Improved platelet survival after cold storage by prevention of glycoprotein Ibα clustering in lipid rafts

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Supplementary Design and Methods

Materials and antibodies

The following products (with sources) were used: arachidonic acid (AA; Bio/Data Corporation, Horsham, PA, USA), GlcNAc, fatty acid free-bovine serum albumin, N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), *D*-glucose and mowiol 4-88 (Sigma-Aldrich, St Louis, MO, USA), Cell tracker green 5-chloromethyl fluorescein diacetate (CMFDA) (Molecular Probes, Invitrogen, Carlsbad, CA, USA), monosialogangliosides GM1 and GM3 (GenWay Biotech Inc, San Diego, CA, USA), GM6001 (Millipore, Billerica, MA, USA), Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and prostacyclin (PGI2; Cayman Chemical, Ann Arbor, MI, USA). Protease-activating receptor-4 (PAR-4) agonist peptide AYPGKV was synthesized at the Netherlands Cancer Institute (Amsterdam, the Netherlands). Antibodies and lectins used for flow cytometry were directed against GPIbα (clone HIP1; BD Pharmingen (San Diego, CA, USA), GPV (clone CLB-SW16; Monosan, Uden, the Netherlands) and goat anti-mouse $F(ab)_2$ conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA), against phosphatidylserine (fluorescein-conjugated lactadherin; Haematologic Technologies Inc. Essex Junction, VT, USA), sialic acid (fluorescein-conjugated sambucus nigra, SNA), galactose (fluorescein-conjugated ricinus communis I, RCA-I), and GlcNAc (fluorescein-conjugated succinylated wheat germ agglutinin, s-WGA; Vector Laboratories, Burlingame, CA, USA). To assess murine platelet activation by flow cytometry we obtained phycoerythrin (PE)-labeled anti-active CD41/CD61 (active form of integrin αIIbβ3; clone JON/A), PE-labeled anti-CD62P (P-selectin; clone Wug.E9), and the corresponding PE-labeled negative control (Emfret Analytics GmbH & Co, Eibelstadt, Germany). The cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) was from Sigma-Aldrich (St Louis, MO, USA). Antibodies for immunoprecipitation were against GPIbα (clone AK2; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and for western blotting against GPIbα (clone SZ2; Beckman Coulter, Brea, CA, USA), GPV (clone CLB-SW16; Monosan, Uden, the Netherlands) and 14-3-3ζ (C-16, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were Alexa Fluor 680-conjugated goat-α-rabbit and goat-α-mouse (Invitrogen, Carlsbad, CA, USA). The antibody used for Förster resonance energy transfer (FRET) analysis of GPIbα co-localization was the recombinant 6B4-Fab fragment directed against amino acids 200-268 of GPIbα.[1] Other tools for FRET analysis were Alexa Fluor-488 and -594 protein labeling kits and cholera toxin subunit B conjugated to Alexa Fluor-594 (CTB-594; Invitrogen). O-sialoglycoprotein endopeptidase (osge) was from Cedarlane Laboratories (Burlington, ON, Canada). Polyclonal sheep-anti-VWF was from Abcam (Cambridge, UK), antibodies against P-selectin and activated αIIbβ3 (PAC-1) were from BD Pharmingen (San Diego, CA, USA)

Platelet isolation and incubations

Human platelets were isolated[2] with free-flow blood collection. The first 2 mL of blood and all collections that showed micro-aggregates were discarded. Procedures were approved by the Medical Ethical Committee of our hospital; the laboratory is certified for ISO-9001:2008. Platelets were resuspended in Hepes-Tyrode's ($2x10^{11}$ cells/L, pH 7.3) and kept at room temperature (10 min), 4 h at 0°C (or indicated) or at 0°C followed by rewarming to 37°C. In a few experiments, D-glucose (100 mM) or GlcNAc (1 μM to 100 mM) was added prior to platelet storage. To lower AA content in the platelet membrane, fatty acid free-bovine serum albumin was present (75 g/L in Hepes-Tyrode, pH 7.3) during the 4 h incubation at 0°C, as applied earlier.[3-6] All incubations were without stirring, unless specifically indicated.

