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

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ABSTRACT

Background
Storing platelets for transfusion at room temperature increases the risk of microbial infection and decreases platelet functionality, leading to out-date discard rates of up to 20%. Cold storage may be a better alternative, but this treatment leads to rapid platelet clearance after transfusion, initiated by changes in glycoprotein Ibα, the receptor for von Willebrand factor.

Design and Methods
We examined the change in glycoprotein Ibα distribution using Förster resonance energy transfer by time-gated fluorescence lifetime imaging microscopy.

Results
Cold storage induced deglycosylation of glycoprotein Ibα ectodomain, exposing *N*-acetyl-D-glucosamine residues, which sequestered with GM1 gangliosides in lipid rafts. Raft-associated glycoprotein Ibα formed clusters upon binding of 14-3-3ζ adaptor proteins to its cytoplasmic tail, a process accompanied by mitochondrial injury and phosphatidyl serine exposure. Cold storage left glycoprotein Ibα surface expression unchanged and although glycoprotein V decreased, the fall did not affect glycoprotein Ibα clustering. Prevention of glycoprotein Ibα clustering by blockade of deglycosylation and 14-3-3ζ translocation increased the survival of cold-stored platelets to above the levels of platelets stored at room temperature without compromising hemostatic functions.

Conclusions
We conclude that glycoprotein Ibα translocates to lipid rafts upon cold-induced deglycosylation and forms clusters by associating with 14-3-3ζ. Interference with these steps provides a means to enable cold storage of platelet concentrates in the near future.

Key words: platelets, cold storage, glycoprotein Ibα, 14-3-3ζ, lipid raft, clustering.

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The online version of this article has a Supplementary Appendix.

Introduction

Platelet concentrates are currently stored at 22-24°C for a maximum of 5-7 days. The major drawbacks of storage at room temperature are the growth of bacteria, which contaminate one in 2,000 platelet units[1] and the decline in platelet viability and function known as platelet storage lesion.[2] Lowering the temperature to 0-4°C may be a better alternative, but this approach introduces new problems as it affects glycoprotein (GP) Ibα. GPIbα is the major subunit of the receptor for von Willebrand factor (VWF), which traps platelets at sites of vessel damage enabling firm attachment by collagen receptors GPVI and integrin α2β1. The GPIbα change precludes introduction of cold storage in transfusion medicine since it induces apoptosis, starts hemostatic responses upon rewarming,[3] and promotes platelet clearance from the circulation.[4]

There is little insight into the GPIbα change inflicted by cold storage. The ectodomain is highly glycosylated with O- and N-linked carbohydrates, all of which are covered by sialic acid. Chilling causes deglycosylation, leading to changes in galactose and *N*-acetyl-D-glucosamine (GlcNAc) exposure. The damaged GPIbα molecules rearrange and become targets for the lectin binding domain of αMβ2 on liver macrophages and for Ashwell-Morell receptors on hepatocytes, thereby initiating platelet destruction.[4,5] Cold storage affects the cytosolic domain, which is released from the membrane skeleton and becomes a target for the adaptor protein 14-3-3ζ. The [14-3-3ζ–GPIbα] association releases phospho-Bad from the [14-3-3ζ - Bad] complex, initiating a fall in mitochondrial membrane potential and caspase-mediated phosphatidylserine expression.[3]

Chilling reduces the binding affinity of AN51 antibody directed against N-terminal amino acids 1-35, reflecting a change in a single GPIbα molecule or steric hindrance when GPIbα molecules form clusters. Added GlcNAc preserves normal AN51 binding and inhibits phosphatidylserine expression, suggesting that interference with the ectodomain affects signaling through the cytosolic tail.[6] Possibly, the ectodomain change is caused by receptor clustering, since GPIbα release from the membrane skeleton[7] and dimerizing GPIbα constructs in Chinese hamster ovary cells[8] enhance VWF signaling.

