Heterogeneity of Platelet Functional Alterations in Patients With Filamin A Mutations

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Objective—We examined platelet functions in 4 unrelated patients with filaminopathy A caused by dominant mutations of the X-linked filamin A (FLNA) gene.

Methods and Results—Patients P1, P2, and P4 exhibited periventricular nodular heterotopia, heterozygosity for truncating FLNA mutations, and thrombocytopenia (except P2). P3 exhibited isolated thrombocytopenia and heterozygosity for a p.Glu1803Lys FLNA mutation. Truncated FLNa was undetectable by Western blotting of P1, P2, and P4 platelets, but full-length FLNa was detected at 37%, 82%, and 57% of control, respectively. P3 FLNa (p.Glu1803Lys and full-length) was assessed at 79%. All patients exhibited a platelet subpopulation negative for FLNa. Platelet aggregation, secretion, glycoprotein VI signaling, and thrombus growth on collagen were decreased for P1, P3, and P4, but normal for P2. For the 2 patients analyzed (P1 and P4), spreading was enhanced and, more markedly, in FLNa-negative platelets, suggesting that FLNa negatively regulates cytoskeleton reorganization. Platelet adhesion to von Willebrand factor under flow correlated with platelet full-length FLNa content: markedly reduced for P1 and P4 and unchanged for P2. Interestingly, von Willebrand factor flow adhesion was increased for P3, consistent with a gain-of-function effect enhancing glycoprotein Ib-IX-V/von Willebrand factor interaction. These results are consistent with a positive role for FLNa in platelet adhesion under high shear.

Conclusion—FLNA mutation heterogeneity correlates with different platelet functional impacts and points to opposite regulatory roles of FLNa in spreading and flow adhesion under shear. (Arterioscler Thromb Vasc Biol. 2013;33: e11-e18.)

Key Words: adhesive functions, filamin A, glycoprotein VI, platelets, signal transduction

Filamins (FLN) are large dimeric actin-binding proteins that stabilize actin filament networks with which they connect to the cellular membrane.1 FLN also bind to a large number of signaling proteins (>70).2 The FLN family consists of 3 dimeric proteins (FLNa, FLNb, and FLNc) produced by distinct genes. FLNa, the most abundant isoform, is encoded by FLNA located on chromosome Xq28 and is a member of the X-linked filamin A (FLNA) gene.

Filamin A mutations are associated with a wide spectrum of rare developmental diseases and cause various malformations of brain, heart, and muscle.3 The most frequent phenotype is periventricular nodular heterotopia (FLNA-PVNH), which can be associated with other features, including thrombocytopenia, patent ductus arteriosus, and Ehlers-Danlos syndrome. Skeletal dysplasia, including the oto-palato-digital syndrome spectrum disorders and terminal osseous dysplasia, familial cardiac valvular dystrophy, and congenital intestinal pseudo-obstruction, has also been described.4,5,6,7

Platelets express predominantly FLNa. FlnaAloxP GATA1-Cre mice that exclusively lack FLNa in platelets are characterized by a macrothrombocytopenia and increased tail bleeding time.5 A thrombopathy was found to alter α-granule secretion, integrin αIIbβ3 activation, and signaling through the collagen receptor glycoprotein VI (GPVI) and the C-type lectin-like receptor 2. In human platelets, FLNa was shown to interact with glycoprotein Ibα (GPIbα) through the Ig-like repeat domain of GPⅠbα.
the principal adhesion receptor of von Willebrand factor (VWF). The association of FLNa with GP Ib requires amino acids 563–571 localized in the cytoplasmic tail of GP Ib.\textsuperscript{10} Using transgenic mice expressing human GP Ib\textalpha mutated at p.Phe568 and p.Try570, a recent report showed that FLNa plays an important role in platelet adhesion and plasma membrane stability under pathological shear rates.\textsuperscript{11} Finally, the coordinated expression of GP Ib\textalpha and FLNa seems to be essential for the production of normal-size platelets.\textsuperscript{12} Because hemorrhage and thrombocytopenia have been reported in patients with FLNa-PVNH,\textsuperscript{6,13} we have recently defined the platelet structural characteristics of 2 patients displaying this phenotype (P1 and P2) and of 1 patient (P3) exhibiting an isolated macrothrombocytopenia caused by a novel FLNa mutation. Anisocytosis with both giant and normal platelets was observed in all 3 patients, as well as an abnormal platelet production.\textsuperscript{14}

