The αIIb p.Leu841Met (Cab3^{a+}) polymorphism results in a new human platelet alloantigen involved in neonatal alloimmune thrombocytopenia

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BACKGROUND: Fetal-neonatal alloimmune thrombocytopenia (FNAIT) diagnosis relies on maternofetal incompatibility and alloantibody identification. Genotyping for rare platelet (PLT) polymorphisms allowed the identification of three families with suspected or confirmed maternofetal incompatibility for the *αIIb-c.2614C>A* mutation (Halle et al., Transfusion 2008;48:14-15).

STUDY DESIGN AND METHODS: A polymerase chain reaction–sequence-specific primers amplification assay was designed to genotype the *αIIb-c.2614C>A* mutation. HEK293 cells expressing αIIb-Leu841 or αIIb-Met841 αIIbβ3 forms were used to probe the reactivity of maternal sera from these families and to study the effects of the substitution on αIIbβ3 expression and functions.

RESULTS: Tested by flow cytometry (FCM), one serum sample specifically reacted with αIIb-Met841 but not with αIIb-Leu841 αIIbβ3. This specificity revealed the αIIb-Leu841 polymorphism as a new alloantigen named Cab3^{a+}. Cross-match testing using FCM also showed the Cab3^{a+} antigen to be expressed at the PLT surface. As for anti-human PLT alloantigen (HPA)-3a (or -3b) and anti-HPA-9bw, detection of anti-Cab3^{a+} alloantibodies appeared difficult and required whole PLT assays when classical monoclonal antibody–specific immobilization of PLT antigen test failed. In our FNAIT set, the immune response to Cab3^{a+} maternofetal incompatibility could induce severe thrombocytopenias and life-threatening hemorrhages. The p.Leu841Met substitution has limited effects, if any, on local αIIb structure, preserving both αIIbβ3 expression and functions.

CONCLUSION: The Cab3^{a+} polymorphism is a new rare alloantigen (allelic frequency <1%) carried by αIIb that might result in severe life-threatening thrombocytopenias. In Sub-Saharan African populations, higher Cab3^{a+} gene frequencies (up to 8.2%; Halle et al., Transfusion 2008;48:14-15) and homozygous people are observed.

Fetal-neonatal alloimmune thrombocytopenia (FNAIT) is characterized by fetal-neonatal platelet (PLT) destruction mediated by maternal alloantibodies specific for alloantigens inherited from the father.[1] The incidence of FNAIT has been shown to be 1 per 800 to 1000 live births and the most feared complications are intracranial hemorrhages leading to death or neurological sequelae.[2] Human PLT alloantigens (HPA) result from several gene polymorphisms (see http://www.ebi.ac.uk/ipd/hpa/ for an up-to-date list) but are mainly carried by the integrin αIIbβ3 (or glycoprotein [GP]IIb-IIIa), a fibrinogen (fg) receptor responsible for PLT aggregation and adhesion.[3,4] However, only few alloantigenic systems or antigens have been described on the αIIb subunit: HPA-3a/b, -9bw, -20bw, and Cab2^{a+}.[5-8] Halle and colleagues[9] described three new αIIb mutations identified in Sub-Saharan African populations after HPA-3 and -9 genotyping discrepancies. Among them the Cab3^{a+} (alias

ABBREVIATIONS: 3D = three-dimensional; 7-AAD = 7-aminoactinomycin; FCM = flow cytometry; fg = fibrinogen; FNAIT = fetal-neonatal alloimmune thrombocytopenia; GP = glycoprotein; HPA = human platelet alloantigen; MAIPA (or MAICA) = monoclonal antibody–specific immobilization of platelet (or cell) antigen; MD = molecular dynamics; NT = not transfected; PB(s) = protein block(s); SSP = sequence-specific primers amplification.

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Received for publication March 13, 2012; revision received May 7, 2012, and accepted May 7, 2012.

doi: 10.1111/j.1537-2995.2012.03762.x

TRANSFUSION 2013;53:554-563.

Ak) allele results from the mutation *c.2614C>A* that leads to the Leu841Met substitution. This mutation had initially been identified in a Beninese woman who presented an intrauterine fetal death. However, the maternal Cab3 serum failed to react with the PLTs from the father by the monoclonal antibody (MoAb)-specific immobilization of PLT antigen (MAIPA) technique, and the cause of the fetal death remained to be identified.

As a national reference laboratory for FNAIT diagnosis, we study approximately 300 families a year. When FNAIT diagnoses are inconclusive by studying common HPA systems (HPA-1, -2, -3, -5, and -15), genotyping is systematically performed for rare HPA polymorphisms and few other αIIbβ3 mutations identified in the laboratory in a context of FNAIT. We discovered two families with obligatory and one suspected Cab3 maternofetal incompatibility. Because crossmatch in MAIPA technique using paternal PLTs failed to detect anti-αIIβ3 alloantibodies, sera were tested against HEK293 cells expressing the $Cab3^{a+}$ form of αIIβ3 in flow cytometry (FCM). One of the three sera specifically reacted with the $Cab3^{a+}$ cells but not with the $Cab3^{a-}$, showing that the Cab3 polymorphism can define a new PLT alloantigen.

MATERIALS AND METHODS

Case reports

Family 1 (Cab3 alias Ak)

An immune thrombocytopenia was suspected to be responsible for an in utero death after 16 weeks of gestation. Initial serologic studies using the MAIPA technique failed to detect maternal antibodies to αIIβ1, GPIb-IX, and αIIbβ3 (GPIIb-IIIa) of the paternal and panel PLTs. Routine DNA genotyping in the HPA-1, -3, and -5 alloantigenic systems revealed a possible maternofetal incompatibility as the mother was HPA-5a/a and the father HPA-5a/b. As no anti-HPA-5b were detected, the FNAIT diagnosis was not confirmed. No incompatibility was observed in HPA-1 and -3 systems.