For isolation of murine platelets, 8-week old strain-, and sex-matched C57BL/6 wild type mice from Harlan (Boxmeer, the Netherlands) were used. The experimental protocols were approved by the local ethics committees for animal experiments. Mice were anesthetized with isoflurane and blood was collected in a 0.1 volume of 130 mM trisodium citrate by cardiac puncture and centrifuged (420 *g*, 3 min, 22°C, no brake). The pellet, together with one third of the red blood cell fraction was collected and centrifuged again (960 *g*, 1 min, 22°C). Platelets were collected and resuspended in Hepes-Tyrode (pH 6.5), washed in 0.1 volume ACD and PGI_2 (2700 *g*, 2 min, 22°C) and resuspended in Hepes-Tyrode (pH 7.3) to a final concentration of $2x10^{11}$ platelets/L.

Ex vivo *murine platelet activation*

To investigate the hemostatic functions of stored mouse platelets after transfusion, we determined activation of integrin αIIbβ3 and P-selectin expression upon stimulation with 0.5 mM PAR-4 agonist peptide. Platelet activation was analyzed immediately after storage in the aforementioned conditions and 24 h after transfusion. Whole blood (25 μL) was diluted (1:20 (v/v) with Hepes-Tyrode buffer (pH 7.3), recalcified (1 mM $CaCl_2$), and subsequently incubated with 5 μL phycoerythrin (PE)-conjugated anti-active integrin αIIbβ3 (5 μL, JON/A) or PE-conjugated anti-P-selectin (5 μL, Wug.E9) for 15 min at 20°C. Samples were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) (20°C), and CMFDA-positive platelets were analyzed by flow cytometry.

Flow cytometric analysis

Characterization of platelets by FACS was based on forward scatter and sideways scatter (FACS-Calibur; BD Biosciences, San Jose, CA, USA). Appropriate antibodies or lectins were added and incubated for 15 min at 37°C. Ten thousand platelets were analyzed for surface expression of GPIbα, PS, sialic acid, galactose, GlcNAc, von Willebrand factor, P-selectin, activation of integrin αIIbβ3, and GPV. For determination of the mitochondrial membrane-potential $\Delta\Psi_m$, platelet suspensions were incubated with JC-1 dye (0.5 μM, 30 min, 37°C),[7] which changes emission from ~590 to ~525 nm upon depolarization. Changes in $\Delta\Psi_m$ were expressed as the ratio of platelets in lower- over upper-right quartiles.[8,9]

Immunoprecipitations and western blots

Platelet suspensions were added to lysis buffer.[2] For immunoprecipitations, 450 μL washed platelets ($5x10^{11}$ platelets/L) were lysed, (15 min, 0°C), centrifuged (10 000 *g*, 10 min, 4°C) to remove cell debris and mixed with 55 μL (10% vol/vol) protein G beads together with antibody (1 μg/mL, 30 min, 4°C, rotating).[7] Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and subjected to western blotting. After blocking with Odyssey Blocking buffer, membranes were incubated with primary antibodies (1 μg/mL) and protein bands visualized with an Odyssey Imaging system (LI-COR Biosciences, Lincoln, NE, USA). Quantification was performed with Image-J software (NIH, Bethesda, MD, USA). Possible lane-to-lane loading variation was corrected by normalization to the immuno-precipitated protein.

Analysis of GPIbα distribution by Förster resonance energy transfer, measured by fluorescence lifetime imaging microscopy

The 6B4-Fab fragment was labeled with Alexa Fluor-488 or -594, which was performed as recommended by the manufacturer. In short, 300 μg of Fab-fragment were incubated with 40 μg of DMSO dissolved amine-reactive Alexa-Fluor dye for 1 h at room temperature in the dark. Labeled Fab fragment was separated from non-reacting dye using 0.5 mL Zeba desalt spin columns (Thermo Scientific, Waltham, MA, USA). Labeling efficiency was determined with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware), and was on average 2.5 Alexa-Fluor label per Fab fragment. The final concentration of the fluorescent 6B4-Fab fragments used for cell labeling varied between 1 and 1.5 μg/mL.

