response. *J. Endocrinol.* 2009;201(2):169–184.

30 Hayward CPM, Fuller N, Zheng S, et al. Human platelets contain forms of factor V in disulfide-linkage with multimerin. *Thromb. Haemost.* 2004;92(6):1349–1357.

31 Adam F, Zheng S, Joshi N, et al. Analyses of cellular multimerin 1 receptors: in vitro evidence of binding mediated by alphaIIbbeta3 and alphavbeta3. *Thromb. Haemost.* 2005;94(5):1004–1011.

32 Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa). *Blood.* 1993;82(1):135–138.

33 Kahn ML, Diacovo TG, Bainton DF, et al. Glycoprotein V-deficient platelets have undiminished thrombin responsiveness and Do not exhibit a Bernard-Soulier phenotype. *Blood.* 1999;94(12):4112–4121.

34 Berger G, Massé JM, Cramer EM. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood.* 1996;87(4):1385–1395.

35 Suzuki M, Miyazaki Y, Miyake T, et al. Uptake of Fibrinogen by Circulating Platelets. *Vox Sang.* 1994;67(2):243–243.

36 Ye F, Kim C, Ginsberg MH. Reconstruction of integrin activation. *Blood.* 2012;119(1):26–33.

37 Zhu J, Boylan B, Luo B-H, Newman PJ, Springer TA. Tests of the Extension and Deadbolt Models of Integrin Activation. *J. Biol. Chem.* 2007;282(16):11914–11920.

38 Zhu J, Luo B-H, Xiao T, et al. Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. *Mol. Cell.* 2008;32(6):849–861.

39 Lanza F, Stierlé A, Fournier D, et al. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa complexes and a glycoprotein IIIa 214Arg----214Trp mutation. *J. Clin. Invest.* 1992;89(6):1995–2004.

40 Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood.* 2011;118(23):5996–6005.

41 Raborn J, Wang W, Luo B-H. Regulation of Integrin αIIbβ3 Ligand Binding and Signaling by the Metal Ion Binding Sites in the β I Domain. *Biochemistry.* 2011;50(12):2084–2091.

42 Springer TA, Zhu J, Xiao T. Structural basis for distinctive recognition of fibrinogen gammaC peptide by the platelet integrin alphaIIbbeta3. *J. Cell Biol.* 2008;182(4):791–800.

43 Zappelli C, van der Zwaan C, Thijssen-Timmer DC, Mertens K, Meijer AB. Novel role for galectin-8 protein as mediator of coagulation factor V endocytosis by megakaryocytes. *J. Biol. Chem.* 2012;287(11):8327–8335.

44 Pacienza N, Pozner RG, Bianco GA, et al. The immunoregulatory glycan-binding protein galectin-1 triggers human platelet activation. *FASEB.* 2008;22(4):1113–1123.

45 Camby I, Mercier ML, Lefranc F, Kiss R. Galectin-1: a small protein with major functions. *Glycobiology.* 2006;16(11):137R–157R.

46 Romaniuk MA, Croci DO, Lapponi MJ, et al. Binding of galectin-1 to αIIbβ3 integrin triggers "outside-in" signals, stimulates platelet activation, and controls primary hemostasis. *FASEB.* 2012;26(7):2788–2798.

47 Zur Stadt U, Rohr J, Seifert W, et al. Familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) is caused by mutations in Munc18-2 and impaired binding to syntaxin 11. *Am. J. Hum. Genet.* 2009;85(4):482–492.

48 Al Hawas R, Ren Q, Ye S, et al. Munc18b/STXBP2 is required for platelet secretion. *Blood.* 2012;120(12):2493–2500.

49 Rohr J, Beutel K, Maul-Pavicic A, et al. Atypical familial hemophagocytic lymphohistiocytosis due to mutations in UNC13D and STXBP2 overlaps with primary immunodeficiency diseases. *Haematologica.* 2010;95(12):2080–2087.

50 Pagel J, Beutel K, Lehmberg K, et al. Distinct mutations in STXBP2 are associated with variable clinical presentations in patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL5). *Blood.* 2012;119(25):6016–6024.

51 Zhao XW, Gazendam RP, Drewniak A, et al. Defects in neutrophil granule mobilization and bactericidal activity in familial hemophagocytic lymphohistiocytosis type 5 (FHL-5) syndrome caused by STXBP2/Munc18-2 mutations. *Blood.* 2013;122(1):109–111.