Family 2

A 33-year-old woman delivered a full-term female neonate with a severe thrombocytopenia at birth (4×10^9/L) associated with petechiae and hematomas. The child was otherwise healthy (Apgar score 10/10). She was transfused with 1 PLT unit that increased her PLT count to 111×10^9/L. On Days 4 and 5 she received intravenous immunoglobulins (IVIGs; 1 g/kg). Her PLT count decreased to 28×10^9/L on Day 10 and she received a new PLT transfusion. The PLT count had increased at 75×10^9/L and the outcome was favorable. Maternofetal incompatibility was diagnosed for HPA-1a, -3a, and -5a antigens, the mother and father being respectively homozygous for the alleles b and a in all three HPA systems. No antibodies directed to the alloantigens HPA-1a, -3a, and -5a were detected.

Family 3

A 30-year-old woman with gestational diabetes gave birth to a full-term male child without clinical signs but thrombocytopenia (39×10^9/L) was discovered on Day 4. He was treated with 1 g/kg IVIGs on Day 7. The PLT count was normalized 2 days later (173×10^9/L) and the outcome was normal. No maternofetal incompatibility was observed in the HPA-1, -3, and -5 systems.

Blood samples

Blood samples from parents and their neonates and volunteer blood donors were collected after informed consent was obtained. Venipuncture samples collected in 5 mmol/L ethylenediaminetetraacetate (EDTA) were referred to our laboratory. PLTs were separated by differential centrifugation and stored until use at 4°C in phosphate-buffered saline (PBS) containing 0.1% sodium azide.

Serology

PLT phenotyping and characterization of the maternal serum alloantibody were performed by using the MAIPA technique.[10] A similar technique using cultured cells (MoAb-specific immobilization of cell antigen [MAICA]) was also performed as previously described.[8]

MoAbs

The following MoAbs were used in this study: Gi9 (Immunotech, Marseille, France) to GPIa-IIa; GRP (from Dr Garrido, Granada, Spain) to GPIb-IX; XIIF9 (CRTS, Bordeaux, France), SZ21 (Immunotech, Marseille, France), and AP2 (complex dependent) to β3; SZ22 (Immunotech), M148 (TEBU, Le Perray en Yvelines, France), and PT25-2 (Takara, Otsu, Japan) to αIIb; Pl2-46 and Pl1-64 (Coger, Paris, France) to αIIb; and Gi5 (Coger) to αIIbβ3.

DNA extraction, sequencing, nucleotide, and amino acid numbering

DNA extraction and direct sequencing of polymerase chain reaction (PCR) products were performed as previously described.[11] For nucleotide numbering the A nucleotide of the ATG start was designated +1 (cDNA ITGA2B GenBank Accession Number *NM_000419.3*). For amino acid numbering, the first amino acid of the mature αIIb protein (NP_000410.2) was designated +1. The allelic variant *NM_000419.3:c.2614C>A* has been deposited on the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/; Accession Numbers ss490181500 or rs149468422).

PCR–sequence-specific primers amplification

The *c.2614C>A* mutation was genotyped by using PCR–sequence-specific primers amplification (PCR-SSP). Briefly, PCR was performed with 50 ng of DNA in 20 μL of a reaction mix containing 1× *Taq* buffer, 2 mmol/L $MgCl_2$, 200 mmol/L of each dNTP, and 0.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Courtaboeuf, France), 3% dimethyl sulfoxide, 4% cresol red, 12% glycerol, 1.2 mmol/L of the specific forward primer, either 5′p-CCCAGGTGGACTGGGGGC-3′OH for $Cab3^{a+}$ or 5′p-CCCAGGTGGACTGGGGGA-3′OH for $Cab3^{a-}$ alleles, 1.2 mmol/L of the common reverse primer 5′p-GGTGG GAGAAGAAGATAAAAACTAAC-3′OH, and 7 μmol/L of each of the internal positive control primers CRP-I 5′p-CCAGCCTCTCTCATGCTTTTGGCCAGACAG-3′OH and CRP-II 5′p-GGGTCGAGGACAGTTCCGTGTAGAAGTGGA-3′OH. PCR was performed using a MasterCycler *ep GradiantS* thermocycler (Eppendorf, Le Pecq, France). A single step at 95°C for 5 minutes to denature genomic DNA and activate the polymerase was followed by 40 cycles of denaturation (95°C, 30 sec), annealing (62°C, 30 sec), and elongation (72°C, 30 sec). A final elongation step (72°C, 7 min) ended the reaction. PCR products were analyzed under ultraviolet (UV) light in presence of GelRed (Interchim, Montluçon, France) after electrophoresis on a 2.5% agarose gel.

Site-directed mutagenesis

The mutation *c.2614C>A* corresponding to the p.Leu841Met substitution was introduced into wild-type αIIb cDNA (from P.J. Newman, Blood Research Institute, Blood Center of Southeastern Wisconsin, Milwaukee, WI) cloned in the eukaryotic expression vector pcDNA3-Geneticin by using an in vitro mutagenesis system (GeneEditor, Promega, Lyon, France) according to the manufacturer's instructions. The *c.2614C>A* mutation was introduced in wild type cDNA sequence by using the mutagenic oligonucleotides 5′p-GGCTGGGGATGGGCA TCCCCCAGTCCACCTTG-3′OH (the mutagenic position is underlined). The mutation was confirmed by direct sequencing.

Cell culture and transfection

HEK293 cells (American Type Culture Collection, Rockville, MD) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% antimycotic-antibiotic reagent (Invitrogen, Cergy-Pontoise, France) in a 5% CO_2 saturating humidity atmosphere. HEK293 cells were transiently transfected according to previously published procedures.[12] HEK293 cells were harvested by using the trypsin-EDTA method and washed three times in PBS buffer containing 1% (wt/vol) bovine serum albumin (PBS-BSA) before use.