Filaminopathy A thus seems as a unique way to examine the role of FLNa in human platelet functions. We now report 4 patients exhibiting FLNa mutations and altered platelet functions. The first 3 patients are the same as in our first study, a fourth patient with FLNa-PVNH being added (P4). For the 3 patients in whom the FLNa mutation is predicted to be truncating, we show that platelets exhibit wild-type FLNa at variable levels and that truncated FLNa is undetectable. Abnormal responses to collagen, including aggregation, secretion, the GPVI signaling pathway, and thrombus formation under flow on collagen, were observed in 3 patients with thrombocytopenia. Similarly, spreading was increased especially for platelets without FLNa, whatever the matrix type. Finally, we found that altered adhesion and altered thrombus growth at low- and high-shear rates paralleled the levels of wild-type FLNa. We thus conclude that in patients with FLNa-truncated molecules, the platelet functional defects depend essentially on the levels of the remaining full-length FLNa. In addition, the mutation for the fourth patient appears as a gain-of-function mutation. Our study thus confirms a central role for FLNa in platelet-adhesive functions.

Materials and Methods

Patients and Platelet Preparation

Four female patients with FLNA mutations were enrolled in this study after informed consent, and blood samples were obtained in accordance with the Declaration of Helsinki. Ethical approval was obtained from INSERM N\textsuperscript{22} RBM 01-14 for the project Network on the inherited diseases of platelet function and platelet production. For P1, with PVNH and a low platelet count (80×10\textsuperscript{9} platelets/L), a heterozygous frameshift point mutation identified in exon 27 of FLNa associated with PVNH was detected.\textsuperscript{15} For P2 who is affected with PVNH–Ehlers-Danlos syndrome, an heterozygous intragenic deletion involving FLNa exons 31 to 48 was observed.\textsuperscript{16} Her platelet count was normal (238×10\textsuperscript{9} platelets/L). For P3, a heterozygous missense p.Glu1803Lys mutation affecting a highly conserved glutamic acid residue localized in Ig-like repeat 16 was detected\textsuperscript{16} and was associated with an isolated low platelet count (40–60×10\textsuperscript{9} platelets/L). P4 is a 12-year-old teenager affected with PVNH associated with a moderately low platelet count (110×10\textsuperscript{9} platelets/L). Gene dosage analysis using array comparative genomic hybridization revealed a heterozygous intragenic deletion involving FLNa exons 20 to 48 (Figure I in the online-only Data Supplement).\textsuperscript{16} All patients underwent analysis for the skewing of X inactivation in lymphocytes: none of them had skewed X inactivation (>70/30; Table I in the online-only Data Supplement).

Platelet Preparation

See Methods in the online-only Data Supplement.

Platelet Aggregation

Platelet aggregation of washed platelets was monitored by measuring light transmission through the stirred suspension of platelets using a Chronolog aggregometer (Coultronics, Margency, France). See Methods in the online-only Data Supplement.

Platelet Dense Granule Secretion

Dense granule secretion was quantified by measuring ATP release during platelet aggregation using a luminometer (Fluostar Optima; BMG Labtech). See Methods in the online-only Data Supplement.

Platelet Spreading

Spreading of platelets was performed as described in Methods in the online-only Data Supplement.

Immunoblotting

Immunoblotting was performed as described in Methods in the online-only Data Supplement.

Thrombus Formation Under Flow

Blood perfusion experiments were as described in Methods in the online-only Data Supplement.

Statistical Analyses

Results were analyzed using the Student t test or 1-way ANOVA followed by a least significant difference multiple comparisons as indicated.

Figure 1. A and B, Level of wild-type and mutated filamin A (FLNa) in platelets for all patients. This figure represents a Western blotting of FLNa using a polyclonal antibody specific for the C-terminal FLNa and a monoclonal antibody specific for the N-terminal FLNa. These results are representative of at least 3 independent experiments.
Results

Only full-length FLNa is detected in platelets of patients with truncating mutations (P1, P2, P4). We first quantified the level of full-length and mutated FLNa in platelets for all patients. Because the patients are heterozygous for FLNA mutations, their platelets may harbor both the full-length and the mutated forms of FLNa. Because P1, P2, and P4 mutations are putatively truncating for FLNa (Figure I in the online-only Data Supplement), a search for such truncated FLNa was initiated by Western blotting. No polypeptides of the sizes predicted for truncated FLNa were detected in P1 (162 kDa), P2 (184 kDa), or P4 (104 kDa) platelets (Figure 1A). In contrast, full-length 280 kDa FLNa was detected in all 3 patients, at different levels, compared with control: 37% for P1, 57% for P4, and 82% for P2 (Figure 1B). Polypeptides below normal FLNa are likely to correspond to degradation products of normal FLNa, because the same patterns are observed for all patients. For P3, who carries the nontruncating missense p.Glu1803Lys mutation, a 79% FLNa content was found in P3 platelets, but the relative ratio of mutated versus full-length FLNa is unknown. Finally, FLNa expression determined by flow cytometry showed that 10% to 20% of FLNa-negative platelets were found in patients P1, P2, and P3, as previously described.14