52 Côte M, Ménager MM, Burgess A, et al. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J. Clin. Invest.* 2009;119(12):3765–3773.

53 Schraw TD, Lemons PP, Dean WL, Whiteheart SW. A role for Sec1/Munc18 proteins in platelet exocytosis. *Biochem. J.* 2003;374(Pt 1):207–217.

54 Smith NL, Chen M-H, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121(12):1382–1392.

55 Italiano JE Jr, Richardson JL, Patel-Hett S, et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood*. 2008;111(3):1227–1233.

56 Chatterjee M, Huang Z, Zhang W, et al. Distinct platelet packaging, release, and surface expression of proangiogenic and antiangiogenic factors on different platelet stimuli. *Blood*. 2011;117(14):3907–3911.

57 Sehgal S, Storrie B. Evidence that differential packaging of the major platelet granule proteins von Willebrand factor and fibrinogen can support their differential release. *J. Thromb. Haemost*. 2007;5(10):2009–2016.

58 Ma L, Perini R, McKnight W, et al. Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. *Proc. Natl. Acad. Sci. U. S. A*. 2005;102(1):216–220.

59 Kamykowski J, Carlton P, Sehgal S, Storrie B. Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet α-granules. *Blood*. 2011;118(5):1370–1373.

60 Van Nispen tot Pannerden H, de Haas F, Geerts W, et al. The platelet interior revisited: electron tomography reveals tubular alpha-granule subtypes. *Blood*. 2010;116(7):1147–1156.

61 Dayananda KM, Singh I, Mondal N, Neelamegham S. Von Willebrand Factor self-association on platelet GpIbα under hydrodynamic shear: effect on shear-induced platelet activation. *Blood*. 2010;

62 Swords NA, Mann KG. The assembly of the prothrombinase complex on adherent platelets. *Arterioscler. Thromb. Vasc. Biol.* 1993;13(11):1602–1612.

63 Watkins SC, Raso V, Slayter HS. Immunoelectron-microscopic studies of human platelet thrombospondin, von Willebrand factor, and fibrinogen redistribution during clot formation. *Histochem. J.* 1990;22(9):507–518.

64 Burkhart JM, Vaudel M, Gambaryan S, et al. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120(15):e73–82.

CHAPTER 7

Summary
Samenvatting
Curriculum vitae
Acknowledgments

SUMMARY

Platelets store a spectrum of functionally distinct proteins in the secretory α-granules, which are released upon platelet activation at sites of injury. This makes platelets a unique vehicle for the delivery of critical proteins that contribute to wound repair. Most of these α-granule proteins are synthesized by the platelet precursor cells, the megakaryocytes (MKs). Platelets and MKs are, however, unique in that they also take up plasma proteins. The mechanisms behind the endocytosis of plasma proteins is, however, still poorly understood. Moreover, only recently the proteins of the granule exocytosis machinery have been investigated in platelets. In the present thesis, we employ protein-protein interaction studies and cell-based internalization studies to get more insight into the mechanisms underlying the endocytosis of plasma proteins in MKs. In addition, we utilize mass spectrometry-based approaches to assess the role of the platelet integrins in α-granule cargo enrichment as well as the mechanism of the release thereof.

In CHAPTER I, a general background regarding platelet biology is presented. An overview is given about the role of platelets in blood clot formation for effective bleeding arrest. In addition, the current knowledge about how platelets internalize the plasma proteins factor V (FV) and fibrinogen is summarized. We further describe rare platelet-related disorders, that are associated with improper protein loading of the α-granules or impaired α-granule release. These diseases include Glanzmann Thrombastenia (GT), in which patients exhibit a functional absence of the most abundant platelet glycoprotein, GPIIbIIIa, Leucocyte-Adhesion-Deficiency type III (LAD-III) characterized by a dysfunctional integrin activation machinery, and Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL-5), in which patients may have an impaired mechanism for the release of α-granules.

In CHAPTER II, the mechanism that contributes to FV endocytosis by megakaryocytes is addressed. Evidence is provided that the lectin galectin-8 (Gal8) mediates the uptake of coagulation FV by megakaryocytes. SPR analysis shows a reversible and dose-dependent binding of Gal8 and FV. Employing a targeted reduction of the expression of Gal8 in the megakaryocyte-like cells (DAMI), anti-Gal8 antibodies, and β-galactosides, we demonstrate that the uptake of FV requires Gal8-FV complex.

