A new form of macrothrombocytopenia induced by a germ-line mutation in the PRKACG gene

Vladimir T. Manchev,1,2,3 Morgane Hilpert,1,2,3 Eliane Berrou,4 Ziane Elaib,4 Achille Aoubi,5 Siham Boukour,1,3,4 Sylvie Souquere,3,6 Gerard Pierron,3,6 Philippe Rameau,3,7 Robert Andrews,8 François Lanza,9 Regis Bobe,4 William Vainchenker,1,3 Jean-Philippe Rosa,4 Marijke Bryckaert,4 Najet Debili,1,3 Remi Favier,1,10 and Hana Raslova1,3

Inherited thrombocytopenias (ITs) are a heterogeneous group of blood disorders characterized by a reduced platelet count in blood. Some of these diseases are exclusively restricted to megakaryocytes (MKs) and platelets, whereas others also affect other tissues. Functional platelet defects are also often associated, and their severity may lead to a high risk of bleeding. Based on the mean platelet volume, ITs have been classified into 3 subgroups with large, normal, or small platelets.

The most important subgroup of inherited platelet disorders is characterized by low platelet counts and the presence of large and giant platelets, designated as macrothrombocytopenia. This subgroup includes 2 types of thrombocytopenia. The first type is characterized by syndromic features associated with mutations in genes encoding myosin II A in myosin heavy chain 9 (MYH9)-related disease, lamin-A (FLNa) in FLNa-related thrombocytopenia, friend leukemia integration 1 (FLI1) in Paris-Trouseau syndrome, and ATP-binding cassette sub-family G member 5 (ABCG5) macrothrombocytopenia associated with sitosterolemia. In contrast, the second type lacks syndromic features and is related to mutations in genes encoding transcription factors such as GATA1 and GF1B and for cytoskeletal proteins or surface receptors such as tubulin (β1, αIIbβ3), and platelet glycoprotein Ib (GPIb)/platelet glycoprotein IX (GPIX).1,2 Recently, next-generation sequencing methodology led to the identification of the NBEAL2 gene altered in Gray platelet syndrome and of the ACTN1 gene affected in congenital macrothrombocytopenia (CMTP).3

Here, using exome sequencing, we identified for the first time a c.222C>G mutation in the PRKACG gene in a family with a novel form of IT. PRKACG encodes the γ catalytic subunit of the cyclic adenosine monophosphate-dependent protein kinase, the mutated allele cosegregating with the macrothrombocytopenia. We demonstrate that the p.74Ile>Met PRKACG mutation is associated with a marked defect in proplatelet formation and a low level in filamin A in megakaryocytes (MKs). The defect in proplatelet formation was rescued in vitro by lentiviral vector-mediated overexpression of wild-type PRKACG in patient MKs. We thus conclude that PRKACG is a new central actor in platelet biogenesis and a new gene involved in inherited thrombocytopenia with giant platelets associated with a thrombocytopenia. (Blood. 2014;124(16):2554-2563)
(Cy) of cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), and the mutation is predicted to lead to a missense p.744le→Met substitution. This mutation is responsible for the autosomal recessive IT characterized by defects in proplatelet formation and platelet activation in the homozygous patients, identifying a new mechanism responsible for thrombocytopenia and thrombocytopathy.

Material and methods

Patients

Blood samples from patients and healthy subjects were collected after informed written consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Ethic Committee of the Institut National de la Santé et de Recherche Biomédicale (INSERM RBM) 01-14.

Samples

Venous blood from the patient was collected in 10% (volume to volume) ACD-A buffer (75 mM trisodium citrate, 44 mM citric acid, and 136 mM glucose, pH4). The platelet-rich plasma was prepared by centrifugation at 80g for 10 minutes. Platelets were pelleted by centrifugation at 2100g for 10 minutes. Platelets were washed in the presence of apyrase (100 μU/mL) and prostaglandin E1 (1 μM) to minimize platelet activation. The number of platelets from the patient and the control was adjusted to similar levels (107 platelets/mL; 150

Free intracellular calcium concentration measurement

Washed platelets (2.5 × 107 platelets/mL) were loaded with Oregon green 488 BAPTA-1-AM (1 μM; Molecular Probes, Eugene, OR) for 45 minutes at 20°C. Platelets were then diluted in Tyrode’s buffer to 2.5 × 107 platelets/mL, and the cytosolic Ca2+ concentration was analyzed using an Accuri C6 (Becton Dickinson) flow cytometer. First, Ca2+ mobilization in response to thrombin was observed in the absence of extracellular Ca2+ (100 μM EGTA). After 3 minutes, Ca2+ influx was induced by adding Ca2+ (300 μM) in extracellular medium.