Collagen- and Convulxin-Induced Platelet Aggregation and Platelet Secretion Are Decreased in Patients With FLNA Mutations

Because the signaling pathway of GPVI was shown to be impaired in FLNa-deficient mouse platelets,8 we next investigated platelet aggregation and secretion induced by type I collagen and convulxin (Cvx) in all patients. Platelet aggregation induced by various concentrations of type I collagen (0.25–2 μg/mL) or Cvx (400 or 800 pmol/L) during 3 minutes. Aggregations of P1 (A), P2 (B), P3 (C), and P4 (D) were expressed as the percentage change in light transmission, with the value of the blank (buffer without platelets) set at 100%. Traces are representative of at least 2 experiments. Dense granule secretion of P1 (A), P2 (B), P3 (C), and P4 (D) was evaluated by assessment of ATP release after aggregation. Results are expressed as the amount of ATP released (pmol).
and P4, 57%), whereas normal aggregation/secretion for P2 (82%). P3 platelets, despite a near-normal FLNa content (79%), exhibited low aggregation/secretion, consistent with a dominant-negative effect of the p.Glu1803Lys mutation.

**Platelet Signaling Induced by Cvx Is Decreased in Patients P1 and P4 With Low FLNa Content or Patient P3 With p.Glu1803Lys FLNa**

We next characterized the signaling pathway of GPVI induced by Cvx (400–800 pmol/L) in unstirred platelets, that is, in the absence of integrin αIIbβ3 signaling. The expression of GPVI and FcγR chain being normal for all patients (Figure II in the online-only Data Supplement), we tested the tyrosine kinase Syk phosphorylation, previously shown to be dependent on FLNa.8 Cvx activation of platelets induced Syk phosphorylation (Syk-P) in nonstirring conditions (Figure 3). Quantification of Syk-P showed it was low in P1, P3, and P4 platelets (48%, 59%, and 40% of control, respectively) but was normal in P2 platelets (103%). The phosphorylation of LAT (LAT-P), a direct substrate of Syk and a key actor in phospholipase Cγ2 activation,17 was also low in P1 (49%), P3 (44%), and P4 platelets (52%), but not in P2 platelets (99%; Figure 3). The level of Syk-P or LAT-P parallels the level of wild-type FLNa in patients with FLNA deletions or nonsense mutation: low for P1 and P4 (37% and 57% wtFLNa) and normal for P2 (82%). The low Syk-P/LAT-P in P3 platelets is consistent with a dominant-negative effect of p.Glu1803Lys mutation.

**Platelet Aggregation Induced by Other Agonists**

We next examined in the 4 patients the effect of FLNA mutations on platelet aggregation and secretion induced by other agonists, such as thrombin (0.1–0.2 U/mL), proteinase-activated receptor 1-activating peptide (PAR1-AP) (5–10 μmol/L), and ADP (5–20 μmol/L). No significant difference between control and patients was observed in platelet aggregation and secretion, whatever the G protein–coupled receptor agonists (Figure III in the online-only Data Supplement), consistent with a specific defect in the GPVI-dependent activation pathway in these patients.

**Platelet Spreading on Collagen, VWF, and Fibrinogen**

FLNa being central in platelet spreading and cytoskeleton reorganization, we next analyzed the spreading of patients’ platelets on different matrices (collagen, VWF, and fibrinogen). Contrary to control platelets, the spreading of patients’ platelets over type I collagen (Figure 4A) was heterogeneous, with both large and small platelets present. The mean platelet area (versus control, 100%) was increased in P1, P3, and P4 reaching 196±25% (P<0.01), 167±12% (P<0.001), and 174±12% (P<0.001), respectively, but was unchanged in P2 (93±3%; Figure 4A). The absence of

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Figure 3. A–D. Platelet signaling induced by convulxin (Cvx). Washed platelets in suspension were activated by Cvx (400 or 800 pmol/L) for 3 minutes in the absence of stirring. Tyrosine phosphorylation of Syk (Syk-P) and LAT (LAT-P) was assessed by immunoblotting with an anti–Syk and anti–β-actin. These results are representative of at least 2 independent experiments.
increased platelet spreading for P2 is consistent with the high amount (82%) of residual wild-type FLNa. For P3, the increased spreading might reflect the mean platelet area 1.5× larger than normal control. Conversely, the increased spreading for P1 and P4 is the likely result of low amounts of FLNa (P1 and P4). The defect may be a direct functional consequence of altered (quantitatively or qualitatively) FLNa and GPVI (collagen-specific) signaling or may be the indirect consequence of comparatively higher adhesion of large versus small platelets.

