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Marked bleeding diathesis in patients with platelet dysfunction due to a novel mutation in *RASGRP2*, encoding CalDAG-GEFI (p.Gly305Asp)

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Abstract

Congenital platelet function disorders are often the result of defects in critical signal transduction pathways required for platelet adhesion and clot formation. Mutations affecting *RASGRP2*, the gene encoding the Rap GTPase activator, CalDAG-GEFI, give rise to a novel, and rare, group of platelet signal transduction abnormalities. We here report platelet function studies for two brothers (P1 and P2) expressing a novel variant of *RASGRP2*, CalDAG-GEFI(p.Gly305Asp). P1 and P2 have a lifelong history of bleeding with severe epistaxis successfully treated with platelet transfusions or rFVIIa. Other bleedings include extended hemorrhage from minor wounds. Platelet counts and plasma coagulation were normal, as was α IIB β 3 and GPIb expression on the platelet surface. Aggregation of patients' platelets was significantly impaired in response to select agonists including ADP, epinephrine, collagen and calcium ionophore A23187. Integrin α IIB β 3 activation and granule release were also impaired. CalDAG-GEFI protein expression was markedly reduced but not absent. Homology modeling places the Gly305Asp substitution at the GEF-Rap1 interface, suggesting that the mutant protein has very limited catalytic activity. In summary, we here describe a novel mutation in *RASGRP2* that affects both expression and function of CalDAG-GEFI and that causes impaired platelet adhesive function and significant bleeding in humans.

Keywords

platelets; signaling; dysfunction; bleeding; *RASGRP2*

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Introduction

Inherited platelet function disorders (IPFDs) are a frequent cause of bleeding in children and adults. The majority of IPFDs affect signaling pathways used by platelet agonists[1]. Mutations affecting platelet granule secretion or important surface expressed adhesion receptors, including α IIb β 3 integrin (Glanzmann thrombasthenia; GT) are also common. The availability of powerful DNA and RNA sequencing technologies has helped to identify novel genes affected in IPFDs. *RASGRP2* is one such gene. *RASGRP2* codes for the calcium-sensing guanine nucleotide exchange factor, CalDAG-GEFI. Studies in mice, dogs and cattle identified CalDAG-GEFI as a critical regulator of integrin signaling in platelets[2–5]. Specifically, CalDAG-GEFI mediates the activation of the small GTPase Rap1, an event required for the near-immediate inside-out activation of platelet integrins required for hemostatic plug formation at sites of vascular injury. Consistent with these studies in animal models, platelets from humans with mutations in *RASGRP2* show marked defects in agonist-induced activation of Rap1 and α IIb β 3 integrin, in particular when stimulated with ADP or low doses of other physiological agonists[6–8]. To date four mutations in *RASGRP2* have been reported, three of which lead to CalDAG-GEFI deficiency in platelets. The only loss-of-function (LOF) mutation reported so far leads to a p.Gly248Trp variant that shows no GEF activity in vitro and in vivo[6,7]. Here we report a novel mutation in *RASGRP2* (c.914G>A), resulting in a conversion of Gly305 to Asp (G305D), which affects both the expression and function of CalDAG-GEFI.

Methods

See supplemental information.

Results and Discussion

Patients

We report two Argentinian boys with a lifelong bleeding syndrome including epistaxis and cutaneous and minor wound bleeding. The overall bleeding score for P1 (9 years old) and P2 (24 years old) were 17 and 13 according to the ISTH-BAT (Table 1). P1 exhibited epistaxis at a very young age, with 5-6 severe episodes per year, some requiring hospitalization with cauterization and/or tamponade, and transfusions of red blood cells and platelets. He also suffered from extensive cutaneous hematomas, bleeding with the appearance of permanent teeth or tooth extraction, and bruising with vaccination. Minor wounds sometimes resulted in bleeding that lasted for more than 20 minutes and required transfusion of red blood cells. P2 also suffered from severe epistaxis between 2 and 15 years of age. Severe bleeding episodes required hospitalization and transfusions of red blood cells and platelets. Other symptoms presented were: bleeding with appearance of new teeth and bruising with vaccinations, extensive cutaneous hematomas, extended bleeding with minor wounds. P2 also had a severe hemorrhagic episode after a deep wound to his leg, which required transfusion of platelets to stop bleeding. P1 had a hemorrhagic episode successfully treated with rFVIIa alone. The mother had epistaxis during childhood but no reported bleeding during her adult life. She was adopted (biological parents not known) and recently died of

acquired pneumonia. Based on the patients' statements, the father has no history of bleeding. Data on the paternal grandparents are not available.