Platelet spreading and F-actin/G-actin quantification

Glass coverslips were precoated with human von Willebrand factor (VWF; 10 μg/mL) in the presence of botrocetin (5 μg/mL) and with fibrinogen (100 μg/mL; HYPHEN BioMed SAS) overnight at 4°C. Then washed platelets (107 platelets/mL; 150 μL) were allowed to adhere at room temperature. After 30 minutes, adherent platelets were fixed with 4% paraformaldehyde in 0.1 M pipеразин-Ν,Ν’-бис(2-этилентиахионида), 2 M glycerol, 1 mM EGTA, and 1 mM MgCl2, pH 6.9, for 15 minutes and then permeabilized in the same buffer containing 0.2% Triton X-100 for 5 minutes. Platelets were stained with Alexa Fluor488-phallolidin and Alexa Fluor594-labeled DNase I (Molecular Probes) and then visualized using an epifluorescence microscope (Eclipse 600; Nikon France). Cell surfaces and F- and G-actin contents were determined using ImageJ version 1.42k (rsb.info.nih.gov/ij).

Targeted exon sequencing (vs-70 Mb)

Library preparation, capture, sequencing, and variant detection were performed by IntegraGen S.A. (Evry, France). Genomic DNA was extracted with the DNA purification kit (Qiagen), captured using Agilent in-solution enrichment methodology with their biotinylated oligonucleotides probes library (Human All Exon v5-70 Mb; Agilent), followed by paired-end 75-base massively parallel sequencing on Illumina HiSEQ 2000. For detailed explanations of the process, see Ginie et al.6 Sequence capture, enrichment, and elution were performed according to the manufacturer’s instructions and protocols (SureSelect; Agilent). Briefly, 3 μg of each genomic DNA was fragmented by sonication and purified to yield fragments of 150 to 200 bp. Paired-end adaptor oligonucleotides from Illumina were ligated on repaired, A-tailed DNA fragments and then purified and enriched by 4 to 6 polymerase chain reaction (PCR) cycles. Five hundred nanograms of these purified Libraries was hybridized to the SureSelect oligo probe capture library for 24 hours. After hybridization, washing, and elution, the eluted fraction was PCR-amplified with 10 to 12 cycles, purified, and quantified by quantitative PCR to obtain sufficient DNA template for downstream applications. Each eluted-enriched DNA sample was then sequenced on an Illumina HiSEQ 2000 as paired-end 75-base reads. Image analysis and base calling were performed using Illumina Real Time Analysis Pipeline version 1.12 with default parameters.

The bioinformatics analysis of sequencing data was based on the Illumina pipeline (CASAva 1.8.2). CASAVa performs alignment of the reads to a reference genome (hg19) with the alignment algorithm ELAND2, and then calls the SNPs based on the allele calls and read depth and detects variants. Only the positions included in the oligonucleotide probe coordinates ≥20 bp were conserved. Genetic variation annotation was performed using the IntegraGen in-house pipeline, which consists of gene annotation (RefSeq), detection of known polymorphisms (dbSNP 132; 1000Genome), and mutation characterization (exonic, intronic, silent, or nonsense).

For each position, the exonic frequencies (homo and heterozygous) were determined from the IntegraGen exome database and the exome results provided by HapMap.

The PRKACG mutation was validated by Sanger sequencing using forward (5’-ACC GCC CAT GG GAC ACC C-3’) and reverse (5’-GAAAC CAC AGGG CCC A-3’ primers).

In vitro MK differentiation

Patient or control CD34+ cells were grown in serum-free medium as previously reported.8 The medium was supplemented with 10 ng/mL thrombopoietin (TPO) (Kirin Brewery) and 25 ng/mL stem cell factor (SCF; Biovitrum AB).

Flow cytometry analysis

MKs were stained with directly coupled monoclonal antibodies anti–CD41-phycoerythrin and anti–CD42a-allophycocyanin (BD Biosciences) for 30 minutes at 4°C. Platelet surface staining of CD41a, CD42a, and CD62P was performed with the PLT Gp/Receptors kit (Bectyx) at room temperature before and after activation by the thrombin receptor agonist peptide (25 μM). For western blot analysis, MKs were sorted according to CD41 and CD42 expression using an Influx flow cytometer equipped with 5 lasers (BD Biosciences).