FCM

Reactivity of maternal sera with HEK293 cells expressing αIIb-Leu841 or mutant αIIb-Met841 forms of αIIbβ3 or PLTs was tested by FCM. Eighty microliters of the washed HEK293 cells (5×10^5) suspension was completed with 20 μL of serum and incubated for 1 hour at 4°C under gentle rocking. After being washed three times with 2 mL of PBS-BSA, cells were suspended in 100 μL of PBS-BSA containing 2 μL of R-phycoerythrin (PE)–conjugated goat anti-human IgGs (Fc specific; Jackson ImmunoResearch Europe Ltd, Newmarket, UK) and 10 μL of fluorescein isothiocyanate (FITC)-conjugated MoAb SZ-21. Cells were incubated for 1 hour at 4°C under gentle rocking, washed twice, and resuspended in 1 mL of PBS-BSA containing 10 μg/mL of 7-aminoactinomycin (7-AAD; Invitrogen). Bound human IgGs were detected at the surface of cells negative for 7-AAD and positive for SZ-21 labeling by using a flow cytometer (Epics-XL, Beckman Coulter, Roissy, France). FCM testing with PLTs was performed under the same experimental conditions but with PBS-BSA buffer containing 3 mmol/L EDTA.

FCM was also used to study fg binding on transiently transfected HEK cells. Briefly, 5×10^5 washed cells were resuspended in 100 μL Tyrode buffer containing 0.1 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 4 mg/mL BSA (TB), and 10 μg/mL 7-AAD and 20 μg/mL MoAbs PT25-2 or M148 or of an irrelevant mouse IgG. Cells were incubated for 20 minutes at 4°C under gentle rocking before adding 10 μL of a 1.5 mg/mL solution of fg conjugated to the fluorochrome A-488 (Molecular Probes, Eugene, OR) and 2 μL R-PE–conjugated goat anti-mouse IgGs (Fc specific; Jackson ImmunoResearch Europe Ltd). Cells were further incubated for 45 minutes, washed once in 2 mL, and resuspended in 1 mL TB before FCM analysis. 7-AAD–negative cells were analyzed for both fg and MoAb binding.

Modeling of the αIIb Phe827-Lys855 peptide

The peptide Phe827-Lys855 of αIIb was modeled with computer software (PyMOL, http://www.pymol.org/),[13] starting from an extended conformation. The p.Leu841Met substitution was also done with PyMOL.[13]

Molecular dynamics simulations

Molecular dynamics (MD) simulations were performed using computer software (GROMACS 4.5.4, http://www.gromacs.org/)[14] with the OPLS-AA force field[15] for Leu841 and Met841 peptides and TIP3P water molecules. The total energy of the system was minimized twice (before and after the addition of ions to neutralize the system). MD simulations were run under the NPT thermodynamic ensemble at the constant physiologic tempera-

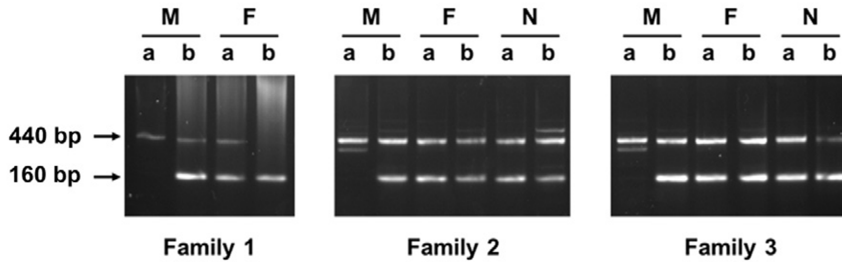

Fig. 1. ***c.2614C>A*** **PCR-SSP genotyping. Genomic DNA from mothers (M), fathers (F), and newborns (N) in Families 1, 2, and 3 were genotyped for the αIIb variants** ***c.2614C*** **(Lane a) or** ***c.2614A*** **(Lane b) by using the PCR-SSP technique. Amplicons were analyzed by electrophoresis on a 2.5% agarose gel and visualized under UV light after staining by GelRed. The lower band (160 bp) when present and the upper band (440 bp), respectively, correspond to the allele-specific amplicons and the internal PCR control. In the three families, mothers were genotyped homozygous** ***c.2614C*** **(Cab3$^{a-/a-}$), and fathers and newborns, heterozygous** ***c.2614C/A*** **(Cab3$^{a-/a+}$).**

ture of 310 K. For both systems, five 50-nanosecond simulations were performed (using different initial velocities) and trajectory analyses were computed on a total of 2250 configurations.

Protein block analysis

Protein blocks (PBs)[16] allow a reasonable approximation of local protein three-dimensional (3D) structures.[17] PBs are a structural alphabet composed of 16 local prototypes, labeled from *a* to *p* (see Fig. 1[18]) and characterized by the φ, ψ dihedral angles of five consecutive residues. The equivalent number of PBs[17] (*Neq*) is a statistical measurement similar to an entropy that represents the average number of PBs a given residue takes. A *Neq* value of 1 indicates that a single PB is observed; on the opposite a value of 16 is equivalent to a random distribution. A logo representation of PB frequency along the peptide was also created with the development version (as of November 2011) of a sequence logo generator (WebLogo, http://weblogo.berkeley.edu/).[19]

Functional studies

Aggregation and adhesion tests were performed as previously described.[8]

RESULTS

Identification of maternofetal Cab3 incompatibility in two new families

In the absence of identified alloantibody and/or common HPA maternofetal incompatibility, immunologic investigation is focused on rare alloantigenic variants. Genotyping is also done for a few other rare polymorphisms identified by our laboratory in a FNAIT context but not shown to be associated with an alloimmune response. By using a newly set up PCR-SSP assay (see Materials and Methods), two new families (Families 2 and 3) presenting a maternofetal incompatibility for the *c.2614A* variant form of the αIIb gene[9] were identified (Fig. 1). All genotypes were confirmed by direct sequencing of PCR products corresponding to the Exon 26 of αIIb (not shown). Family 1 corresponds to the index case where the *c.2614C>A* mutant was discovered. PCR-SSP confirmed the mother to be homozygous for the *c.2614C* αIIb allele (Lane M/b) and the father heterozygous *c.2614C/A* (Lanes F/a and F/b). In Families 2 and 3 similar amplification patterns were obtained with mothers homozygous *c.2614C* and fathers heterozygous *c.2614C/A*. In Families 2 and 3, newborn DNA samples were genotyped heterozygous *c.2614C/A* (Lanes N/a and N/b). The *c.2614C>A* mutation was also identified in two other families. In one case, the *c.2614C>A* mutation was not related to FNAIT because the mother was heterozygous and in the second case, the newborn DNA was not available to confirm a maternofetal incompatibility (results not shown). From 2001 to 2011, the *c.2614C>A* mutation was identified in five heterozygous individuals from genotyping of 752 parents from families with FNAIT, giving an allelic frequency of 0.33%.

