L718P mutation in the membrane-proximal cytoplasmic tail of β3 promotes abnormal αIIbβ3 clustering and lipid microdomain coalescence, and associates with a thrombasthenia-like phenotype

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Supplementary Methods

Exon-trap analysis

The exon-trap vector system (Mo Bi Tec GmbH, Göttingen, Germany) was used to analyze whether sequence variations in intron 21 altered the splicing pattern of the transcript. Genomic DNA from a control and the patient was used for PCR-amplification of a 874-bp DNA fragment encompassing exons 21 and 22 and the adjacent regions of introns 20 and 22, using the oligonucleotide pair: sense-intron 20: 5'-tatatgatgctctgtaatttc-3', and antisense-intron 22: 5'-tcagacgggggaagggtggt-3'. The PCR products were cloned into the BamH I-Not I digested exon-trap vector, and constructs were transfected into K562 cells. Forty-eight hours after transfection, total RNA was extracted and RT-PCR was performed.

Immunoprecipitation analysis

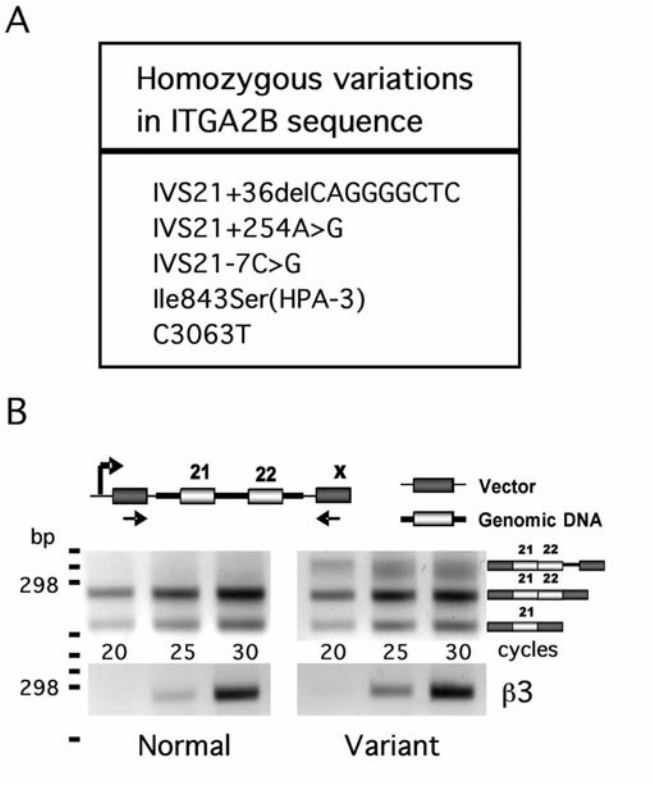
For immunoprecipitation analysis of αvβ3 and αIIbβ3 in transiently transfected K562 cells, 300 μg of solubilized protein were pre-cleared and incubated overnight at 4°C with 3 μg/mL of anti-αvβ3, anti-αIIb or anti-β3 monoclonal antibodies. The immunoprecipitates were treated for 2 h with protein A-Sepharose CL-4B, washed and eluted by boiling in 25 μL of non-reduced loading buffer, and analyzed by western blotting with either anti-β3 or a mix of anti-αIIb and anti-β3 monoclonal antibodies.