Platelets were prepared for immunohistochemistry and FRET/fluorescence lifetime imaging microscopy (FLIM) analysis by fixation with 2% paraformaldehyde for 30 min at room temperature. Platelets were subsequently fixed to glass slides by cytospin centrifugation (Shandon Cytospin 3, Astmoor, UK) and dried for 10 min. After three wash steps in phosphate-buffered saline (PBS), cells were blocked with 1% bovine serum albumin in PBS (30 min) and incubated with 6B4-488, 6B4-594 (both 1 μg/mL) or cholera toxin B-594 (2 μg/mL) for 1 h at 37°C. Cells were washed again, embedded in mowiol and stored at -20°C until further use. Wide field microscopy was performed using an Axio Observer Z1 microscope equipped with an AxioCam MRm CCD camera using a Zeiss 100x/1.3 EC PlanNeoFluar oil immersion lens (Carl Zeiss MicroImaging GmbH, Gottingen, Germany).

Clustering of GPIbα and translocation to lipid rafts was determined by FRET using FLIM, as previously described[10] with minor adaptations. Compared with conventional FRET approaches this technique has the advantage that it is insensitive to variations in the concentration and emission intensity of fluorophores (for a review see Wallrabe *et al.* 2005).[11] A Nikon PCM 2000 confocal scanning laser microscope (CSLM) was equipped with a fluorescence lifetime imaging module (LiMo Nikon Instruments, Badhoevedorp, the Netherlands),[12] which captures four images representing the total fluorescent intensity in four consecutive time gates of approximately 2 ns each (*Online Supplementary Figure S1A*; donor and B; donor + acceptor). The analyzed surface area was a quadrant of 50x50 μm and contained approximately 50 platelets. The four-gate intensity decays recorded for each pixel were fitted with a monoexponential decay using the LiMo software to generate lifetime images. An intensity threshold was set to exclude pixels with a photon count too low to accurately fit exponential decay. The fluorescence lifetimes were plotted in a histogram that was fitted with a Gaussian function (using GraphPad Prism 5; San Diego, CA, USA) to determine the average lifetime (*Online Supplementary Figure S1C*). To determine variation in FRET efficiency, the lifetimes of three randomly chosen quadrants were quantified and a Student's t-test was performed to determine the statistical significances (*Online Supplementary Figure S1D*). The lifetime values were subsequently used to calculate the FRET efficiency, defined as

$$FRET\ efficiency = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100\%$$

where τ is the lifetime in ns in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor.

6B4-Fab binding to platelets

Alexa Fluor-488 and -594 conjugated 6B4-Fab (6B4-488 and 6B4-594, respectively) binding to platelets was tested in an enzyme-linked immunosorbent assay system in which the Fab fragment was added, in a 1:2 (vol:vol) dilution series into wells pre-coated with platelets. Unbound Fab fragment was removed and binding was quantified using a Spectramax M2e microplate reader (Molecular Devices, Sunnyvale, CA, USA). Respective excitation and emission wavelengths for analysis of 6B4-488 binding were 494 and 520 nm, whereas they were 590 and 619 nm for analysis of 6B4-594 binding.