Characterization of the new PLT alloantigen Cab3^{a+}

During diagnosis, maternal sera were tested against PLTs from their respective husbands and/or PLTs from the panel by using the MAIPA assay. None of the maternal sera from the three families gave a positive reaction with α2β1, GPIb-IX, αIIbβ3, or HLA antigens (data not shown).

The *c.2614C>A* mutation resulting in the αIIb p.Leu841Met substitution,[9] maternal sera from the three families were tested against HEK293 cells expressing either the αIIb-Leu841 or the αIIb-Met841 forms of αIIbβ3 by using FCM. Figure 2 shows that the maternal serum from Family 2 reacted with the αIIb-Met841 form of αIIbβ3 but not with the variant αIIb-Leu841 (gray bars). As control, a serum sample containing an anti-HPA-3a alloantibody was shown to similarly react with both cell types (open bars). An unreactive negative control serum was also used (black bars). The maternal serum from Family 2 reacting specifically with the αIIb-Met841 form of αIIbβ3 defined a new PLT alloantigen named Cab3^{a+}. Maternal sera from Families 1 and 3 did not react with any of the HEK293 tested (not shown). The maternal serum from Family 2 although reactive with Cab3^{a+} HEK293 cells in FCM failed to react in MAICA (not shown).

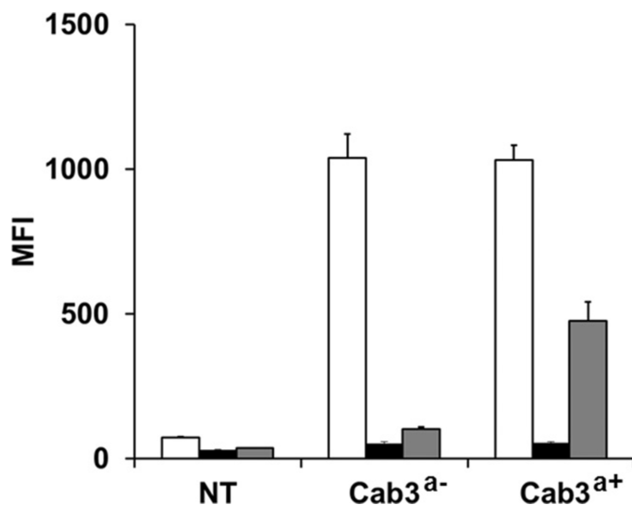

Fig. 2. Serum reactivity with HEK293 cells expressing the Cab3^{a+} αIIbβ3 form. The reactivity of a characterized anti-HPA-3a alloantibody (□), of a control serum (■), and of the maternal serum from Family 2 (▩) were tested in FCM with untransfected HEK293 cells (NT) or expressing the Cab3^{a-} or Cab3^{a+} forms of αIIbβ3. Bound human IgGs were detected by using a polyclonal goat anti-human IgG conjugated to FITC. Mean fluorescence intensities (MFI) were measured for living cells not labeled by the viability dye 7-AAD. The maternal serum from Family 2 specifically reacted with cells expressing the Cab3^{a+} forms of αIIbβ3.

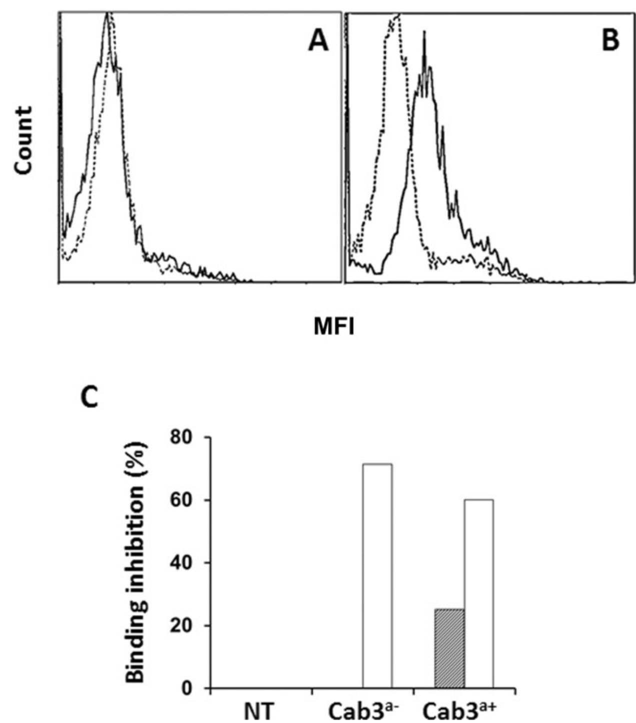

Fig. 3. Cab3 serum reactivity with the paternal PLTs. PLTs from mother and father (Family 2) were incubated with sera from a control donor or from the mother. Bound antibodies were detected by using an anti-human IgG conjugated to PE and FCM. (A) Maternal (········) and paternal (——) PLTs presented a similar labeling with the control serum that also corresponded to the labeling of the maternal PLTs when incubated with its own serum (B, ········). By comparison, the maternal serum exhibited a higher reactivity with the paternal PLTs (B, ——). (C) Before FCM testing, serum from Mother 2 (▩) or a serum containing an anti-HPA-3a alloantibody (□) were adsorbed onto HEK293 cells untransfected (NT) or expressing Cab3^{a-} or Cab3^{a+} αIIbβ3. Serum reactivity inhibition was expressed as a percentage of the serum reactivity without adsorption. The maternal serum is specifically inhibited by adsorption onto cells expressing Cab3^{a+} αIIbβ3.