To test these hypotheses, we examined platelet spreading on VWF and fibrinogen. On both matrices, platelet area versus control (100%) was increased for P1 and P4: 159±11% (P<0.001) and 181±11% (P<0.01) for fibrinogen and 133±9% (P<0.01) and 181±16% (P<0.01) for VWF (Figure 5A). In contrast, the spreading of P2 and P3 platelets over VWF and fibrinogen was comparable with control. For P3, the normal spreading over VWF or fibrinogen, despite an enhanced mean platelet area (x1.5), might reflect a lower capacity of spreading compared with control. The absence of increased spreading for P2, whether on VWF, fibrinogen, or collagen, remains consistent with its near-normal content in FLNa. The increased spreading for P1 and P4 platelets, whatever the matrix type, confirms the effect of low FLNa content.
We thus examined platelet spreading with regard to FLNa content, as determined by immunofluorescence with an anti-C-terminal FLNa antibody, specific for full-length FLNas and Alexa Fluor488-labeled phalloidin. P4 patient was examined. Truncated FLNa was not detected by double labeling with anti-N-terminal and anti-C-terminal FLNa antibodies. Interestingly, the surface area of FLNa-negative P4 platelets (50–60% of adhering platelets) was markedly increased on all matrix types: 201±14% compared with control (100%) on collagen, and 220±17% and 225±16% on VWF and fibrinogen, respectively (Figure 5B). In contrast, the surface area of FLNa-positive P4 platelets was not different from control on collagen, VWF, or fibrinogen. In parallel, the size of FLNa-negative or FLNa-positive P4 platelets was measured by fluorescence-activated cell sorter analysis. No significant difference was observed between these two populations (FLNa+: 133% of control; FLNa−: 142% of control; Figure 5C).

Altogether, these results suggest that absence of FLNa drastically augments spreading, consistent with FLNa exhibiting a negative regulation on cytoskeletal reorganization during spreading. In addition, the specific increase in spreading on collagen of FLNa-positive P3 platelets suggests that the GPVI/Syk pathway is more dependent on FLNa than the GPIb/VWF or the αIIbβ3/fibrinogen pathways.

**Thrombus Formation Under Flow**

Platelet cytoskeletal regulation is likely to play a role in thrombus formation.11–18 We thus investigated thrombus formation on type I collagen at 300 s⁻¹ and 1500 s⁻¹ (Figure 6A and 6B). For these experiments, platelet counts were normalized. After 5 minutes of perfusion at 300 s⁻¹, the area covered by P1, P3, and P4 platelets was lower than control (100%), reaching 54±10% (P<0.05), 45±2.5% (P<0.05), and 62±4.4% (P<0.001), respectively (Figure 6A). For P1 and P3, lower thrombus area correlated with defective platelet secretion as indicated by the absence of additive effect of the ADP or ATP scavenger apyrase on thrombus formation (Figure IV in the online-only Data Supplement). P4 was not tested. In contrast, the area covered by P2 was normal (115±5%).

Under the shear conditions of arterial flow (1500 s⁻¹), thrombus formation on collagen-coated surface was comparable with 300 s⁻¹ for all patients’ platelets (Figure 6B). Altogether, collagen-induced thrombus formation correlates with the defect in FLNa: altered for patients exhibiting a quantitative (P1 and P4) or qualitative (P3) defect in platelet FLNa and unaltered when near-normal level of FLNa is observed (P2).

Because of the known interaction of FLNas with GPIbα, we extended our study to the investigation of thrombus formation on a VWF matrix. Shear rates at 1500 s⁻¹ and 5000 s⁻¹ were tested to mimic normal and pathological arterial flows, respectively. Total GPIbα expression assessed by flow cytometry analysis was normal for P1, P2, and P4 (results not shown), but increased for P3.13 At 1500 s⁻¹, thrombus formation was similar for P1 and P2 compared with donors controls (115±10.0% and 115±2.3%), moderately decreased for P4 (75±9±3.8%; P<0.01), but was significantly increased for P3 (152±61.1%; P<0.01; Figure 6C). At 5000 s⁻¹, corresponding to a pathological shear rate, platelets from P1 and P4 covered only 29.2±3.4% (P<0.001) and 48.9±2.1 (P<0.001) of the VWF-coated surface covered by control platelets, whereas the covered surface was increased, up to 171.5±20.3% (P<0.01), for P3 and was normal for P2 (104±2.7%; Figure 6D).