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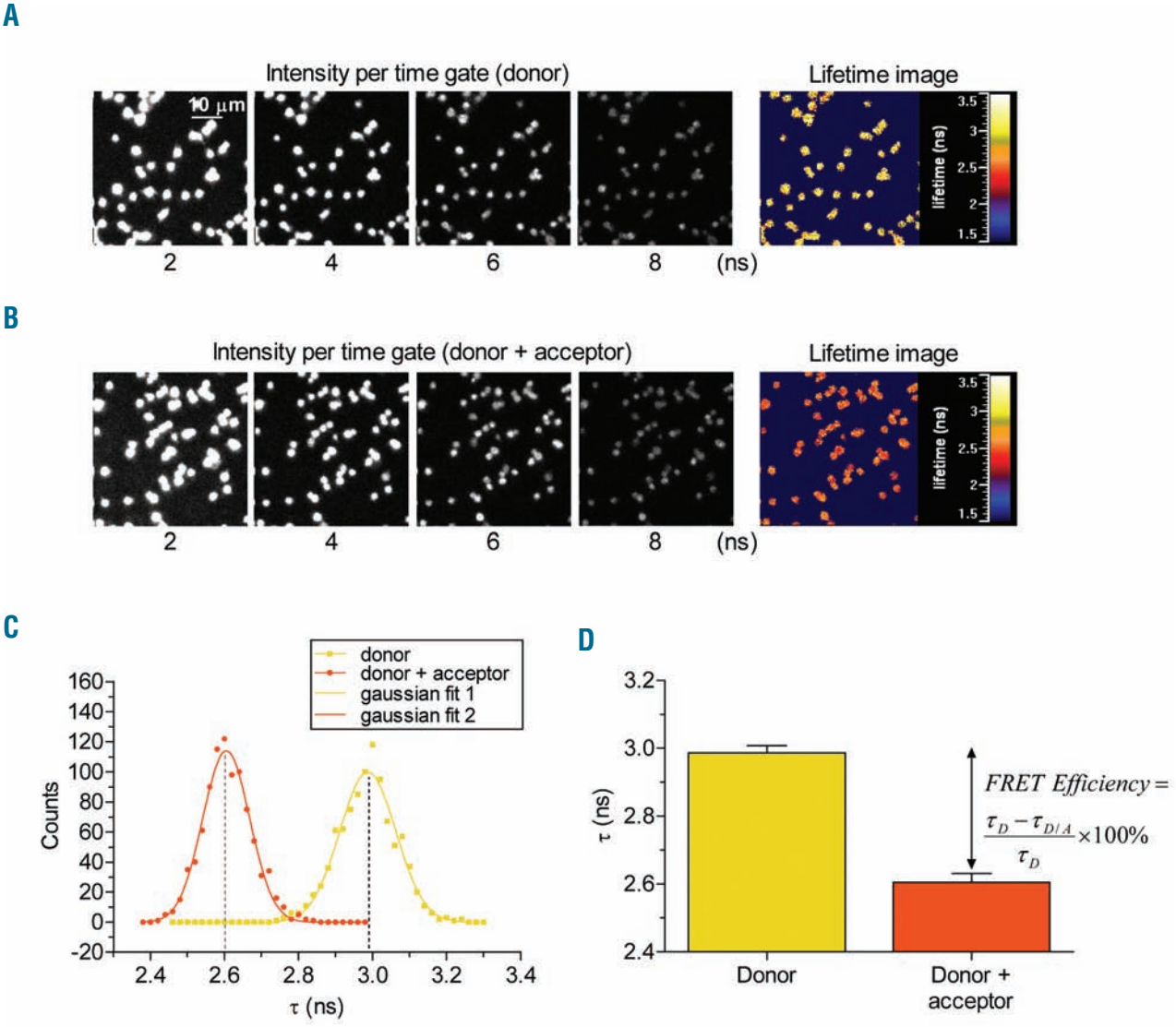