Cross-match testing by using FCM

New blood samples from Family 2 were available to perform autologous and cross-match testing by FCM (Fig. 3). Compared to a serum control (Fig. 3A), the maternal serum presented a positive reactivity with the paternal PLTs (cross-match) but not with her own PLTs (Fig. 3B). However, FCM does not allow the distinction between different serum specificities. As maternofetal incompatibilities to HPA-1a, -3a, and -5a variants exist in this family, the specific presence of anti-Cab3^{a+} alloantibodies was studied by using cross-match testing with the serum adsorbed onto HEK293 cells expressing αIIbβ3 (Fig. 3C). Adsorption onto HEK293 cells expressing Cab3^{a+} but not Cab3^{a-} αIIbβ3 inhibited the serum reactivity with paternal PLTs. As controls, the reactivity of a serum containing an anti-HPA-3a was inhibited by both Cab3^{a-} and Cab3^{a+} cells, while NT cells had no effect. These results showed that the anti-Cab3^{a+} alloantibody reacted with an epitope expressed at the PLT surface. As HEK293 cells naturally express α2β1 (HPA-5a) and potentially HLA antigens, and αIIbβ3 (HPA-1a and -3a) following transfection, adsorption experiments suggested an absence of antibody to HPA-1a, -3a, and -5a or HLA antigens in the maternal sera. These results correlated with the negative MAIPA obtained with the paternal or panel PLTs (data not shown). As observed with the former sample, the new serum was similarly and specifically reactive with Cab3^{a+} HEK293 cells in FCM while it remained negative in MAIPA or MAICA (not shown).

αIIb-Leu841Met substitution and anti-HPA-3a alloantibody reactivity

The Cab3 polymorphism p.Leu841Met locates two amino acids upstream of the common p.Ile843Ser (HPA-3) polymorphism of αIIb.[5] The anti-HPA-3a alloantibody previously used as positive control similarly reacted with HEK293 cells expressing the Cab3^{a+} or Cab3^{a-} forms of αIIbβ3 (Fig. 2, open bars). These results suggested that the p.Leu841Met substitution did not affect the epitope of this

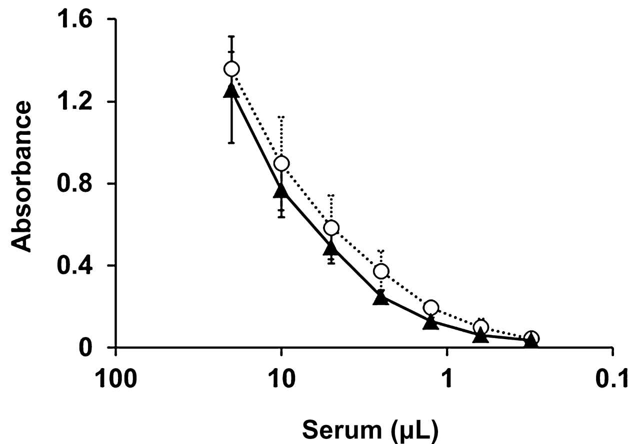

Fig. 4. Anti-HPA-3a reactivity with the Cab3^{a+} αIIbβ3 form. Serial dilutions of a serum containing an anti-HPA-3a were tested in MAICA with HEK293 cells expressing the Cab3^{a-} (○) or Cab3^{a+} (▲) forms of αIIbβ3. The anti-HPA-3a serum reacted similarly with HEK293 cell independently of the Cab3 variant form of αIIbβ3 they expressed.

anti-HPA-3a alloantibody. Nonetheless, this test could have been performed at saturating amounts of alloantibody, masking a decreased affinity. To test this hypothesis, serial dilutions of the serum were tested in MAICA (Fig. 4). The serum reacted similarly with HEK293 cells expressing the Cab3^{a+} or Cab3^{a-} αIIbβ3 forms whatever the dilution tested. To support this observation, HPA-3a/a and Cab3^{a-} PLTs and HPA-3a/a but Cab3$^{a+/a-}$ PLTs also reacted similarly with this anti-HPA-3a in phenotyping tests by using MAIPA. In the same experiment, HPA-3b–homozygous PLTs used as negative control were not reactive and HPA-3a/b heterozygous PLTs gave a decreased signal (results not shown).

The p.Leu841Met substitution and local αIIb structure

Like HPA-3 (p.Ile843Ser) and HPA-9 (p.Val837Met), the Cab3 polymorphism locates in Loop 827 to 855 of αIIb whose 3D structure remains to be determined.[20] Nonetheless, to assess if the p.Leu841Met substitution would alter local 3D structure, structure modeling of amino acid sequences 829 to 853 for each Cab3 variant was done and submitted to long MD simulations. N_{eq} analyses (see Materials and Methods) were performed on the structures obtained from MD simulations (Fig. 5A). As expected for an unsolved crystallographic structure, N_{eq} analyses showed high structure flexibility ($N_{eq} > 2$) for both variants. Interestingly, the p.Leu841Met mutation locates in a very flexible sequence ($N_{eq} \approx 4$) and did not modify the Neq profile. Furthermore, neither the PBs adopted by residues nor the secondary structure of the αIIb 829 to 853 peptidic sequence seemed significantly affected by the p.Leu841Met substitution (Fig. 5B). So this substitution would have little effect, if any, on the Peptide 829 to 853 backbone structure.

Cab3 polymorphism and αIIbβ3 expression and function

Similar mutant complex expression in transfected HEK293 cells and PLT phenotyping analyses revealed that the p.Leu841Met substitution did not significantly affect the expression of αIIbβ3 (Fig. 2 and data not shown). In absence of homozygous Cab3^{a+} PLTs, the p.Leu841Met substitution effect on αIIbβ3 functions was studied by using transfected HEK293 cells.