Cell adhesion assay under flow

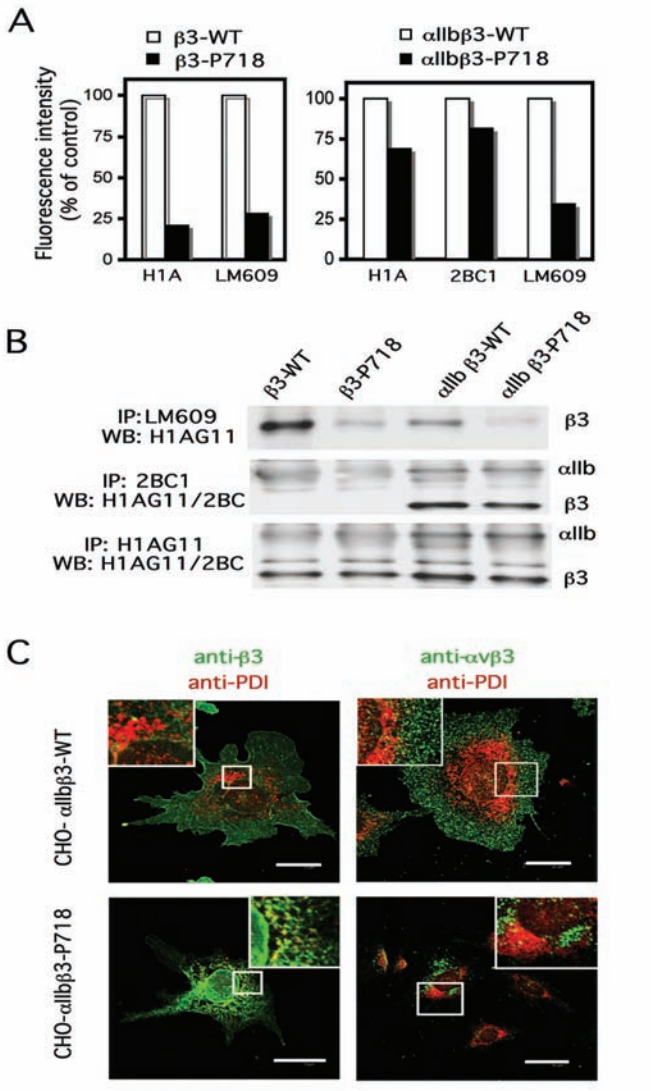
Cell adhesion under flow was assessed using a flow chamber assembled to plates coated with 50 μg/mL fibrinogen. Cells were resuspended at a concentration of 10^6/mL and perfused through the flow chamber at shear rates ranging from 1 to 10 dyne/cm^2.

Cross-linking experiments

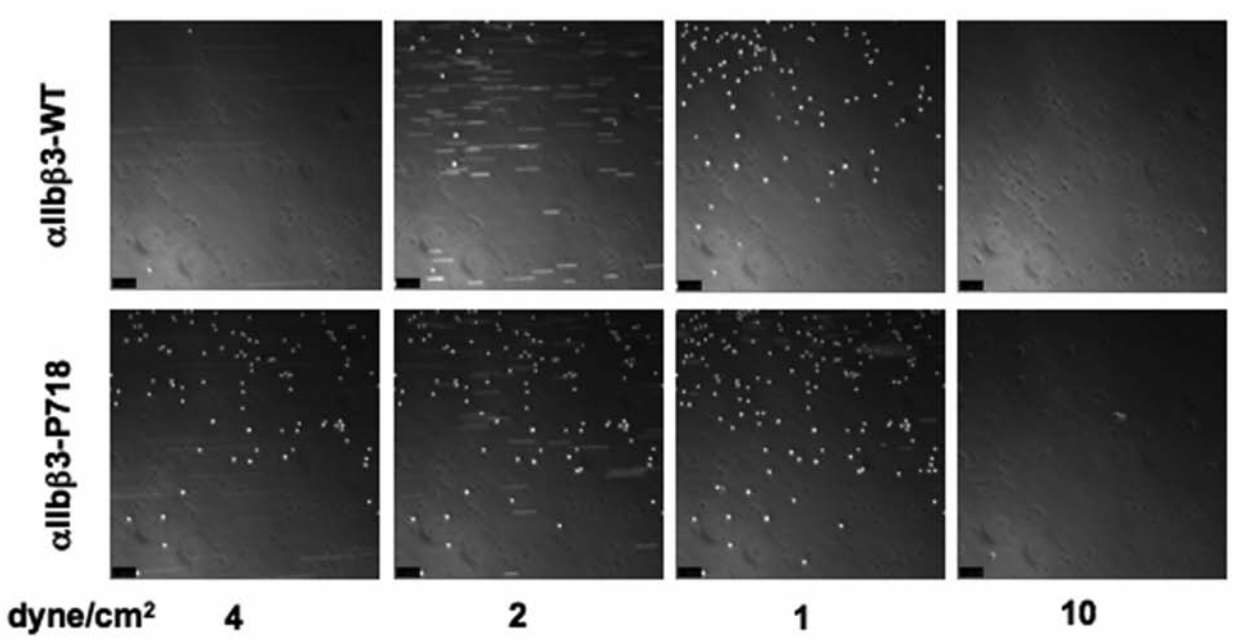
Stably transfected CHO cells were grown to confluence on 100-mm tissue culture plates coated with 50 μg/mL fibrinogen, rinsed and preincubated with KRH buffer (50 mM HEPES, 128 mM NaCl, 1.3 mM $CaCl_2$, 5 mM $MgSO_4$, 5 mM KCl, pH 7.4) for 30 min at 4°C, followed by cross-linking with 0.5 mM disuccinimidyl suberate (DSS, Pierce, Rockford, IL, USA) in KRH buffer for 15 min. Cells were then washed with cold Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) and lysed in 1% Triton X-100 buffer. Five hundred micrograms of protein lysates were immunoprecipitated with anti-αIIb or anti-β3 as described above.