Ploidy analysis

At day 10 of culture, Hoechst 33342 (10 μg/mL; Sigma-Aldrich) was added in the medium of cultured MKs for 2 hours at 37°C. Cells were then stained with directly coupled monoclonal antibodies: anti–CD41-phycoerythrin and anti–CD42a-allophycocyanin (BD Biosciences) for 30 minutes at 4°C.9 Ploidy was measured in the CD41+CD42+ cell population by means of an Influx flow cytometer (BD Biosciences) and calculated as previously described.7

Quantification of proplatelets bearing MKs

To evaluate the percentage of MKs forming proplatelets (PPTs) in liquid medium, CD41+ cells were sorted at day 6 of culture and plated in 96-well plates at a concentration of 2000 cells per well in serum-free medium in the presence of TPO (10 ng/mL). MKs displaying PPTs were quantified between
and cell transduction were performed, as previously described.9 Control CD34+ cells (10^6/mL) were prestimulated for 24 hours with TPO, interleukin-3, SCF, and Fms-like tyrosin kinase 3-ligand and transduced with concentrated lentiviral (wild-type or mutated PRKACG) particles for 12 hours at a multiplicity of infection of 10, followed by a second transduction. Cells were then cultured in the presence of TPO and SCF alone. CD41− GFP− cells were sorted by flow cytometry (FACS Vantage; BD Biosciences) 6 days after transduction. The CD41− GFP− cells were further assessed for the ability to form PPTs or for the diameter of PPT-like structures by immunofluorescence assay.

Western blot assays

MKs were sorted by CD41 and CD42 expression at day 12 of culture, pelleted, and sonicated in 2× Laemli buffer (100 mM Tris-HCl, pH 6.8, 0.05% bromophenol blue, 4% sodium dodecyl sulfate [SDS], and 20% glycerol) supplemented with 100 mM dithiothreitol. Washed unstimulated platelets were lysed in SDS denaturing buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 mM phenylarsine oxide, 1% SDS, 5 μg/mL leupeptin, and 10 μg/mL aprotinin, pH 7.4). The proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The membranes were incubated with various primary antibodies: affinity-purified rabbit anti–GPIb/β-phospho-Ser166 and total rabbit anti-GP Ib antibody both previously described,9 mouse anti–β-actin and anti-HSC70 antibodies (Sigma-Aldrich), mouse anti-PRKACG antibody (Abcam), and rabbit anti-Filamin A antibody (Cell Signaling), followed by horseradish peroxidase-linked secondary antibodies. Protein blots were analyzed using Image Quant LAS 4000 (GE Healthcare), and protein expressions were quantified using ImageQuant TL 8.1 software.

Electron microscopy

Blood (200 μL) was centrifuged at 2100g for 10 minutes in Eppendorf microtubes. Plasma was removed, and the pellet was fixed with 1.5% glutaraldehyde (Fluka Chemie) in 0.1 M phosphate-buffered saline, pH 7.2, for 1 hour at room temperature. The platelet disk was cut into small slices and washed 3 times in 1× phosphate-buffered saline. For morphological examination, fixed platelets were postfixed in 1% osmic acid, dehydrated in ethanol, and embedded in Epon by standard methods. Sections were observed with a Technai 12 transmission electron microscope (FEI).

Statistical analyses

Data are presented as means ± standard deviation (SD) or ± standard error of the mean (SEM) as indicated. Statistical significance was determined by a 2-tailed Mann-Whitney test or unpaired Student t test with Welch’s correction. P < .05 was considered statistically significant.