FCM analysis using fg conjugated to A-488 showed that both αIIb-Leu841 and αIIb-Met841 forms of αIIbβ3 could bind soluble fg (Fig. 6, open bars) after activation by MoAb PT25-2.[21] Neither irrelevant mouse IgG (gray bars) nor MoAb M148 to αIIb, a complex inhibitor[22] (black bars), induced fg binding. MoAbs M148 and PT25-2 similarly bound HEK293 cells expressing Cab3^{a-} or Cab3^{a+} αIIbβ3 (not shown).

HEK293 cells expressing Cab3^{a-} and Cab3^{a+} αIIbβ3 could also aggregate in presence of soluble fg and complex activation by MoAb PT25-2 but not by irrelevant IgG (Fig. 7). Under identical experimental conditions, not transfected (NT) cells did not form large aggregates and BSA did not support cell aggregation whatever the cell type or MoAb used (not shown).

HEK293 cells expressing Cab3^{a-} or Cab3^{a+} αIIbβ3 also presented similar saturable adhesion onto fg (Fig. 8A, black diamonds and open squares curves). HEK293 cells may express low levels of αvβ3; however, NT cells did not adhere (black circles curve) while cells transfected by the β3 coding plasmid alone (open triangles curve) only adhered in a limited extent. Finally, adhesion of HEK293 cells expressing Cab3^{a-} or Cab3^{a+} αIIbβ3 was inhibited in presence of MoAb AP2[23] (Fig. 8B, black bars) but not in presence of a control IgG (open bars). These results indicated that the Leu841Met substitution did not significantly affect expression and major functions of αIIbβ3.

DISCUSSION

The *c.2614C>A* mutation of αIIb resulting in the p.Leu841Met substitution was initially described in a context of FNAIT without identified alloantibody by using MAIPA (Family 1 “Case Report” section and Halle et al.[9]). Genotyping for rare polymorphisms in unsolved FNAIT allowed detection of the αIIb *c.2614A* allele in three new families with potential (one case) or confirmed (two cases) maternofetal incompatibility. In our panel the *c.2614A* variant is a rare polymorphism presenting an allelic frequency of 0.33% that contrasts with the higher frequencies

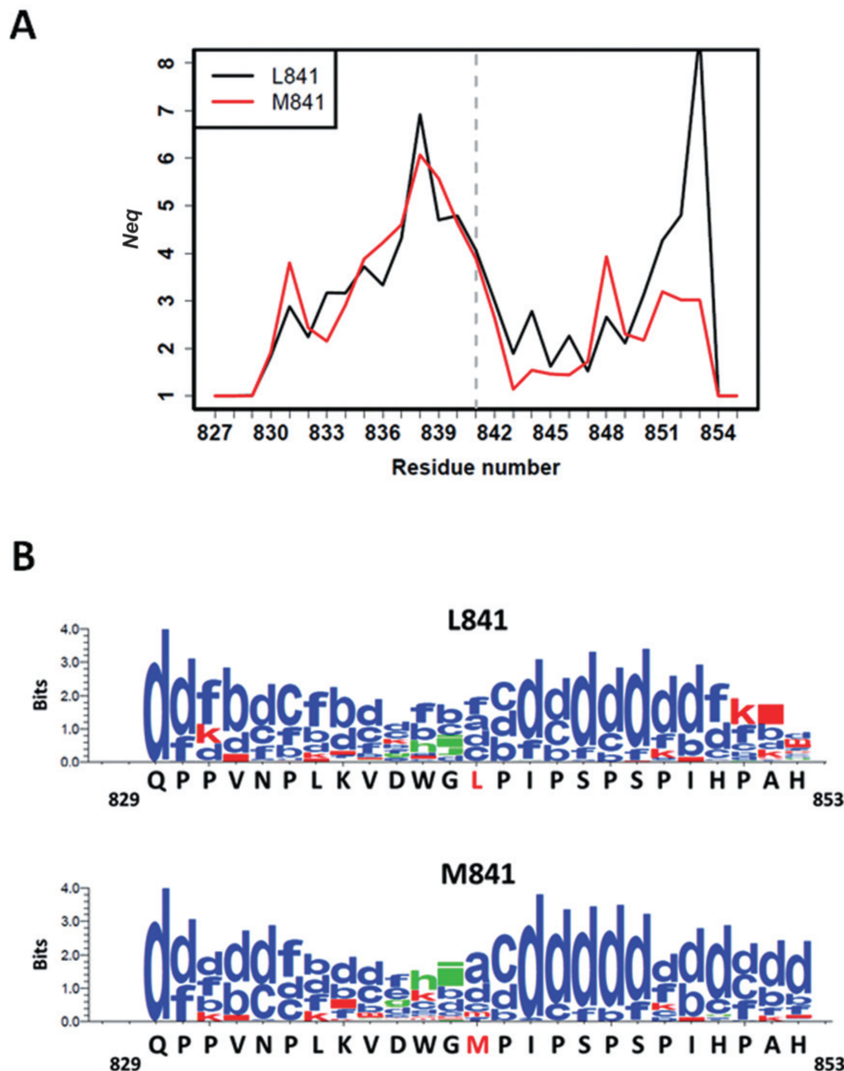

Fig. 5. Structure analysis of the Leu841 and Met841 forms of the αIIb Peptide 829 to 853. (A) N_{eq} values for each amino acid of the αIIb Peptide 829 to 853 were computed from MD structures of the Leu841 (black line) and the Met841 (red line) variants. The dashed gray line indicates N_{eq} values of amino Acid 841. Note that Residues 827, 828, 854, and 855 were not assigned by PBs analysis. (B) PBs (represented by their letter code) that a residue can adopt in the Leu841 and Met841 peptidic sequences. The letter size is proportional to the PB frequency observed in the 2250 structures obtained during MD simulations. Blue, red, and green letter colors, respectively, correspond to β-strand, α-helix, and coils secondary structures. The p.Leu841Met substitution did not significantly affect the structure of the αIIb 829 to 853 peptidic sequence.