Altogether, these results lead to the conclusion that (1) the status of thrombus formation, particularly at 5000 s⁻¹ parallels that of platelet wild-type FLNa: low for P1 and P4 and normal for P2; and (2) the increased thrombus formation for P3 may be the consequence of increased GPIbα exposure at the platelet surface. Another hypothesis, not exclusive from the first one, is that increased thrombus formation is the result of a gain-of-function mutation (pGlu1803Lys), possibly linked to the proximity of the interaction site between FLNa and the GPIbα cytoplasmic domain.

**Discussion**

Recent results obtained using transgenic mice support a major role for FLNa in platelet functions. However, because these results were obtained in mouse models, we wished to test the role of FLNa in a human platelet context by examining the impact on platelet functions of FLNA gene mutations responsible for filaminopathy A. The 4 patients examined in this report are women, heterozygous for the FLNA mutations, 3 of which predicted to lead to truncated FLNa polypeptides, because of premature termination induced by a frameshift point mutation (P1) or by a gene deletion (P2 and P4). A fourth mutation (P3), p.Glu1803Lys, is predicted not to affect FLNA length (Figure I and Table I in the online-only Data Supplement). Because patients are women and FLNA is located on the X chromosome, both wild-type and mutant alleles are expected to be coexpressed. Interestingly, no truncated peptide was detected in platelets. This may be consistent with a nonsense mRNA decay mechanism that may be enhanced by the nonrenewal of transcripts in anucleated
platelets. In addition, the long half-life of circulating platelets may accelerate the decay of truncated polypeptides that may have been translated in megakaryocytes (MK). Platelet proteins exhibit a degradation pattern (Figure 1). However, this seems to correspond to degraded normal FLNa since (1) seen with both N terminus–specific or C terminus–specific antibodies, whereas all truncations delete the C-terminal end of FLNa, (2) patterns are nonspecific, because observed with different mutations or even with some controls (see Figure 1 and P1, P4, and control), and (3) also visible in P3, whose mutation is nontruncating (p.Glu1803Lys).

A question raised by our observation is the varying levels of wild-type FLNa among patients. Because of X inactivation and X clonal expression in MK lineages, the FLNA gene in our female patients should undergo allelic exclusion. This would lead to 2 populations of platelets, each containing an FLNa originating either from the normal (FLNa+) or the defective allele (FLNa–), but not both. Although this seems to be the case for patient P4, where 50% to 60% of adhesion and spreading of platelets do not contain FLNa (FLNa–), only 20% of FLNa-negative platelets were observed for P1 or P2 platelets. This suggests that at least in those 2 patients, FLNa expression is skewed toward the normal allele, because the defective allele leads to less-efficient MK maturation or platelet production or to higher platelet clearance. Indeed, in mice, 95% of platelets isolated from FlnA−/− females contained FLNa. The reason why platelet numbers are normal for P2, with a total amount of FLNa near normal (82%), is that, in this patient, there is a higher expression of the unaffected FLNA allele compared with controls. Alternatively, platelet production from the mutant FLNA allele MK lineage is severely altered, but P2 platelet counts remain within the normal range because the MK lineage containing the wild-type FLNA allele happens to produce high platelet numbers. This is clearly not the case for P1, because total FLNa is low (37%) despite an 80% ratio of FLNa-containing platelets, as previously described; this indicates that in this patient, the expression and stability of normal FLNa are diminished compared with controls. These marked differences in FLNa expression levels are likely because of differential consequences of the mutations at the level of platelet production or clearance.

Platelets from P1, P3, and P4 patients exhibited altered aggregation and secretion depending on collagen receptors but not on G protein–coupled receptor (thrombin or ADP receptors). This defect on platelet secretion is the consequence of FLNA mutations and not of an abnormal dense granule storage pool (results not shown). Furthermore, thrombus formation on collagen that is dependent on secretion in conditions of low shear rates (Figure IV in the online-only Data Supplement) was also diminished. In these cases, the low level of full-length FLNa in P1 and P4 platelets may explain altered GPVI signaling, because FLNa acts as a signaling scaffold for GPVI through interaction with Syk. For P3, it is possible that p.Glu1803Lys, an Ig repeat 16 mutation, interferes with the Ig repeat 5 engaged in signaling of GPVI–collagen interaction. Our data are consistent with previous reports in mouse platelets shown in FlnA−/− mice that the presence of FLNa is essential for GPVI signaling. Of note, the limited platelet functional alterations (aggregation, secretion, thrombus formation) for P2 are consistent with the near-normal levels of full-length FLNa (82%).