Formation of a [14-3-3ζ-GPIbα] complex requires release of arachidonic acid (AA) from membrane phospholipids, which transfers 14-3-3ζ to the cytoplasmic tail of GPIbα.[9] AA is released by cytosolic phospholipase A_2 ($cPLA_2$) upon cold-activation of the stress kinase P38-mitogen-activated protein kinase (P38MAPK).[10] Cooling of platelet suspensions reveals the first signs of P38MAPK activation and [14-3-3ζ-GPIbα] association at 10°C. This is the phase transition temperature at which membrane phospholipids shift from the liquid-crystalline phase into the gel phase and nanoscale cholesterol-rich domains coalesce to microscale signaling platforms known as lipid rafts.[11-13] The latter property is of specific interest for GPIbα, since at physiological temperature GPIbα depends on raft association to become a signaling receptor for VWF.[14]

In the present study, we investigated the effect of cold storage on GPIbα distribution using Förster resonance energy transfer (FRET), measured by time-gated fluorescence lifetime imaging microscopy (FLIM). This technique allows determination of the co-localization of two fluorescent molecules within 1-10 nm. We used Alexa Fluor-conjugated Fab-fragments of 6B4 antibody against GPIbα[15] to measure [GPIbα-GPIbα] associations and cholera toxin subunit B (CTB) against the lipid raft marker GM1 ganglioside to assess [GPIbα-GM1] associations.

Design and Methods

Materials, platelet isolation and incubations, and molecular techniques

A detailed description of the materials, platelet isolation and incubations and molecular techniques can be found in the *Online Supplementary Design and Methods*.

Analysis of glycoprotein Ibα distribution by Förster resonance energy transfer, measured by time-gated fluorescence lifetime imaging microscopy

A detailed description of the GPIbα distribution analyzed by FRET/FLIM can be found in the *Online Supplementary Design and Methods*. In brief, 6B4-Fab fragments were conjugated to Alexa Fluor-488 or -594 (6B4-488 and 6B4-594, respectively), of which the labeling efficiency on average was 2.5 dye per Fab. Fixed platelet samples were labeled with the Fabs (1 μg/mL) or CTB-594 (5 μg/mL) and clustering of GPIbα and translocation to lipid rafts was determined by FRET using FLIM. The analyzed surface area was a quadrant of 50x50 μm and contained approximately 50 platelets. The fluorescence lifetimes of the donor fluorophore (6B4-488) were determined in the absence and presence of acceptor fluorophore (6B4-594 or CTB-594) and 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 nanoseconds in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor. To determine variation in FRET efficiency, the lifetimes of three randomly chosen quadrants were quantified and analyzed for statistical significance.

Platelet survival in vivo

Platelet survival in mice was analyzed as described previously.[9]

Statistics

Data are means ± SEM (n=4), or as indicated. Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA, USA) software. Statistical differences between platelets stored at room temperature and other incubations were analyzed by the Mann-Whitney test. *P*-values less than 0.05 [(* or *_) and between incubations (l-*-l)] were considered statistically significant.

Results

Cold storage induces clustering of glycoprotein Ibα

To understand the change in the GPIbα ectodomain that introduces macrophage recognition and apoptosis induction, platelets were incubated at 0°C, fixed and incubated with antibody 6B4 Fab fragments directed against amino acids 200-268. To enable FRET-FLIM measurements, the 6B4-Fab fragment was conjugated to Alexa Fluor-488 (6B4-488; donor) and -594 (6B4-594; acceptor). Conjugation did not alter the binding capacity to GPIbα (*Online Supplementary Figure S2A*). Platelets were incubated with 6B4-488, 6B4-594 or their combination to label 50%

of GPIbα with the donor and 50% with the acceptor. Figure 1A shows that dual labeling led to a uniform distribution of both probes. The average lifetime of 6B4-488 was determined for platelets stored at room temperature and in the cold (4 h at 0°C) in the absence and presence of acceptor 6B4-594 (Figure 1B,C). Platelets stored at room temperature showed little reduction in lifetime, resulting in a FRET efficiency of 1.8±0.8%. Cold storage significantly increased FRET efficiency to 8.8±0.8% indicating that the average distance of GPIbα molecules decreased to <10 nm (Figure 1C). Thus, cold storage induces clustering of GPIbα.