observed in Aka Pygmies (8.3%) or other Sub-Saharan African populations (2.4%-4.4%).[9] The French population presents a significant rate of racial admixture and it should be noted that the ethnical origin of the families studied here was unknown. So in regard to a possible African ancestry (according to names), the allelic frequency of the Cab3[a+] variant would be higher in such a population. Nonetheless this variant corresponds to a rare polymorphism in the global French population (<1%). Although associated with the HPA-3a allele in our FNAIT set, the *c.2614A* variant can be found in HPA-3b homozygous subjects.[9]

As initially observed for the index case (Family 1), maternal sera from Families 2 and 3 did not react in cross-match testing by using MAIPA for the antigens studied (αIIβ1, GPIb-IX, αIIbβ3, or HLA). Nonetheless, using FCM and transfected HEK293 cells a single serum (Family 2) was shown to react with the Cab3[a+] αIIbβ3. A major drawback of whole cell assays is potential positivity due to anti-HLA, HEK293 cells expressing HLA antigens.[24] This hypothesis was not confirmed as the serum did not react with untransfected or Cab3[a−] αIIbβ3 HEK293 cells. So this anti-Cab3[a+] serum reactivity revealed the αIIb-Met841 as a new alloantigen. Furthermore, cross-match testing using fresh blood samples and FCM assays indicated that the Cab3[a+] antigen is also expressed at the PLT surface.

The maternal serum from Family 2 failed to react with Cab3[a+] HEK293 cells in MAICA, as in cross-match testing using MAIPA. Similarly, some anti-HPA-3a or -3b or anti-HPA-9a alloantibodies are not detected in MAIPA but can be identified in whole cell assays like PLT immunofluorescence test, FCM, and mixed passive hemagglutination or solid-phase red cell adherence.[25-30] Such difficulties are attributed to "labile" epitopes disrupted by detergent treatment applied in solid-phase assays and /or by PLT storage.[31] This hypothesis would also apply for antibodies to Cab3[a+] whose polymorphic site in position 841 neighbors residues 843 and 837 responsible for the systems HPA-3 and -9. These antigens belong to a flexible αIIb loop (Residues 827-855) that is unsolved in the αIIbβ3 structure.[20] Such flexibility would facilitate the labile nature of the epitopes independently of other sensitive variables like glycosylations, as observed for HPA-3.[31,32]

In the families with confirmed or potential Cab3[a+] maternofetal incompatibility, severe thrombocytopenias (4×10^9 and 39×10^9/L) and one fetal death were observed. In two cases, the Cab3[a+] incompatibility was associated with an HPA-5b (Family 1) or a triple HPA-1a, -3a, and

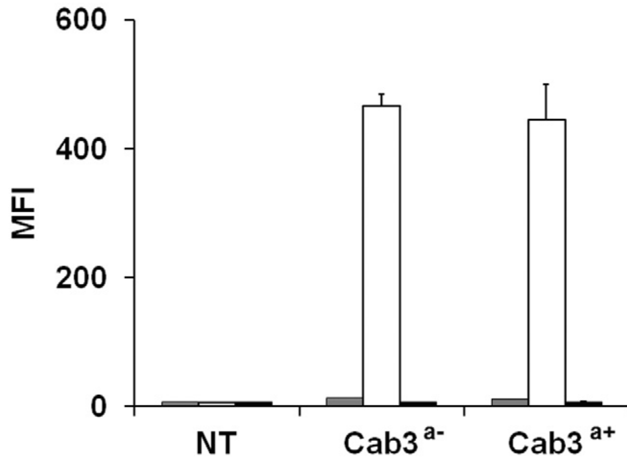

Fig. 6. Cab3[a+] αIIbβ3 form bound fg. Not transfected (NT) cells or HEK cells expressing the Cab3[a–] or Cab3[a+] forms of αIIbβ3 were incubated with a mouse control IgG (■), the complex activating MoAb PT25-2 (□), or the complex inhibitor (of fg binding) MoAb M-148 (■). After control IgG and MoAb incubations, fg conjugated to A-488 was added and its binding was measured by FCM. Both cell types similarly bound soluble fg.

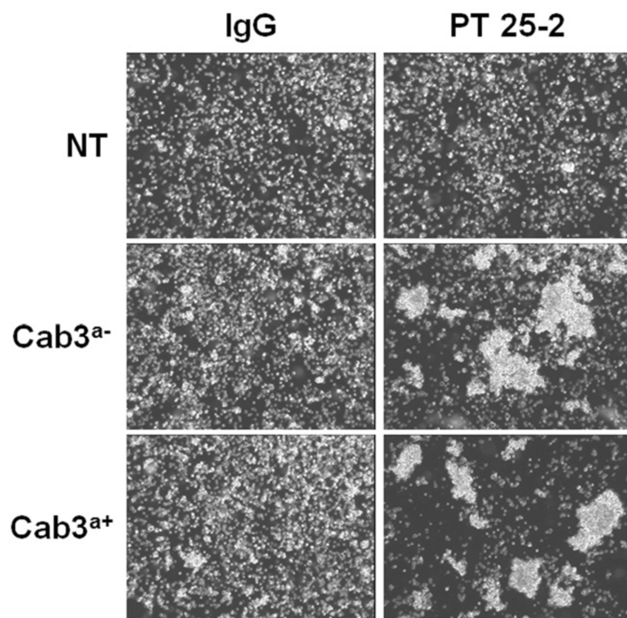

Fig. 7. Cab3[a+] αIIbβ3 form supported HEK293 cell aggregation. Not transfected (NT) cells or HEK293 cells expressing the Cab3[a–] or Cab3[a+] forms of αIIbβ3 were incubated with either a mouse control IgG or the complex activating MoAb PT25-2 in presence of fg and calcium under gentle rocking. Both Cab3[a–] and Cab3[a+] forms of αIIbβ3 similarly supported HEK293 cell aggregation.