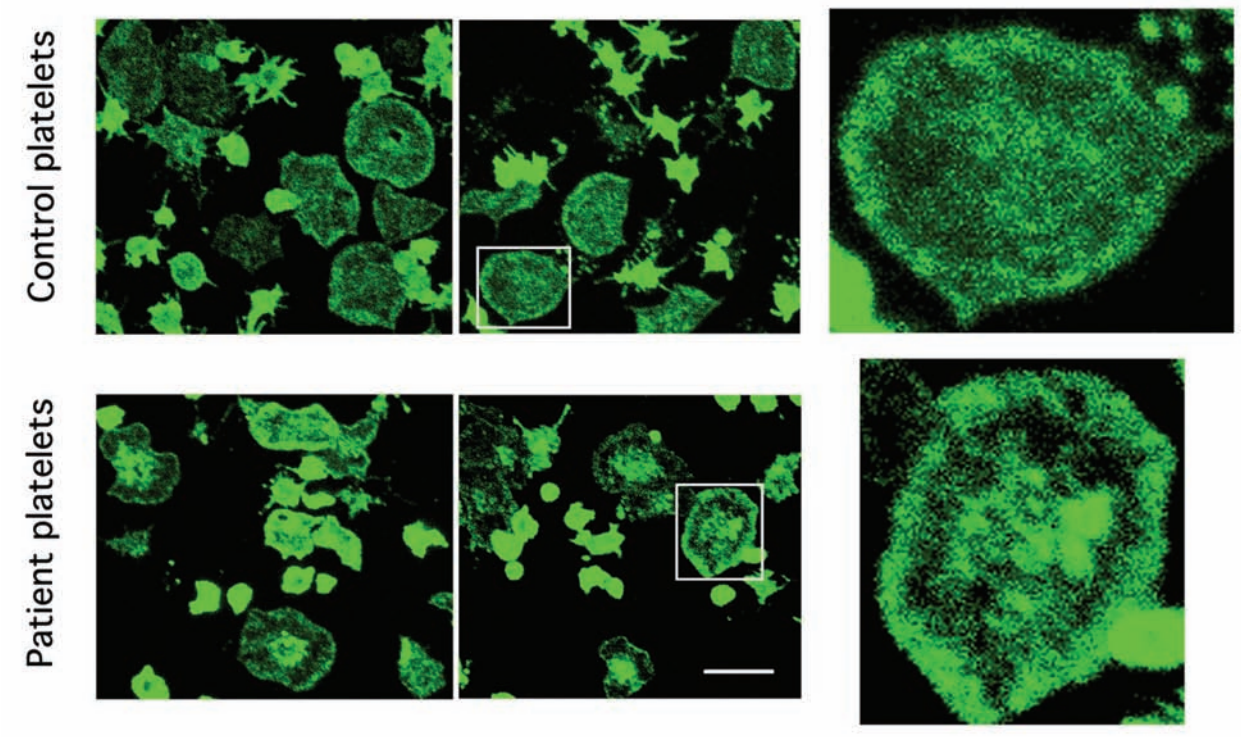
Online Supplementary Figure S1. Identification of sequence variations in the *ITGA2B* gene and exon trapping analysis. (A) Homozygous polymorphic variations found in the *ITGA2B* gene. (B) Exon trapping analysis of αIIb genomic fragments. Fragments of genomic DNA encompassing the exon 21 sequence corresponding to each of the two allelic forms of αIIb were transiently transfected into K562 cells and the splicing patterns were deduced by agarose gel and sequencing analysis as described in the *Online Supplementary Methods*. Data are representative of three separate transfection experiments.