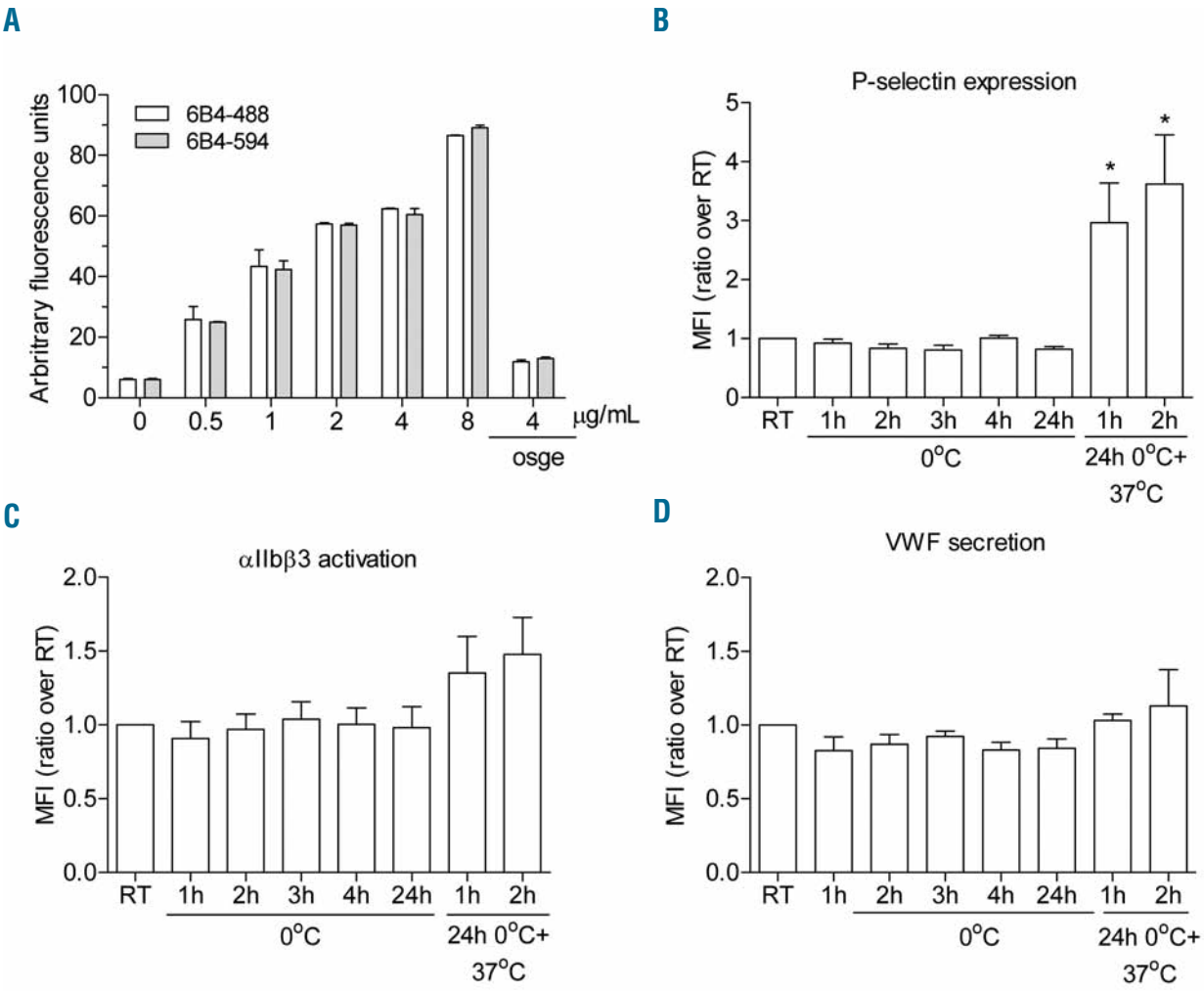
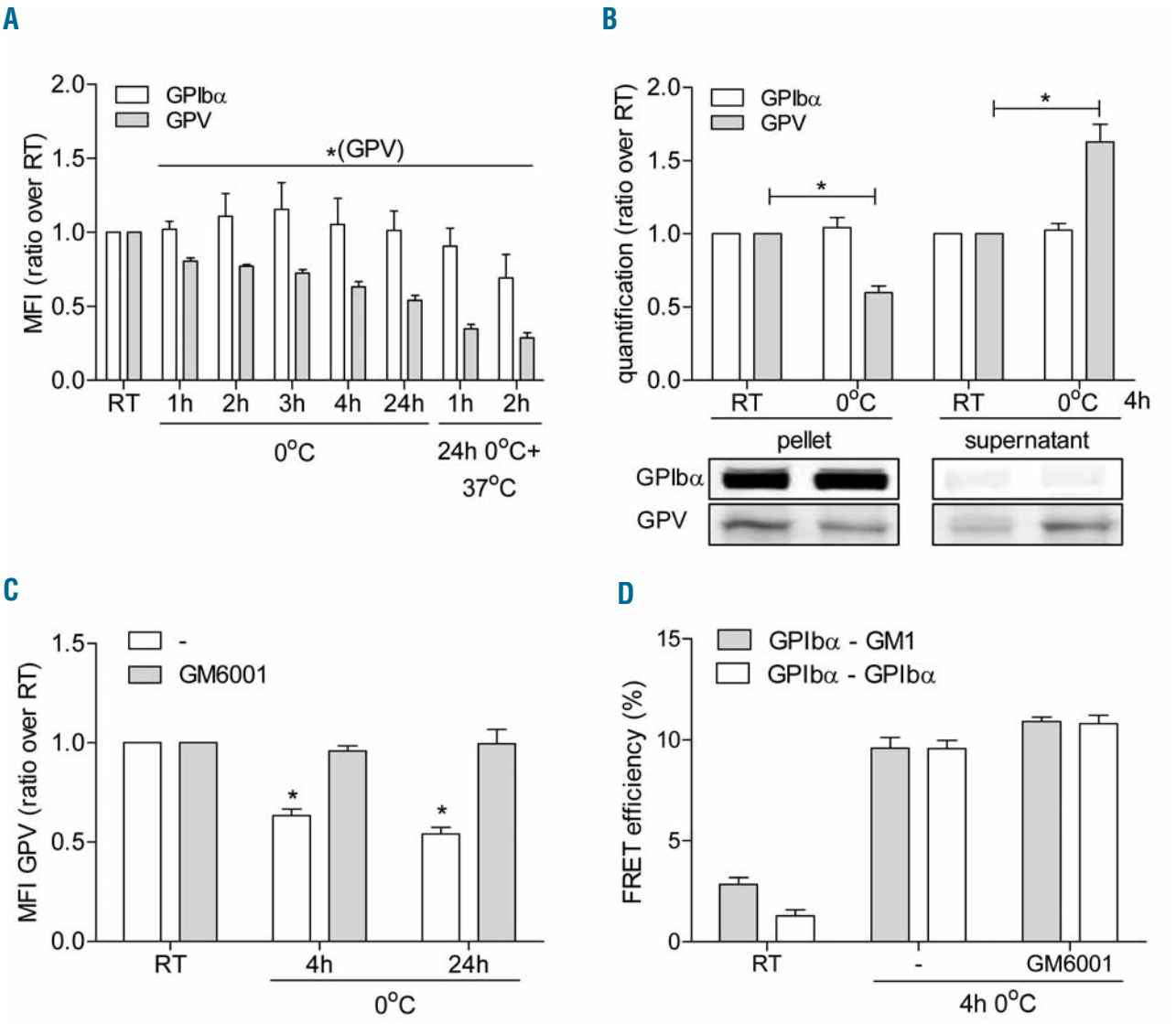