5a (Family 2) incompatibility. Nonetheless, any corresponding alloantibodies were detected in maternal sera. In Family 3, the single identified incompatibility concerned Cab3[a+]. These observations suggest that

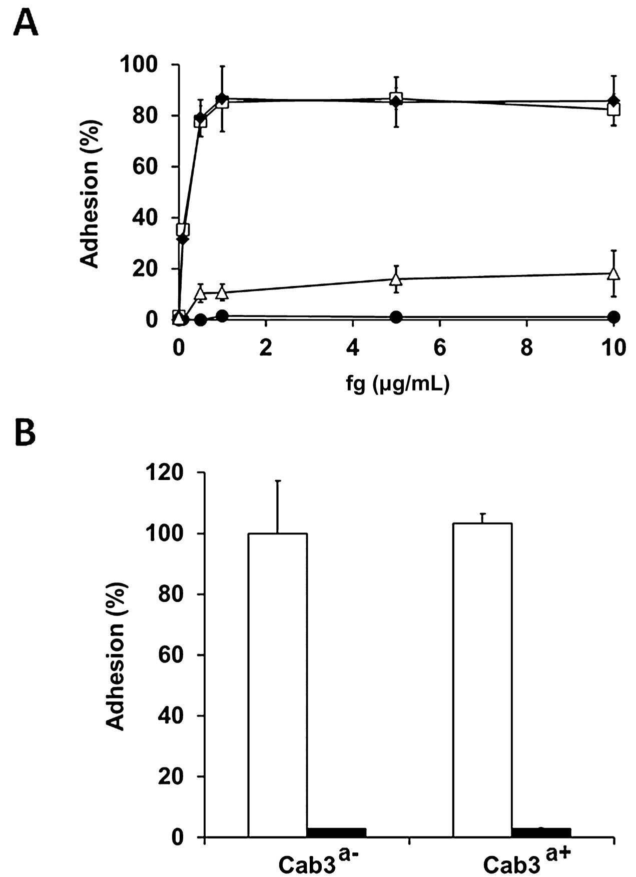

Fig. 8. Cab3[a+] αIIbβ3 form mediated HEK293 cell adhesion. (A) HEK293 cells not transfected (●), transfected with the β3 coding plasmid alone (△), or cotransfected with plasmids coding for β3 and for Cab3[a–] (□) or Cab3[a+] (◆) αIIb were layered onto adsorbed fg (0 to 10 μg/mL) or MoAb AP2 (10 μg/mL). Adherent cells were stained by crystal violet and cell adhesion was quantified by measuring absorbance. Cell adhesion was expressed as the percentage of cells bound onto fg to total AP2 bound cells. Adhesions of cells expressing the Cab3[a–] and Cab3[a+] forms of αIIbβ3 were similar. (B) Adhesion of cells expressing the Cab3[a–] or Cab3[a+] forms of αIIbβ3 onto 1 μg/mL fg was measured in presence of a control mouse IgG (□) or MoAb AP2 (■). Cell adhesion was expressed as a percentage of adherent cells in presence of control IgG or MoAb AP2 to total adhesion obtained in presence of control IgG. Adhesion of both cell types was specifically inhibited by the anti-αIIbβ3 MoAb AP2.

anti-Cab3[a+] alloimmunization could lead to severe and life-threatening thrombocytopenias like in FNAIT with HPA-3a or -3b or -9bw incompatibilities.[29,30,33,34] However, a random study of a large population would be necessary to clearly establish a link between FNAIT and the Cab3[a+] variant. Unfortunately clinical data from the African populations studied where the Cab3[a+] variant is more frequent[9] are not available to our knowledge.

FCM studies with MoAbs directed to αIIbβ3 (P2, SZ21, M148) showed that both Cab3 complex forms are similarly expressed at the surface of transfected HEK293 cells while HPA-1a and -3a phenotyping of PLTs Cab3[a+] heterozygous or Cab3[a–] homozygous also suggested similar αIIbβ3 expression. Interestingly, the p.Leu841Met substitution did not impair an antibody binding to HPA-3a despite close proximity of the polymorphic sites. This anti-HPA-3a alloantibody can be directed to a distinct epitope[31,35] (O-link glycosylation being involved in some cases[32]) or its epitope would not be significantly affected as suggested by structural studies of Peptide 827 to 853. Because no homozygous PLTs were available, functional studies performed with transfected HEK293 cells indicated that the p.Leu841Met substitution did not impair fg binding, cell aggregation, or adhesion. To support this hypothesis, leucine and methionine are similarly hydrophobic[36] and statistical analysis of a large set of crystallographic and mutagenesis data on various proteins revealed that both amino acids can be substituted without affecting folding and functions.[37] These observations suggested a minor effect, if any, of the p.Leu841Met change on local αIIb structure and complex functions.

CONCLUSION

Looking for rare αIIbβ3 allelic variants in unsolved FNAIT, the αIIb *c.2614A*[9] variant was identified in four new families. This rare polymorphism (allelic frequency of 0.33% in our panel) is responsible for a p.Leu841Met substitution. FCM studies using transfected HEK293 cells allowed detection of an alloantibody to the αIIb-Leu841 variant of αIIbβ3, defining this polymorphism as a new alloantigen named Cab3[a+]. Using FCM, cross-match testing revealed that the Cab3[a+] antigen is expressed at the PLT surface. This anti-Cab3[a+] alloantibody like some antibodies to HPA-3a (or -3b) and HPA-9bw require whole cell assays when MAIPA test fails to detect them. The p.Leu841Met substitution affects neither local αIIb structure nor αIIbβ3 expression and functions. The Cab3[a+] case underlined the necessity to closely study any polymorphism identified in a context of FNAIT even when current techniques (MAIPA) have failed to identify an alloantibody. Future studies are required to further characterize the anti-Cab3 immune response and potential immunogenicity of the Cab3[a–] allele.

ACKNOWLEDGMENTS

We thank Dr Alexandre G. de Brevern for helpful discussions. This work was granted access to the HPC resources of CINES under the allocation 2011-c2011076723 made by GENCI (Grand Equipement National de Calcul Intensif).

CONFLICT OF INTEREST

The authors have no disclaimers to make or conflict to disclose.

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