Results

Family description

A severe thrombocytopenia (5 × 10^9/L) was initially identified after a bleeding episode in a 21-year-old woman of West Indian

**Table 1. Clinical characteristics of PRKACG-related thrombocytopenia patients**

<table>
<thead>
<tr>
<th>Family member</th>
<th>Age at diagnosis (years)</th>
<th>Current age (years)</th>
<th>Platelet count (×10^9/L)</th>
<th>MPV (fL)</th>
<th>Hemoglobin (g/dL)</th>
<th>Leukocytes (×10^9/L)</th>
<th>Bleeding score</th>
<th>TPO level (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>44</td>
<td></td>
<td>210</td>
<td>10.3</td>
<td>11</td>
<td>4.5</td>
<td>NB</td>
<td>35</td>
</tr>
<tr>
<td>II-1</td>
<td>4</td>
<td>23</td>
<td>5</td>
<td>ND</td>
<td>9</td>
<td>5</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>II-2</td>
<td>2</td>
<td>26</td>
<td>8</td>
<td>ND</td>
<td>12.3</td>
<td>5.6</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>III-1</td>
<td>21</td>
<td>229</td>
<td>10.8</td>
<td>12.9</td>
<td>7.1</td>
<td>NB</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>III-2</td>
<td>3</td>
<td>276</td>
<td>10.4</td>
<td>10.4</td>
<td>8</td>
<td>NB</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

Normal TPO level, <30 ng/L; MPV, mean platelet volume; NB, no bleeding; ND, not done.
(proband II-1) at the age of 4 years. She presented with no syndromic features but many bleeding episodes during infancy, including epistaxis, spontaneous hematomas, menorrhagias inducing anemia, and, notably, 3 consecutive bleeding ruptures of ovarian cysts involving life prognosis and requiring platelet and blood cell transfusion. The World Health Organization bleeding score is 4 in this patient.
Genealogical studies (Figure 1) showed that her brother (II-2) also exhibited severe thrombocytopenia ($8 \times 10^3$ platelets/L; Table 1) and life-long moderate bleeding, epistaxis, and cutaneous hematomas (World Health Organization bleeding score of 3). Her mother (I-1) had a normal platelet count. Data from her father (I-2) were not available because he died prior to this study. However, his son (II-4) from his second wife (I-3) did not display any platelet defects.

### Platelet and MK morphology

May-Grünwald-Giemsa staining of peripheral blood smears of patients II-1 and II-2 (Figure 2A) showed 90% giant and macrocytic platelets. This was confirmed by electron microscopy. The diameter of platelets was 4.86 and 4.98 μm for II-1 and II-2, respectively, compared with 2.84 and 2.97 μm for control and I-1 platelets, respectively (Figure 2B-C). The morphology of the patient’s bone marrow cells revealed the presence of MK clusters, which is absent in normal bone marrow (Figure 2D). An in vitro study of patient MKs derived from peripheral blood CD34+ cells in the presence of TPO and SCF revealed no differences in percentage of mature CD41+CD42+ cells and in the ploidy level between patients II-1 and II-2 and a healthy donor (Figure 2E). Similar results were obtained for family members I-1 and III-1 (data not shown). Altogether, these results suggest no defect in MK differentiation and ploidization.
Patient platelets exhibit defective activation

Because the patients exhibit a bleeding score of 3 to 4, suggesting a profound defect in platelet function, we first analyzed the effects of stimulating platelets. Quantification of integrin αIIbβ3 and GPIb/IX/V by flow cytometry showed that their levels in the unstimulated patient platelets was 2.7- and 2-fold higher than in control platelets, respectively (Figure 3A-B), consistent with their large size. After stimulation with protease-activated receptor 4-activating peptide (25 μM), GPIb/IX/V underwent poor activation-dependent internalization (18% of the level of resting platelets) in patient platelets compared with control platelets (44.2% of the level of resting platelets; Figure 3A). Moreover, although surface expression of integrin αIIbβ3 in control platelets, as expected, was upregulated (193% compared with unstimulated control platelets), no change in αIIbβ3 surface expression was observed for patient platelets after stimulation (Figure 3B). Finally, no surface expression of P-selectin, a marker of granule mobilization, on activation was observed in patient platelets (Figure 3C). Altogether, these results are consistent with patient platelets being unable to undergo activation.

Consistent with altered activation, Ca2+ mobilization from internal stores and Ca2+ influx elicited by added extracellular Ca2+ were much lower in patient platelets upon stimulation with thrombin (50 and 100 μU) than in control platelets (Figure 3D).