Several lines of evidence have demonstrated that collagen-induced tyrosine phosphorylation of proteins, including immunoreceptor tyrosine-based activation motif (ITAM)-containing FcRγ chain and Syk tyrosine kinase, is essential for platelet activation. Using the model of FlnA-deficient mice, FLNa has been shown to contribute to Syk spatial distribution at the cytoplasmic surface of the plasma membrane. For all patients, the platelet functional defect is not a consequence of a defect in collagen receptors, because the levels of GPVI and FcRγ were normal. In contrast, the GPVI signaling pathway was affected in P1, P3, and P4 platelets, as confirmed by the drastic decrease in Cvx-induced phosphorylation of Syk and LAT. Our hypothesis is that the low level of full-length FLNa for P1 and P4 leads to low level of FLNa-associated Syk that, in turn, poorly recruits ITAM motifs of FcRγ. For P3, either the p.Glu1803Lys mutation or the abnormal localization of FLNa found for this patient in our previous study, or both, affects GPVI signaling.

Surprisingly, spreading of P1 and P4 platelets was increased on collagen, VWF, and fibrinogen. Platelet distribution of FLNa on collagen shows that the surface area was largely increased in FLNa-negative platelets for P4. Unexpectedly, FLNa-positive platelets also exhibited increased surface area, although to a lesser extent than FLNa-negative platelets, suggesting that the level of full-length FLNa in FLNa-positive platelets is probably lower than in control platelets. The other possibility is that there is functional interference between mutant and wild-type allele-bearing MK and platelets within the bone marrow or circulating blood. Furthermore, Syk has been reported to be critical for lamellipodia formation on various matrices, such as collagen, VWF, and fibrinogen. The impairment in Syk activation raises the question of lamellipodia formation in our patients’ platelets (P1, P3, and P4). In fact, full lamellipodia formation occurred in FLNa-negative platelets (results not shown), suggesting that lamellipodia formation (in addition to not requiring Flna) does not require full phosphorylation of Syk. The other hypothesis is that the engagement of Syk in spreading is different in mouse and human platelets, for example, through different signaling pathways. Finally, a normal phosphorylation of Syk cannot be completely excluded in conditions of platelet spreading (not explored in our conditions), which would suggest that an alternate Syk-P pathway bypasses FLNa in human (but not in mouse) platelets during spreading.

For P3, it is possible that the limited increase in spreading on collagen is only the consequence of the large size of platelets (×1.5). In turn, because spreading on VWF and fibrinogen is comparable with control, and thus not increased proportionally to size, this suggests that p.Glu1803Lys FLNa exhibits a stronger negative regulation than wild-type FLNa on spreading on VWF and fibrinogen. The differential effect between collagen and VWF or fibrinogen may point to a differential engagement of FLNa in these conditions. Next, thrombus formation on a VWF matrix in physiological and pathological conditions was examined. In contrast to a recent report showing that GP Ibα was degraded in platelets from FlnA−/− PF4-Cre mice, the cell surface expression of GP Ibα assessed by flow cytometry analysis was normal for P1, P2, and P4 (results not shown) and was increased for...
P3, suggesting that the level of remaining FLNa is sufficient to promote normal platelet surface expression of the GPIb complex. In agreement with the model of mice expressing human GP Ibα mutated in the FLNA interaction site, thrombus formation on VWF was normal at 1500 s⁻¹ for P1 and P2 platelets and only slightly decreased for P4 platelets. Thus, low FLNa content does not preclude normal adhesion in conditions of artherosclerotic shear. In contrast, the decrease in thrombus formation at 5000 s⁻¹ for patients with a low level of full-length FLNa (P1 and P4) confirms that a normal level of FLNa is essential for platelet adhesion under pathological shear rates. Surprisingly, for P3, thrombus formation on a VWF matrix was increased in both physiological and pathological conditions. This gain-of-function effect might be the consequence of increased GP Ibα expression (x2.2). The other possibility is that the mutation (Ig-like repeat 16) located between the site of FLNa–GP Ibα/VWF interaction (Ig-like repeat 17) and the calpain site affects the binding to GP Ibα. The less peripheral distribution of FLNa in large versus normal-size P3 platelets, suggesting that GP Ibα–Glu1803Lys–FLNa would be less available to an interaction with GP Ibα, may be compensated for by the likely excess of FLNa over GP Ibα, leaving enough peripheral molecules to interact with membrane GP Ibα.