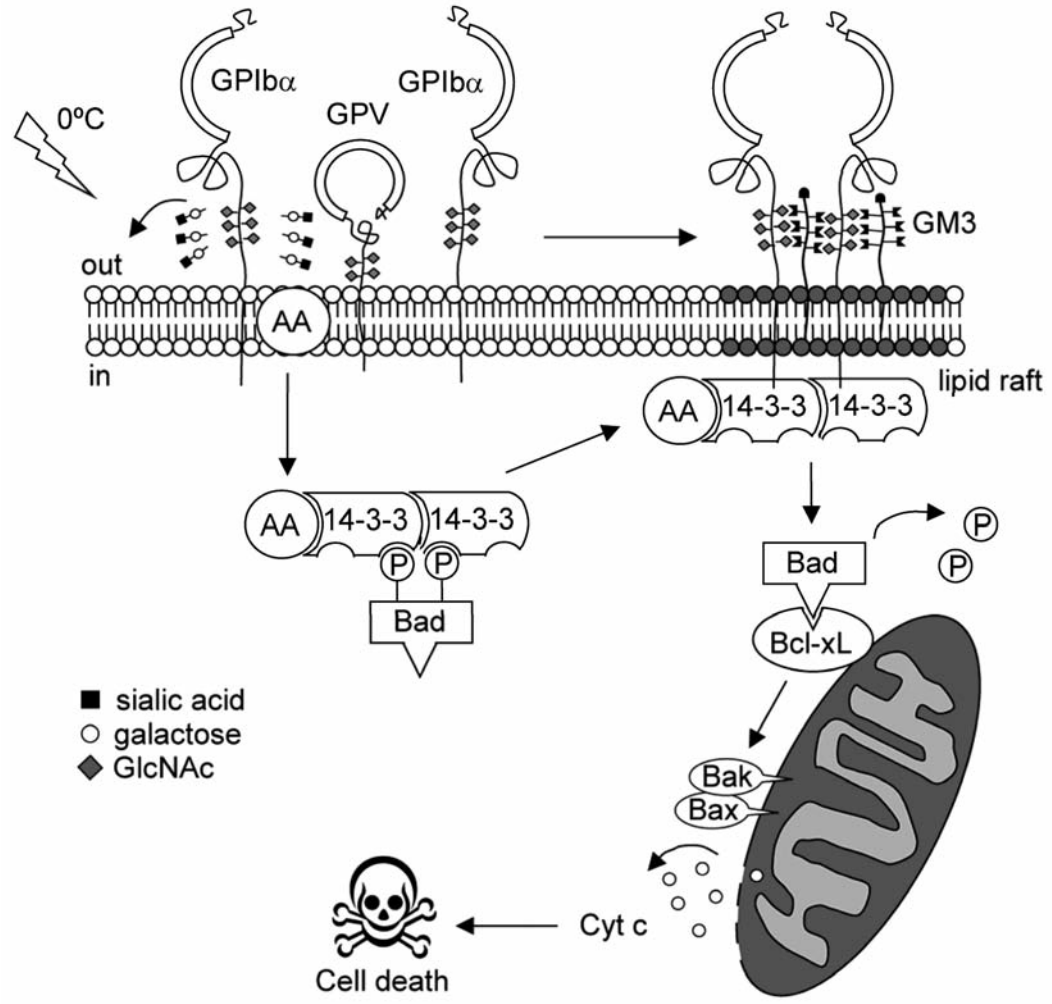
Online Supplementary Figure S1. Analysis of FRET/FLIM data. Intensity images from the four consecutive 2 nanoseconds (ns) time gates and the lifetime image of platelets stored for 4h at 0°C labeled with 6B4-488 (donor) in the absence (A) and presence (B) of acceptor probe 6B4-594. (C) Determination of the average τdonor value by Gaussian fitting. (D) The average donor lifetime τ ± SEM (n=6) in the absence or presence of acceptor for >50 platelets per measurement. The formula for calculating FRET efficiency is also shown.