In CHAPTER III, mass spectrometry analysis of the platelets of GT and LAD-III patients is employed to assess the role of integrins on platelets cargo enrichment. The results show that GPIIbIIIa only contributes to the uptake of fibrinogen, and that activation of this integrin is not required to facilitate fibrinogen endocytosis. Novel differentially expressed proteins that may be associated with the investigated diseases are identified as well. In this chapter, we further identify by mass spectrometry analysis, the genetic defect of a previously uncharacterized GT patient. The results demonstrate that mass spectrometry analysis represents a powerful tool for effective diagnosis of rare platelet disorders.

In CHAPTER IV, the platelet secretory efficiency is investigated in FHL-5 patients that may exhibit impaired α-granule exocytosis because of mutations in mammalian-uncoordinated-2 (Munc18-2). This protein regulates the complex formation of the so-called SNARE proteins, which are critical for granule release process. We use mass spectrometry, flow cytometry and electron microscopy to identify the protein product of a Munc18-2 splice-site variant (1247-1G>C) and the effect thereof on platelet degranulation. This Munc18-2 variant has been established to cause impaired degranulation of neutrophils. In spite of previous suggestions,

flow cytometry, electron microscopy and mass spectrometry studies reveal that the splice-site variant does not significantly affect platelet degranulation.

In CHAPTER V, the potential role of the other interaction partners of Gal8, i.e. GPIIbIIIa and GPIb-GPIX-GPV, in FV endocytosis is evaluated. The platelets of the LAD-III and GT patients show that the level of GPV and not of GPIIbIIIa correlates with the level of FV in the platelet lysates. Biochemical approaches show that Gal8 can act as a cross-linker between FV and GPV. The results of this chapter suggest that GPV may act as the Gal8 counter-receptor to promote FV endocytosis.

In CHAPTER VI, the major findings of the present thesis are summarized and discussed. We highlight the role of carbohydrates as players in the process of α-granule protein loading and provide new directions for future researches. In addition, we discuss the value of the proteomic approaches for the identification of rare platelet disorders and novel proteins that may be associated with the disease. Potential implications of these findings and perspectives for future studies in the platelet field are also discussed.

SAMENVATTING

Bloedplaatjes bevatten een spectrum aan functioneel verschillende eiwitten in hun opslaggranula, de α-granula, die worden uitgescheiden na activatie van bloedplaatjes bij een verwonding. Dit maakt het bloedplaatje tot een uniek vehikel voor de aanvoer van cruciale eiwitten die bijdragen aan wondherstel. De meeste α-granula-eiwitten worden gesynthetiseerd door de voorlopercellen van bloedplaatjes, de megakaryocyten (MKs). Bloedplaatjes en MKs zijn echter uniek in het feit dat ze ook in staat zijn om eiwitten uit plasma op te nemen en daarna op te slaan. Het mechanisme achter deze opname, zogenaamde endocytose, is echter verre van duidelijk. Bovendien is de machinerie die granula-exocytose medieert in bloedplaatjes pas zeer recent onderwerp van studie geworden. In dit proefschrift hebben we door het bestuderen van eiwit-eiwit interacties en cellulaire internalisatie geprobeerd inzicht te verkrijgen in het onderliggende mechanisme van endocytose van plasma-eiwitten in MKs. Daarnaast hebben we massaspectrometrie gebruikt om zowel de rol van bloedplaatjes-integrines in de toename α-granula-inhoud te bestuderen als het mechanisme waarlangs deze wordt uitgescheiden.

In Hoofdstuk I wordt een algemene achtergrond geschetst van de biologie van bloedplaatjes. Er wordt een overzicht gegeven van de rol die bloedplaatjes spelen tijdens de bloedstelping via de vorming van een bloedplaatjesprop. Daarnaast wordt de huidige stand van zaken wat betreft de internalisatie van de plasma-eiwitten factor V en fibrinogeen samengevat. Er worden zeldzame bloedplaatjesdysfuncties beschreven die samenhangen met verstoorde eiwitopname in α-granula of met defecten gedurende de uitscheiding van α-granula. Hieronder vallen Glanzmann Thrombastenia (GT), waarbij patiënten geen functionele versie bevatten van het meest voorkomende bloedplaatje glycoproteïne, GPIIbIIIa, Leukocyte Adhesion Deficiency type III (LAD-III) gekarakteriseerd door een defect integrine activatie mechanisme en als laatste Familial Hemaphagocytic Lymphohistiocytosis type 5 (FHL-5), een ziekte waarin patiënten mogelijkerwijs leiden aan een verstoorde uitscheiding van α-granula.