This study clearly demonstrates that platelet functions are altered in patients with FLNA mutations. Abnormal response to collagen affecting aggregation, secretion, thrombus formation, and GPVI signaling was associated with a low level of full-length FLNa. Spreading on various matrix types was also abnormal, especially in platelets devoid of FLNa. Finally, thrombus growth at low and high shear rates was associated with the normal level of FLNa. In conclusion, FLNa is essential for human platelet adhesive functions.

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Disclosures

None.

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Supplemental Material

Equine type I collagen was obtained from Kordia (Leiden, The Netherlands). Human purified von Willebrand Factor (VWF) was a kind gift of the Laboratoire Français de Fractionnement et Biotechnologies (LFB, Courtaboeuf, France). Fibrinogen was obtained from HYPHEN BioMed SAS (Andresy, France). Convulxin and the monoclonal antibody directed against GPVI (3J24.2) were produced as previously described.1,2 The thrombin receptor-derived peptide (SFLLRN-NH2: PAR1-AP) was purchased from Bachem (Weil am Rhein, Germany). ADP was obtained from Kordia. D-Phe-Pro-Arg chlormethylketone dihydrochloride (PPACK) was from Calbiochem-VWR (Fontenay-sous-Bois, France). Thrombin, leupeptin, aprotinin, apyrase grade VII, prostaglandin E1 and rhodamine-6G were from Sigma-Aldrich (Saint-Quentin Fallavier, France). The monoclonal antibody directed against Syk polyclonal antibodies directed against the phosphorylated form of linker for activation of T cells (LAT) and against the Fc Receptor (FcR) γ-chain were purchased from Millipore (Billerica, MA). The monoclonal antibody directed against the phosphorylated forms of Syk, was obtained from Cell Signaling Technology (Danvers, MA). The polyclonal antibody specific for C-terminal FLNa was obtained from Abcam (Cambridge, UK). The monoclonal antibody specific for N-terminal FLNa was obtained from Millipore (Billerica, MA). Peroxydase-conjugated AffiniPure secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Supplemental Methods

Platelet preparation
Samples from patients and control random donors were always processed in parallel. The quantification of FLNa levels in controls showed that variability of FLNa content between random controls did not exceed 6%. Venous blood from patients and control was collected in 10% (vol/vol) ACD-A buffer (75 mM trisodium citrate, 44 mM citric acid, 136 mM glucose, pH 4) for experiments with washed platelets or in PPACK (80 µM) for flow experiments using whole blood. Platelet-rich plasma was obtained by centrifugation (100g for 15 minutes at 20°C) and platelets were isolated by differential centrifugations as previously described.3 The platelet pellet was resuspended in Tyrod’s buffer (5 mM HEPES, 137 mM NaCl, 2 mM KCl, 12 mM NaHCO3, 0.3 mM NaH2PO4, 1 mM MgCl2, 2 mM CaCl2 and 55 mM glucose, pH 7.4).

Platelet aggregation
Platelet aggregation of washed platelets was monitored by measuring light transmission through the stirred suspension of platelets (2.5x108 platelets/mL) during 3 minutes using a Chronolog aggregometer (Coultronics, Margency, France). Platelet aggregation was triggered by adding fibrillar equine type 1 collagen, convulxin (Cvx), thrombin, PAR1-AP or ADP as described in Results. Platelet aggregation was assessed as the percentage change of light transmission with respect to the blank (buffer without platelets) set at 100%.

Platelet dense granule secretion
Dense granule secretion was quantified by measuring ATP release during platelet aggregation. After 3 minutes, platelet aggregation was stopped by adding cold ethylenediaminetraacetic acid (EDTA) (16 mM) followed by centrifugation (12 000g, 1 minute). ATP release was quantified by an ATP determination kit using luciferase and its
substrate D-luciferin according to the manufacturer’s instructions. Light emission was assessed using a luminometer (Fluostar Optima; BMG Labtech). Dense granule secretion was expressed as pmoles of ATP released.

**Platelet spreading**
Glass coverslips were pre-coated with type I collagen (50 µg/mL), VWF (10 µg/mL) or fibrinogen (100 µg/mL) overnight at 4°C. Then washed platelets (10⁷ platelets/mL; 150 µL) from patients or control donors were allowed to adhere at room temperature. After 30 minutes, unbound platelets were removed and adherent platelets were fixed with 4% paraformaldehyde in cytoskeleton buffer (0.1 M PIPES, 2 M glycerol, 1 mM EGTA, 1 mM MgCl₂ pH 6.9) for 15 minutes, then permeabilized in the same buffer containing 0.2% Triton X-100 for 5 minutes. Platelets were stained with Alexa Fluor488-labeled phalloidin (1/500) and anti-C-terminal FLNa and then visualized under an epifluorescence microscope (Nikon, Eclipse 600). Cell surfaces were analyzed using the Image J software (rsb.info.nih.gov/ij).