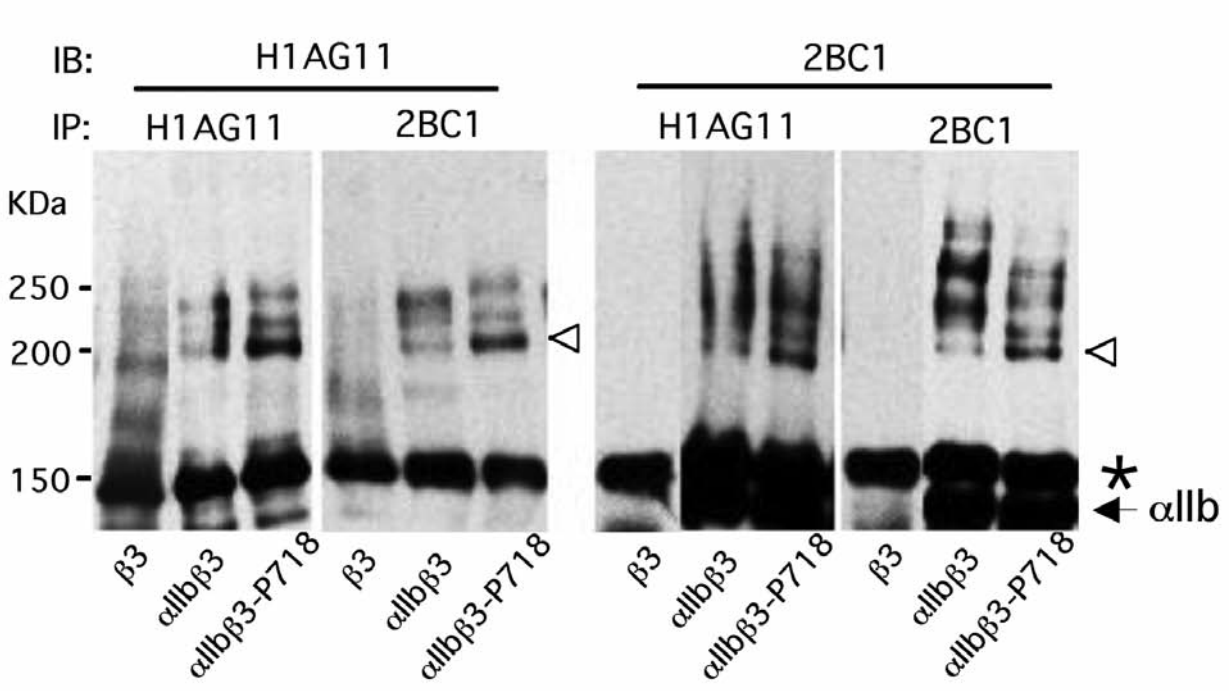
Online Supplementary Figure S2. Differential processing of mutant αIIbβ3-P718 and αvβ3-P718 integrins in transfected cells. Flow cytometry (A) and immunoprecipitation (B) analysis of β3 integrins in transiently transfected K562 cells. Cells were nucleofected with normal or mutated β3 cDNA alone or together with normal αIIb cDNA and, 48 h after transfection, the surface or total cellular content of αIIbβ3, αvβ3 or both β3 integrins were determined with the indicated monoclonal antibodies as described in the *Design and Methods* section. Bars represent the mean fluorescence intensity values expressed as percentage of the control. Representative results of three separate experiments are shown. (C) Intracellular retention of αvβ3-P718. CHO cells stably expressing αIIb and either normal or mutant β3 subunits were adhered to Fg-coated dishes, fixed, labeled with anti-PDI and either anti-β3 (H1AG11) or anti-αvβ3 (LM609) monoclonal antibodies, and visualized in a confocal microscope with a x63 objective. Bars: 20 μm. Representative images of two separate immunofluorescence experiments are shown.



Online Supplementary Figure S3. Cell adhesion to fibrinogen-coated surface at different shear rates. Phase-contrast microphotographs with a x10 objective showing CHO cells adherence after 1 min under shear of 4, 2, or 1 dyne/cm², assessed as described in the *Online Supplementary Methods*. The same field was used to record adhesion of CHO-αIIbβ3-P718 cells after detachment of CHO-αIIbβ3-WT cells under flow at a shear stress of 10 dyne/cm². The experiment was performed four times with similar results.



Online Supplementary Figure S4. Confocal immunofluorescence analysis of αIIbβ3 distribution in fibrinogen-adherent platelets. Washed platelets were seeded on fibrinogen-coated coverslips for 15 min at 37°C and then fixed, labeled with anti-αIIbβ3 monoclonal antibodies 2BC1, and analyzed by confocal microscopy with a x100 objective. The images show integrin distribution in sections near the adherent surface of the platelets. Bars: 10 μm.



Online Supplementary Figure S5. Immunoprecipitation analysis of cross-linked αIIbβ3. Confluent CHO cells expressing β3 or normal or mutant αIIbβ3 were treated with 0.5 mM DSS as described in the *Online Supplementary Methods*. Protein lysates (500 μg) were immunoprecipitated with anti-β3 (H1AG11) or αIIbβ3 (2BC1) monoclonal antibodies and the elution products were separately analyzed by western blotting with both monoclonal antibodies. Arrowheads point to immunoprecipitated products of approximately 200 KDa in cells expressing the mutant αIIbβ3 -P718 integrin. The asterisk points to bands corresponding to the recognition of immunoprecipitation monoclonal antibodies by the secondary antibody in western analysis. The results are representative of more than three experiments.