In Hoofdstuk II is het mechanisme dat bijdraagt aan opname van factor V door megakaryocyten bestudeerd. Bewijs wordt aangeleverd dat het lectine Galectine-8 (Gal8) de opname van FV door megakaryocyten medieert. SPR analyse laat zien dat er een reversibele en dosis-afhankelijke binding bestaat tussen Gal8 en FV. Door middel van depletie van Gal8 expressie in megakaryocyt-achtige cellen (DAMI), anti-Gal8 antistoffen en β-galactosiden laten we zien dat de opname van FV de vorming van een Gal8-FV complex vereist.

In Hoofdstuk III wordt door middel van massaspectrometrische analyse van bloedplaatjes van GT en LAD-III patiënten de rol van integrines in de eiwitopname door bloedplaatjes bestudeerd. De resultaten laten zien dat GPIIbIIIa bijdraagt aan de endocytose van fibrinogeen, maar dat activatie van deze integrine niet vereist is voor fibrinogeen-opname. Nieuwe eiwitten die specifiek tot expressie komen in "zieke" of "gezonde" bloedplaatjes en die mogelijkerwijs betrokken zijn bij deze aandoeningen worden eveneens geïdentificeerd. Tevens identificeren we in dit hoofdstuk via massaspectrometrische analyse het genetische defect van een tot nog toe ongekarakteriseerde GT patiënt. Deze resultaten laten eveneens zien dat massaspectrometrie een krachtige techniek is voor diagnose van zeldzame plaatjesdysfuncties.

In Hoofdstuk IV is het secretoire fenotype van de bloedplaatjes van FHL-5 patiënten onderzocht die mogelijk een verstoorde α-granula-secretie zouden hebben wegens mutaties in Mammalian-

uncoordinated-2 (Munc18-2). Dit eiwit reguleert de formatie van een complex van zogeheten SNARE eiwitten, die een kritieke rol vervullen tijdens secretie. Met behulp van massaspectrometrie, flow-cytometrie en elektronenmicroscopie hebben we zowel het eiwitprodukt van een Munc18-2 "splice-site" variant (1247-1G>C) geidentificeerd als het effect ervan op plaatjesdegranulatie. Van deze Munc18-2 variant is eerder vastgesteld dat deze leidt tot verstoorde degranulatie van neutrofielen. In tegenstelling tot eerdere suggesties in die richting, wijzen onze flow-cytometrische, elektronenmicroscopische en massaspectrometrische studies er echter niet op dat de bestudeerde variant de degranulatie van bloedplaatjes aanmerkelijk verstoort.

In Hoofdstuk V wordt de mogelijke rol van andere interactiepartners van Gal8, zoals GPIIbIIIa en GP1b-GPIX-GPV, in de endocytose van FV geëvalueerd. Bloedplaatjes van LAD-III en GT patiënten laten zien dat het gehalte aan GPV, en niet GPIIbIIIa, samenhangt met het niveau aan FV in het lysaat van de bloedplaatjes. Met behulp van biochemische experimenten wordt aangetoond dat Gal8 als brug tussen FV en GPV kan fungeren. De resultaten van dit hoofdstuk suggereren dat GPV, als bindingspartner van Gal8, de endocytose van FV kan versterken.

In Hoofdstuk VI worden de belangrijkste bevindingen van dit proefschrift samengevat en bediscussieerd. De rol van suikers als spelers tijdens de opname van eiwitten in α-granula wordt uitvoerig behandeld en nieuwe richtingen voor verder onderzoek worden aangegeven. Daarnaast wordt de potentie van proteomics voor de karakterisering en identificering van zeldzame bloedplaatjes-afwijkingen en de daarbij betrokken eiwitten belicht. Tenslotte worden de mogelijke implicaties van deze bevindingen en perspectieven voor toekomstig onderzoek op dit terrein bediscussieerd.