Finally, we analyzed platelet spreading with VWF (10 μg/mL) and fibrinogen (100 μg/mL) and quantified actin polymerization by measuring the F-actin/G-actin ratio. Immunofluorescence imaging showed that patient platelets were heterogeneous in morphology (Figure 3E). The majority of the platelets were giant, whereas some of them were of normal size and aspect. Moreover, patient platelets exhibited ruffles, which were absent from the control platelets. Quantification showed that the extent of patient platelet spreading was 2.2- and 2.5-fold that of control platelets with VWF and fibrinogen, respectively (Figure 3F). This larger size is the likely consequence of the larger diameter of the patient platelets vs control platelets. In contrast, a low F-actin/G-actin ratio of patient platelets was observed with VWF (44% of the control), whereas the ratios were comparable with fibrinogen (Figure 3G) between control and patient platelets.

Altogether, these results show an unexpected defect in activation and in cytoskeleton reorganization of patient platelets.

Homozygous PRKACG and GNE mutations are present in affected family members

The absence of genetic defects in GPlbx, GPlbβ, and GPIX (expressed as a GPIb-IX complex) excluded Bernard-Soulier syndrome (BSS), and the absence of neutrophil inclusions excluded an MYH9-related disease. Therefore, to identify a genetic factor leading to this new form of macrothrombocytopenia, we performed whole-exome sequencing of family members I-1, II-1, II-2, II-3, and III-1. A total of 57 447 single nucleotide variations (SNVs) and 6781 insertion-deletions (indels) were detected in I-1; 58 351 SNVs and 6968 indels in II-1; 57 341 SNVs and 6832 indels in II-2; 58 129 SNVs and 7077 indels in II-3; and 57 863 SNVs and 7012 indels in III-1. After excluding variants present in public databases at a frequency of >1%, by keeping only nonsynonymous mutations and by imposing an autosomal recessive mode of transmission for the mutation (I-1 and II-3, homozygous for the mutation; II-1 and II-2, homozygous for the mutation; II-3, homozygous wild type due to the consanguinity between I-1 and I-2 pedigrees and the fact that pedigrees II-4 and III-1 have a normal platelet count), only 2 variations remained. Both are localized on chromosome 9.

The first one, c.1675G>A, is in the gene GNE and corresponds to the substitution of the amino acid glycine for arginine at position 559 (p.559G>R-NM_005476). The second mutation, c.222C>G, was found in the PRKACG gene and substitutes isoleucine for methionine at position 74 (p.74I>M-NM_002732; Figure 4). None of the mutations were found in the SNPdb database, indicating they are not polymorphisms. Furthermore, when analyzed using PolyPhen2, both the GNE p.559G>R mutation and the PRKACG p.74I>M mutation were predicted to be damaging with high probability. Additionally, both residues are well conserved through evolution, further suggesting a strong impact for the mutations. GNE encodes a bifunctional enzyme that initiates and regulates the biosynthesis of N-acetylneuraminic acid, and PRKACG encodes the γ isofrom of the catalytic subunit (Cγ) of cAMP-dependent PKA. Among PKA substrates, FLNa and GPIbβ are present in MKs and platelets and are potential candidates involved in thrombocytopenia. Indeed, genetic alterations of FLNa and GPIbβ were found to be associated with macrothrombocytopenia, whereas GNE mutations result in myopathy or sialuria without thrombocytopenia. Because neither myopathy nor sialuria was detected in the patients, we further focused on PRKACG.

PRKACG homozygous mutation leads to a functional defect of PKA

To ascertain the role of PRKACG mutation in the induction of this new autosomal recessive macrothrombocytopenia with giant platelets, we first investigated mutant protein function. First, we found that the PRKACG mutation did not lead to the degradation of PRKACG in MKs or platelets (supplemental Figure 1). Then, the phosphorylation status of GPIbβ at Ser166 in patient MKs was investigated using CD34+ progenitors cultured in the presence of TPO and SCF and CD41+CD42+ MKs for 12 days. Quantification showed no difference in P-Ser166–GPIbβ levels between homozygous patients and controls in mature MKs (Figure 5A). Moreover, phosphorylation of GPIbβ at Ser166 in homozygous patient platelets was comparable to control platelets (Figure 5B). These results suggest that...
PRKACG is not responsible for GPIbβ phosphorylation in mature MKs and platelets. Finally, because the phosphorylation of FLNa by PKA at Ser2152 has been suggested to protect FLNa from proteolysis,15,16 we examined the level of total FLNa in MKs and platelets. FLNa was almost completely absent from mature MKs (harvested at day 12 of culture) (Figure 5C) and from platelets (Figure 5D) of homozygous patients compared with controls, strongly suggesting that PRKACG is required for FLNa phosphorylation.