Online Supplementary Figure S2. Binding of 6B4-Fab fragments to platelets and activation during cold storage. (A) Platelets were incubated with indicated concentrations of Alexa Fluor-488 and -594 conjugated 6B4-Fabs (6B4-488 and 6B4-594, respectively). Unbound 6B4-Fab fragments were removed and the fluorescence intensities were determined. To demonstrate specific binding to GPIbα, platelets were preincubated with 80 µg/mL O-sialoglycoprotein endopeptidase (osge; 30 min, 37°C), which selectively cleaves the *N*-terminal part of GPIbα, and again analyzed for 6B4-Fab binding. (B-C-D) Flow cytometric analysis of (B) P-selectin expression, (C) activation of αIIbβ3 and (D) surface-bound VWF during cold storage of platelets and subsequent rewarming. (B-D) Data statistically compared to platelets stored at room temperature (RT).



Online Supplementary Figure S3. Surface expression of GPIbα and GPV during cold storage of platelets. (A) Flow cytometric analysis of surface expression of GPIbα (open bars) and GPV (gray bars) during cold storage and rewarming of platelets at indicated times. (B) Assessment of surface expression and shedding of GPIbα and GPV by immunoprecipitation. Platelets were kept at room temperature (RT) or stored for 4 h at 0°C, subsequently fixed and collected by centrifugation. The presence of GPIbα and GPV was measured in pellet and supernatant. (C) Shedding of GPV is prevented by the broad-spectrum matrix metalloproteinase inhibitor GM6001. Platelets were stored for 4 and 24 h at 0°C in the presence of DMSO (control; open bars) or 100 μM GM6001 (gray bars). (D) GPV shedding does not change [GPIbα-GM1] (gray bars) and [GPIbα-GPIbα] (open bars) associations, analyzed by FRET-FLIM. Pre-incubation with 100 μM GM6001 does not prevent the cold-induced redistribution of GPIbα. (A-C) Data are expressed as the ratio of treated platelets over RT platelets.



Online Supplementary Figure S4. Schematic representation of cold-induced GPIbα clustering in lipid rafts. (1) Cold triggers the removal of sialic acid and galactose from glycans on GPIbα leading to exposure of GlcNAc residues. (2) The GlcNAc residues associate with raft component GM3, mediating GPIbα sequestration in lipid rafts, and surface expression of GPV is reduced. (3) In parallel, AA is released from membrane phospholipids and transfers 14-3-3ζ to the GPIbα cytoplasmic tail inducing clustering. (4) Association of 14-3-3ζ to GPIbα lowers 14-3-3ζ bound to pro-apoptotic Bad, resulting in dephosphorylation/activation. (5) Bad displaces Bak and Bax from pro-survival Bcl-xL, thereby activating permeabilization of the mitochondrial membrane, cytochrome C (Cyt c) release and apoptosis.