CURRICULUM VITAE

Claudia Zappelli was born on the 18th of April 1983 in Naples, Italy.
In 2003 she graduated from high school and started the bachelor study "Biotechnology" at the University of Naples Federico II (Italy). Her first internship of one year was at the Department of Molecular Medicine and Medical Biotechnology under the supervision of Prof. Dr. E. De Vendittis. Her bachelor thesis was focused on the "Determination of the activity of Thioredoxin/ Thioredoxin reductase system in Sulfolobus Solfataricus". After graduating in 2006 with maximum vote she started her Master "Medical Biotechnology" at the University of Naples Federico II (Italy). In July 2008 she completed her Master Degree (cum laude) in Medical Biotechnology. Her thesis was based on the scientific results obtained during the two years internship at the University of Naples, Federico II in a research group led by Prof. Dr. M.R. Ruocco (Department of Molecular Medicine and Medical Biotechnology). She studied the involvement of Mn-SOD in mitochondrial impairment and apoptosis induced by Diclofenac, a nonsteroidal anti-inflammatory drug. In January 2009, she started her PhD student project in the Department of Plasma Proteins in Sanquin Research (Amsterdam, The Netherlands) under the supervision of Dr. A. B. Meijer and Prof. Dr. K. Mertens. The results of the research performed are described in this thesis.
In July 2013 she won a fellowship (grant: POR Campania FSE 2007-2013) and worked in the Department of Pharmacy at the Univeristy of Salerno, Italy under the supervision of Dr. F. Dal Piaz and Prof. Dr. N. De Tommasi. Since March 2014 she is working as Marketing and Sales Manager at VitaLab in Naples, Italy.

ACKNOWLEDGEMENTS

Pursuing a PhD was for me a truly life-changing experience. I worked hard to get to the point where I am now, writing my "thank you's", but I definitely could not get this far without the help and support of many people. Though it will not be enough to express my gratitude in words, I would still like to give my many, many thanks to the following people.

First of all, I would like to say a very big thank you to Sander. Without your guidance, encouragement and feedback this PhD would not have been achievable! Although I didn't start as your PhD student you never doubted I could do this and you always showed great enthusiasm for new lines of research. You gave me the freedom to pursue various projects without objection and this immensely helped me to grow. It was not easy in the beginning, yet I managed to come this far. I hope this thesis pays off all your hard work supervising me.

Koen, thank you for your valuable input and fruitful discussions during our regular meetings. Your criticism and your keen eye for details in a way always motivated me to do better each time. It is only at the end, when I saw the whole picture, that I realized how important is to be continuously pushed further and stimulated to look from a different perspective. Thank you!

When I left Naples I never imagined that during my PhD I would also find the most special and amazing group of colleagues I could wish for! Without you I would be just another sad and lonely PhD student! We've all been there for each other and have discovered many tools and means to overcome frustrations and failed experiments.

First of all I would like to thank my paranymphs: Nicoletta and Magdalena.

Nicoletta you are so much more than a friend...a sister that shared with me all the joys and failures of the last ten years! Avere accanto la propria migliore amica Italiana durante il PhD all'estero è una fortuna che in pochi al mondo possono vantare...e io l'ho avuta! Credo ti ricordi le mie urla di gioia quando mi dicesti che accettavi l'offerta e partivi anche tu...non dimenticherò mai quel giorno! Averti lì con me ha avuto un valore aggiunto che non potrò mai esprimere con le parole....i grazie non sarebbero mai abbastanza! Thank you for always listening to me, supporting me and encouraging me. Ti voglio bene!

Magda, having you seated next to me during the last, crazy, hectic months of my PhD was the best gift I could wish for! You were such a valuable support! Although you were just starting your own project, you always found the time to discuss with me my experiments, giving new ideas and a fresh, bright new perspective....thank you for that! "Don't worry, it will all end and it will be a success" , these few, simple words are, for a stressed PhD, essential to regain confidence and you patiently repeated to me almost every day. Thank you for your genuine friendship!

Lydia (Lydiucci), how can I explain here my immense gratitude to you? When things get rough, and during the PhD this happens regularly, a good laugh can make your day! And with you Lydiucci I laughed so much, with our crazy stories no one else could get a grip on....how many times we started with a failed experiment and ended laughing our heads off!! You are a wave of good energy, a generous and honest person! I feel very lucky to have met a wonderful person like you Lydiucci! Thank you for everything and I will visit you soon in Santiago, I promise!

Eszter, people like you are (more than) hard to find. You were and remain my best role model for a scientist and a mentor. Your scientific curiosity, energy and enthusiasm are contagious! If this wasn't enough you are also a great, sincere and reliable friend! I have great memories of you and Peter in Naples....Capri...uncountable ice creams...thank you for all this!!!

Ola, we started this experience almost at the same time and throughout all 4 long, intense years you were around in every crucial step of my PhD. You are a hard worker and a talented researcher and I am sure you are doing great in NY! However most of the best moments we shared are outside the lab...we lived many intense moments, lots of laughs and the trip to Naples...so many memories! Thank you for being there for me..I miss you badly and I wish you all luck you deserve!