For patient 2, all tested blood parameters including white blood cell count, red blood cell count, and platelet count and volume were within the normal range. Patient 1 exhibited at the time of examination an elevated platelet count and anemia (Table 1). Both P1 and P2 also exhibited normal fibrinogen levels (Table 1) and coagulation function (not shown). Important membrane receptors such as α IIB β 3 and GPIb were expressed at normal levels (not shown). Significant defects, however, were detected when platelet function was tested by PFA-200, standard aggregometry, or flow cytometry. PFA-200 closure times to collagen/epinephrine or collagen/ADP were >300 seconds for both P1 and P2 (Table 1). Platelet aggregation was markedly reduced in P1 and P2 in response to ADP, epinephrine (Epi), collagen (Col), and the PAR1 receptor agonist, TRAP-6 (Figure 1). The aggregation response of P1 and P2 platelets was only minimally affected when the cells were activated with thrombin, arachidonic acid (AA), or ristocetin. As this aggregation profile is consistent with recent reports on platelet aggregation in patients with mutations in *RASGRP2*[6–8] and CalDAG-GEFI signaling is known to be driven by changes in cytoplasmic calcium levels[9,10], we also tested the aggregation response of the patients' platelets to the calcium ionophore, A23187, and the protein kinase (PK)C agonist, phorbol 12-myristate 13-acetate (PMA). Like platelets from mice lacking CalDAG-GEFI[9], the patients' platelets did not aggregate to A23187 but showed a normal response to PMA (Figure 1A). P1 and P2 platelets also showed marked defects in fibrinogen binding (α IIB β 3 activation) and surface expression of P-selectin (CD62P, alpha granule release) and CD63 (lysosomal granules) when stimulated with ADP and analyzed by flow cytometry (Figure 1B). In contrast, α IIB β 3 activation and granule secretion were only mildly affected in PMA-activated platelets. Integrin activation in leukocytes, other blood cells that express CalDAG-GEFI, was not assessed, as our patients did not show a predisposition to infections. DNA analysis using the ThromboGenomics high-throughput sequencing platform for selected genes with a known role in platelet function[11] identified a novel homozygous c.914G>A transition in exon 9 of the *RASGRP2* gene for P1 and P2 (Figure 1C). P3, the mother of P1 and P2, was identified as a carrier for the mutation. Immunoblotting demonstrated that the resulting p.Gly305Asp variant of CalDAG-GEFI is expressed at markedly lower levels in platelets from P1 and P2 when compared to controls (Figure 1D). While we were unable to purify the p.Gly305Asp variant of CalDAG-GEFI for testing in our cell-free activity assay[7], we were able to map the affected residue in very close proximity to Gly248 (Figure 1E), a residue shown to be critical for the GEF activity of CalDAG-GEFI[6]. Thus, it appears likely that the residual p.Gly305Asp protein detected in P1 and P2 platelets does not have full GEF activity.

To date there is only one report of a loss-of-function mutation in *RASGRP2* in humans, while protein expression was completely abolished in other patients recently reported by us and others[7,12] as well as patients reported in a companion manuscript published in this issue of *Platelets*. Our studies strongly suggest that a defect in both CalDAG-GEFI expression and function is responsible for the marked defect in platelet function and hemostasis observed in P1 and P2.

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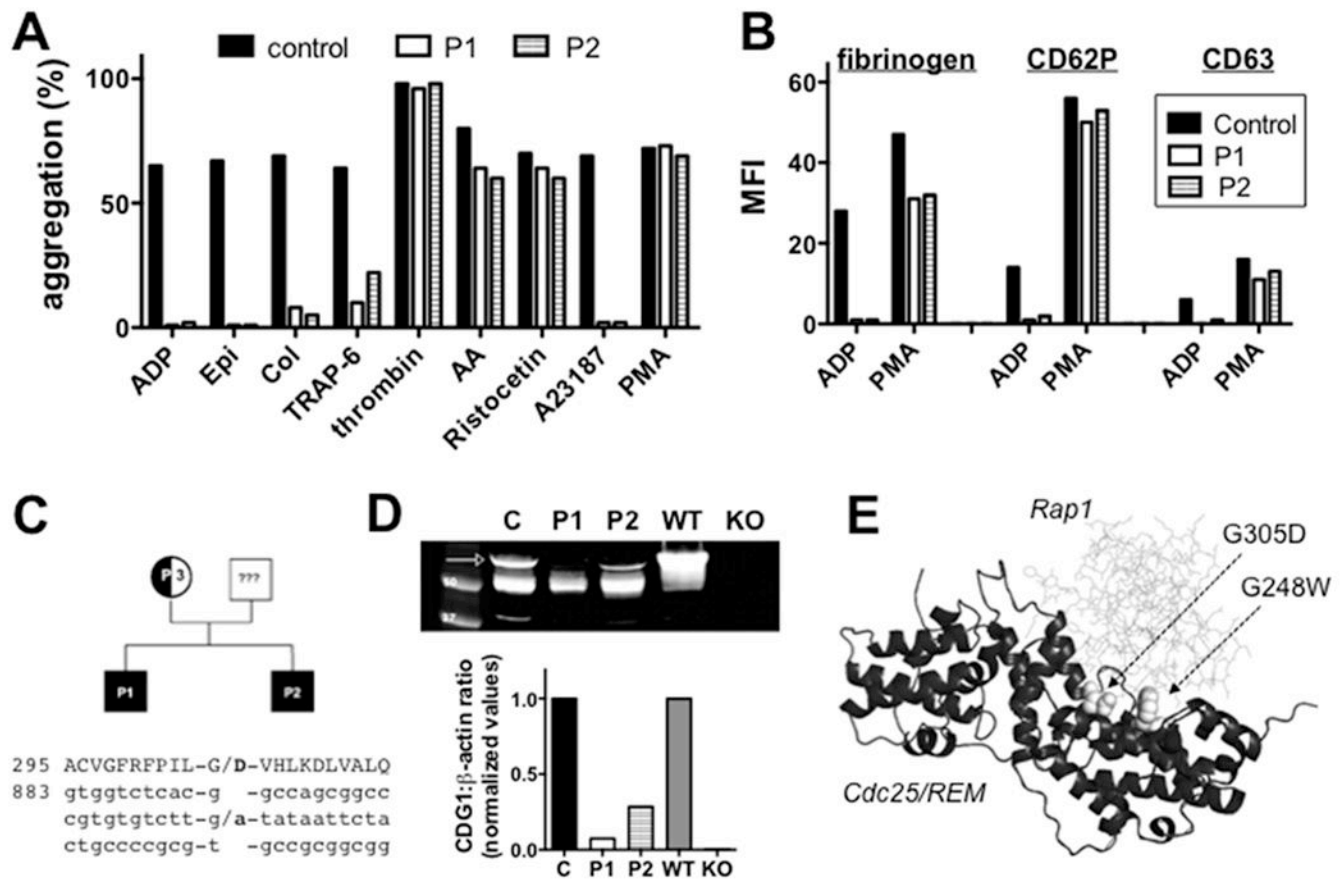