**Immunoblotting**
Washed platelets (2.5x10⁸/mL; 300 µL) were stimulated with Cvx (800 pM) in the absence of stirring. After 3 minutes, the platelets were lysed in SDS denaturing buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 µM phenylarsine oxide, 1% SDS, 5 µg/mL leupeptin, 10 µg/mL aprotinin, pH 7.4). The proteins were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with various primary antibodies (see Results Section). Immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (Pierce, Rockford, IL). Images of the chemiluminescent signal were captured using G:BOX Chemi XT16 Image Systems and quantified using Gene Tools version 4.0.0.0 (Syngene, Cambridge, UK).

**Thrombus formation under flow**
Blood perfusion experiments were performed in a parallel plate perfusion chamber. PPACK (80 µM) anticoagulated blood from patients or controls was incubated with rhodamine 6G (10 µg/mL) for 5 minutes at 37°C, then perfused on glass coverslips pre-coated overnight at 4°C with fibrillar equine type I collagen (50 µg/mL) or human VWF (50 µg/mL) at various shear rates (300 s⁻¹ to 5000 s⁻¹) with a syringe pump (Fisher Scientific, Illkirch, France) as previously described. Thrombus formation was recorded with an inverted epifluorescence microscope (Nikon Eclipse TE2000-U) coupled to the Metamorph 7.0rl software (Universal Imaging Corporation) and was quantitated by assessment of the mean percentage of the total area covered by thrombi.

Supplemental Figures

Figure I: Results of FLNA mutation searches in Patient 4
Custom array-CGH analysis was performed. Both hybridization experiments with fluorochrome swapping show the same deletion (153531400-153590150, minimum size: 58.75 kb; 153530306-153592216, maximum size: 61.91 kb) including the 3’terminal part of FLNA from exon 20 to exon 48 (black arrow), the intergenic region and TKTL1 (exon 2 to 13). Genomic coordinates are indicated on the left (according to the human reference sequence GRCh37/hg19). FLNA is shown as a box on the left, and Log2 values of the ratio of fluorescence patient-reference are indicated above the plot.
Figure II: Quantification of GPVI and Fc Receptor (FCR) γ-chain in platelets
Western blotting with antibody against GPVI and Fc Receptor (FCR) γ-chain in platelets was assessed. These results are representative of at least three independent experiments.

Figure III: Platelet aggregation and secretion induced by other agonists.
Platelet aggregation and secretion were initiated by adding various concentrations of thrombin (0.1 or 0.2 U/mL), PAR1-AP (5 or 10 µM) or ADP (5 or 20 µM) during 3 minutes. Aggregations of P1, P2 and P4 were expressed as the percentage change in light transmission, with the value of the blank (buffer without platelets) set at 100%. Traces are representative of at least two experiments. Dense granule secretions of P1, P2 and P4 were evaluated by assessment of ATP release after aggregation. Results are expressed as the amount of the ATP released (pmoles).

![Graph showing platelet aggregation and secretion](image)

**Figure IV: Role of ADP on thrombus formation**

Apyrase (5 U/ml) was added to blood during 5 minutes and then perfused on collagen matrix (50 µg/mL) at 300 s⁻¹. After 5 minutes (300 s⁻¹) thrombi were observed under an epifluorescence microscope (original magnification x20). Total area covered by platelets was expressed as the mean ± SEM of three independent experiments. * p<0.05 (unpaired Student t test)

**Table S1: Clinical and functional parameters of the FLNa patients**

<table>
<thead>
<tr>
<th>Patients</th>
<th>FLNA Mutations</th>
<th>Putative FLNa protein</th>
<th>X-inactivation in lymphocytes</th>
<th>PNH</th>
<th>Platelet count x 10⁹/L</th>
<th>Aggregation Collagen/VCVx</th>
<th>Spreading VWF FG</th>
<th>Spreading collagen</th>
<th>VWF adhesion 5000s⁻¹</th>
<th>Remaining wt FLNa (%)</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td>Frame-shift c.4573_4612A&gt;G, p.Tyr1525X exon 27</td>
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<td>terminus exon 29 junction IgG14-15 182KD</td>
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<td>NL</td>
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PNH = Periventricular nodular heterotopia