The phosphorylation of many substrate proteins by PKA depends on cAMP. PKA regulates the concentration of cAMP as part of a feedback mechanism.17 First, PKA can induce degradation of cAMP through activation of phosphodiesterases, which catalyze the conversion of cAMP into AMP. Second, PKA can inhibit cAMP synthesis by inhibiting adenylyl cyclase, which catalyzes the conversion of ATP into cAMP. Hence, we hypothesized that the p.74I→M PRKACG mutation alters PKA activity, thereby leading to a high intracellular level of cAMP. Therefore, we measured the cAMP levels in platelets isolated from one external (C1) and one internal control (II-3) and from 2 patients homozygous (II-1 and II-2) and 1 heterozygous for the PRKACG p.74I→M mutation (III-1). Error bars represent mean ± SD of triplicate. Experiments were performed 2 times with similar results. *P < .05, 2-tailed Mann-Whitney test.

PPT formation is defective in patient MKs and is rescued by wild-type PRKACG overexpression

Finally, because the thrombocytopenia was not associated with defective MK differentiation or ploidization (Figure 2D-E), we investigated the ability of patient MKs to produce PPTs. A 2.5-fold lower percentage of PPT-bearing MKs (CD41+CD42+) was evidenced in patients homozygous for the mutation (II-1 and II-2) compared with controls or with patients heterozygous for the mutation (I-1 and III-1; Figure 6A). To confirm the implication of the homozygous PRKACG p.74I→M mutation in the PPT formation defect, we cloned the wild-type PRKACG cDNA into a lentiviral vector and transduced CD34+ cells isolated from peripheral blood of homozygous patients II-1 and II-2. Overexpression of wild-type
PRKACG significantly increased PPT formation by MKs of both patients II-1 and II-2 (Figure 6B). The mutant PRKACG cDNA was used in parallel as a negative control. No rescue in PPT formation was observed in the MKs of patients II-1 and II-2, which already express endogenous mutant PRKACG. Moreover, after overexpression of wild-type PRKACG in patient MKs, the average diameter of platelet-like structures occurring along the PPTs decreased from 3.67 to 1.68 μm for patient II-1 and from 4.17 to 2.01 μm for patient II-2 (Figure 6C-E). This result strongly suggests that the defect in PKA is responsible for the thrombocytopenia.

Discussion

Here we report a new autosomal recessive macrothrombocytopenia characterized by a severe thrombocytopenia (5-8 × 10^9 PLT/L) and a severe thrombopathy with a bleeding score of 3 to 4. Exome sequencing revealed homozygous mutations in the coding exons of 2 genes: GNE and PRKACG. The corresponding GNE p.559G>R and PRKACG p.74I>M missense mutations were predicted by PolyPhen2 to be damaging with high probability, at positions with well-conserved residues, and not corresponding to polymorphisms. GNE is predominantly expressed in the liver and placenta. GNE mutations can result in 2 human disorders: myopathy with mutations in epimerase and/or kinase domains or sialuria linked to the mutations in the epimerase domain.13,14 The GNE p.559G>R mutation located within the kinase domain was reported in 1 patient with myopathy in association with the V572L mutation on the other allele, which is the most frequent mutation associated with this disorder.18 No thrombocytopenia was ever reported in patients with myopathy and, conversely, the patients described here have no signs of myopathy, suggesting that the GNE p.559G>R mutation induces no major functional defect. Thus, the PKA mutation appears more relevant to explain this thrombocytopenia. PKA is composed of
2 catalytic subunits and 2 regulatory subunits. After cAMP binding to the regulatory subunits, the catalytic subunits dissociate and phosphorylate their substrates. The 3 catalytic subunit isoforms, Ca, Cb, and Cγ, are present in platelets, with a twofold lower level for Cγ compared with Ca and Cb. Among the major platelet PKA substrates, 2 groups were identified. The first group includes signaling regulators (Rap1B, Rap1GAP2, Go13, IP3-R, TRPC6, and GP Ibβ), whereas the second includes actin-binding proteins (VASP, LASP, HSP27, FLNa, and caldesmon). We focused our attention on 2 PKA substrates: FLNa and GP Ibβ. Indeed, genetic alterations of these substrates led to different forms of macrothrombocytopenia. Mutations in the FLNa gene localized on chromosome X result in a moderate thrombocytopenia with a mix of normal and giant platelets and with a defect in PPT formation. We demonstrated here that the level of total FLNa in PKA patient platelets was drastically low. This could be a consequence of a decrease in FL Na phosphorylation at Ser2152 in platelets and MKs due to the defect in PKA activity. This nonprotection of FLNa from proteolysis could then lead to thrombocytopenia. Indeed, we previously showed in patients with mutated FL Na that low levels of remaining FLNa in platelets (30%) are correlated with abnormal fragmentation of the cytoplasm, leading to a defect in PPT formation.