Ruben and Javier, two very different personalities but both very important for" La Zappelli"..!!

Ruben, I met you in the ISTH and almost immediately I felt that you were so much more than the arrogant alpha-male I was prepared to meet! You are a great scientist, able to work in an "out of the box" manner, always giving generously your time and insights! Outside the lab you are great fun and we shared many crazy moments! It will be my pleasure having you here in Naples.

Javier (Javi), my brother alter-ego, I could write this acknowledgement in 6 different languages and you would understand them all! You are an incredible talented person, a sincere and sensible guy! Ti auguro tanta fortuna e tutta la felicita' che meriti! Grazie per il tuo grande supporto!

When I left Sanquin there were 4 Italian PhDs around, but you Lussy, were the very first one! I remember your kind welcoming mail in which you invited me and Niki for an "Italian lunch" at the cantine! I still laugh sometimes remembering our nice habit of visiting each other's department for exchanging advice and complaints about doing a PhD...so typically Italian! Our bond became much more intense now that we are back in our country! I know I can count on you and that you have a big heart, although you are most of the time afraid to show it! Grazie di tutto, ti auguro di trovare tutto cio' che desideri, lo meriti!

Simon and Wouter, first of all sorry for pronouncing your names wrong for 4 years!!! Having you two around during the first years of my PhD was a great bless!! You two are such dedicated workers and incredible researchers, always there for stimulating talks and questions! I am sure I will see your names in many important scientific publications! A big thank you also for all the good times outside the lab, I miss you both! Ciao!

I thank all the present and past members of PE department: Esther, Bieuwke and Maartje for many dinners, borrels and discussion together, Dorothee (an extremely nice and helpful person), Kathinka (a funny and friendly girl with muddy shoes and sweet with dogs), Herm-Jan (a kind and supportive person), Robin (a smart talkative guy with an innate curiosity) Eduard (a sociable and positive guy), Marion and Gerty (kind, helpful and supportive secretaries) and all other PhDs that just started when I left: Good luck! A big thank you also to Eveline (for being a dear friend and the nice trip to Amersfoort) and Ilze (a nice and helpful person who was pretty supportive during the first year of my PhD when we worked side by side).

Carmen, Jaqueline Mariette, Paul, Marleen and all other technicians. Your excellent work has made an invaluable contribution towards my PhD. I am not sure I can count the number of

samples I gave you for my MS analysis, what an incredible patience! You are all hard workers able to cope with generations of frustrated PhDs, always giving valuable advice and support!

Fiamma, I would like to thank you as part of the PE Department, although you didn't officially belong to it. Io, te e Niki, tre napoletane in giro Amsterdam....i miei pilastri...grazie per tutti i bellissimi momenti condivisi, tvb!

I also feel indebted to Laura and Iris from BCR department. Laura your personality and enthusiasm are contagious, you always gave me tons of feedback and scientific observations that greatly stimulated me to expand my scientific knowledge! Thank you for being a key player during the last year of my PhD!

Iris, a wonderful person with great lab skills. You always found the time to help me out and taught me many aspects of platelet biology....thank you!

Now I would like to thank my mom, dad, and brother to who I dedicate this thesis. My hard-working parents have sacrificed their lives for my brother and myself and provided unconditional love and care. I love you immensely, and I would not have made it this far without you. Lello you have been my best friend all my life, thank you for all your advice and support. I know I always have my family to count on when times are rough.

To my ever supportive partner in life, Fabrizio. Without your love and endless encouragement this thesis wouldn't have been written. Thank you for understanding that a PhD has crazy time-tables and that being on Christmas eve in the lab can also be possible! I truly thank you for sticking by my side, even when I was irritable and depressed. Thank you for the undying love you have provided me and for believing in me, no matter what. Sei la cosa piu' preziosa che ho, ti amo.

Last but not least I want to thank all my Italian supporters. Stefania, ci conosciamo da una vita e condividiamo un'amicizia sincera e profonda. Grazie per esserci sempre stata e aver sempre creduto in me! Geggiu e tutta la famiglia Pepino/Tursi, siete una pilastro fondamentale per me. Mi avete sempre supportato e dato forza anche quando non ci credevo piu', grazie mille! Roby, sei una persona dolcissima e sincera....grazie per non avermi mai fatto sentire sola neanche in Olanda! Un grazie a Tina, Fabrizio e tutto il Lab, 22 : non dimenticherò l'affetto con cui mi avete accolto, Grazie di cuore!