Figure 1: Altered platelet function in patients with novel mutation in *RASGRP2*.

(A,B) Impaired aggregation, integrin activation and granule secretion in platelets from patients (P1, P2) with lifelong bleeding diathesis. (A) Aggregation response of control and patients' platelets activated with ADP (10 μ M), epinephrine (Epi, 50 μ M), collagen (Col, 8 μ g/mL), TRAP-6 (20 μ M), thrombin (0.1 U/mL, washed platelets), arachidonic acid (AA, 0.5mM), ristocetin (1.2mg/mL), A23187 (2.5 μ M), or PMA (4 μ M). (B) Flow cytometric analysis of fibrinogen binding to α IIB β 3 (left panel), surface expression of P-selectin (CD62P, middle panel) and surface expression of LAMP-3 (CD63, right panel) for PRP activated with ADP (10 μ M) or PMA (4 μ M) followed by labeling with fibrinogen-FITC or the indicated antibodies. Results are shown as mean fluorescence intensity (MFI) after subtracting the MFI measured in unstimulated platelets. (C) Family pedigree (top panel) and localization of the c.914G>A transition and G305D aa substitution within *RASGRP2* (bottom panel). Filled and partially filled black symbols indicate subjects homozygous and heterozygous for the mutation. The father was not genotyped. (D) Upper panel: Immunoblot analysis for CalDAG-GEFI in platelet lysates from a healthy human (control), P1, P2, a control mouse (WT) and a *Rasgrp1*^{-/-} mouse (KO). White arrow indicates the band for CalDAG-GEFI. Bottom panel: densitometric quantification of band intensities. The CalDAG-GEFI to β -actin band intensity ratio is shown. Ratios determined in P1/P2 and KO samples were normalized towards human control and WT mouse samples, respectively. (E) A homology model of the Cdc25/REM domain of CalDAG-GEFI (dark grey, cartoon

representation), interacting with Rap1B (light grey, lines representation). This model was generated in pymol and is based on the crystal structure of Epac2 in complex with Rap1B[7,13]. Arrows point to the homologous positions of CalDAG-GEFI G305D and G248W, shown in sphere representation (white).

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Table 1:

Blood parameters and clinical bleeding symptoms in patients homozygous for the CalDAG-GEFI p.Gly305Asp variant.

	P1	P2	Normal Values	
WBC ($\times 10^9/L$)	8.9	9.9	4.0-10.0	
RBC ($\times 10^{12}/L$)	3.9	5.2	4.2-5.4	
Hb (g/dL)	8.7	16.3	11.5-16	
Ht (%)	28.7	46.4	40-53	
Platelets ($\times 10^9/L$)	397.0	196.0	150-350	
MPV (fL)	9.7	9.9	7-10.5	
Fibrinogen (g/L)	320.0	345.0	200-400	
PFA200 CT (s)	Collagen/Epinephrine	>300	>300	<125
	Collagen/ADP	>300	>300	<160
Bleeding scores	Epistaxis	6	4	
	Cutaneous	3	2	
	Minor wounds	4	4	
	Oral cavity	4	3	
Final Bleeding score	17	13		

Clinically significant bleeding for the indicated sites was graded on severity scale from 0 (absence of symptoms) to 4. The final bleeding score reflects the sum of the severity of all bleeding symptoms reported by the patient.

Abbreviations: CT, closure time; Hb, hemoglobin; Hct, hematocrit; MPV, mean platelet volume; P1, patient 1; P2, patient 2; PFA-100, platelet function analyzer 100; RBC, red blood cell; WBC, white blood cell.