In platelets, GP Ib-IX-V, the major receptor for VWF, is constitutively associated with FLNa. This FLNa-GP Ibα interaction is required for adhesion of platelets onto VWF at high shear conditions, probably via dimerization and/or links with the actin networks, and seems to regulate the mechanical stability of the platelet plasma membrane. In the patient homozygous for the PRKACG p.741le>Met mutation, a decrease in F-actin polymerization was observed after platelet spreading on the VWF matrix. This defect is the likely consequence of the absence of stabilization of actin networks by the remaining FLNAs.

GP Ib-IX-V is another PKA substrate, essential for platelet activation and formation. Indeed, mono-allelic mutations in GP IBβ are at the origin of autosomal dominant macrothrombocytopenia in BSS characterized by the presence of large platelets. In platelets, PKA mediates GP Ibβ phosphorylation at Ser166, which leads to the negative regulation of VWF binding to GP Ib-IX-V. Moreover, phosphorylation of GP Ibβ inhibits actin polymerization during platelet activation through reorganization of the GPIb-IX–associated membrane skeleton. In platelets from the patient homozygous for the PRKACG p.741le>Met mutation, GP Ibβ phosphorylation was normal; thus, the defect in actin polymerization is not the result of altered GP Ibβ but more likely of the degradation of FLNa.

Is the markedly altered activation of platelets, as evidenced by impaired Ca2+ mobilization, no αIIBβ3 and P-selectin externalization, and poor GP Ib internalization, consistent with PKA inactivation? An attractive hypothesis is that increased intracellular cAMP, observed in patient platelets and a long-time recognized physiological negative regulator of platelet responses, alters platelet functions. However, another hypothesis, not exclusive from the first one, is that the defect in FLNa alters actin cytoskeletal reorganization on platelet stimulation, inducing defective externalization or internalization of receptors or even Ca2+ translocation. Further experiments are required to test these hypotheses.

Acknowledgments

The authors thank the patients and their families for participation in this study, Genethon (Evry, France) for the simprRLL-PGK-GFP lentivirus vector, and the Laboratoire Français de Fractionnement et Biotechnologies (LFB, Courtaboeuf, France) for human purified von Willebrand factor. This work was supported by French grants from the Agence Nationale de la Recherche (Jeunes Chercheurs) (to H.R.) and the Ligue Nationale Contre le Cancer (équipe labellisée 2013) (to H.R.). R.F., N.D., and W.V. are recipients of a research fellowship from Assistance Publique-Hôpitaux de Paris-Institut National de la Santé et de la Recherche Médicale (R.F.), Centre Hospitalier Universitaire Bordeaux (N.D.), and Institut Gustave Roussy-Institut National de la Santé et de la Recherche Médicale (W.V.).

Authorship

Contribution: V.T.M., M.H., E.B., S.S., G.P., and P.R. performed experiments and analyzed data; Z.E. and S.B. performed experiments; A.A. performed clinical and biological follow-up of patients; R.A., F.L., and W.V. discussed results and wrote the paper; J.-P.R. designed the work, performed and supervised experiments, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Hana Raslova, INSERM, UMR1009, Gustave Roussy, 114 rue Edouard Vaillant, 94805 Villejuif Cedex, France; e-mail: hraslova@igr.fr.

References

5. Gries A, Melnikov A, Maguire J, et al. Solution hybrid selection with ultra-long oligonucleotides...


A new form of macrothrombocytopenia induced by a germ-line mutation in the PRKACG gene

Vladimir T. Manchev, Morgane Hilpert, Eliane Berrou, Ziane Elaib, Achille Aouba, Siham Boukour, Sylvie Souquere, Gerard Pierron, Philippe Rameau, Robert Andrews, François Lanza, Regis Bobe, William Vainchenker, Jean-Philippe Rosa, Marijke Bryckaert, Najet Debili, Remi Favier and Hana Raslova