To determine how fast cold incubation affected the distance between GPIbα molecules, platelets were incubated at 0°C. The FRET efficiency increased significantly after 1 h and rose further at longer incubations reaching 14.9±0.4% after 48 h (Figure 2A). To mimic post-transfusion conditions, cold-stored platelets (24 h, 0°C) were rewarmed to 37°C. This treatment did not affect the FRET efficiency, indicating that GPIbα clustering was irreversible (Figure 2B). The FRET efficiency increase was accompanied by an increase in [14-3-3ζ-GPIbα] association (Figure 2C) and depolarization of the mitochondrial membrane (Figure 2D). Cold storage was not accompanied by secretion of the α-granule marker P-selectin or activation of integrin αIIbβ3 (*Online Supplementary Figure S2B,C*), but subsequent rewarming to 37 °C increased P-selectin expression about 3-fold. Notably, neither cold storage nor rewarming increased the amount of surface-bound VWF (*Online Supplementary Figure S2D*). Together, these results demonstrate that cold storage induces a time-dependent redistribution of GPIbα, leading to irreversible clustering in the absence of VWF binding.

Clustering of GPIbα is initiated by cold-induced deglycosylation and initiates apoptosis events

One of the factors that might cause a change in the distribution of GPIbα is the loss of sugar residues during cold incubation.[4,16] GPIbα is a heavily glycosylated receptor, containing O- and N-linked glycans. The core consists of GlcNAc residues, covered by galactose residues which in turn are covered by terminal sialic acid. Earlier work indicated that short-term cold storage leads to increased GlcNAc exposure and long-term cold storage to increased galactose exposure.[4] The neuraminidase inhibitor N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) inhibited galactose exposure.[17] Using lectins that specifically bind sialic acid, galactose and GlcNAc, we examined whether cold storage leads to changes in glycan structure. Figure 3A shows that during cold storage both sialic acid and galactose were released simultaneously, leading to GlcNAc exposure. Pre-incubation with DANA fully inhibited the loss of sugars. Previous studies showed that the cold-induced GPIbα change revealed by AN51 binding and the [14-3-3ζ-GPIbα] association initiating apoptosis were blocked by addition of GlcNAc at a final concentration (100 mM) that prevented destruction of cold-stored platelets by macrophages.[3,16] In contrast, the same concentration of glucose had no effect.[3] We investigated the effect of these treatments on the GPIbα–GPIbα association (Figure 3B). DANA and GlcNAc completely abolished the cold-induced FRET efficiency increase, but glucose had no effect. These findings indicate that the exposure of GlcNAc residues on GPIbα ectodomain drives GPIbα clustering, with which excess GlcNAc interferes. DANA and GlcNAc, but not glucose, also inhibited the change in mitochondrial membrane potential (ΔΨm) and phosphatidylserine exposure, which accompany cold storage (Figure 3C). A titration experiment showed that optimal inhibition by GlcNAc was already induced at 50 μM, highlighting the specificity of GlcNAc interference (Figure 3D). Together, these data suggest that the GPIbα–GPIbα association initiates signaling to mitochondrial injury and phosphatidylserine exposure.

Cold-induced deglycosylation triggers glycoprotein Ibα to associate with lipid rafts

At physiological temperature, VWF binding triggers the association of GPIbα with membrane patches enriched in cholesterol and sphingomyelin, which are known as lipid rafts.[14,18,19] To investigate whether cold-induced GPIbα clustering involved raft association, the FRET-FLIM technique was used to determine co-localization of GPIbα, labeled with 6B4-488 (donor), and the raft marker GM1 ganglioside (GM1), labeled with CTB conjugated to Alexa Fluor-594 (CTB-594; acceptor). Figure 4A shows the presence of GM1 on the platelet surface, in a distribution overlapping with staining of GPIbα. FRET-FLIM analysis of the [GPIbα-GM1] and [GPIbα-GPIbα] associations revealed that platelets stored at room temperature have little GPIbα co-localized with GM1 (Figure 4B). Cold storage raised the FRET efficiency of the [GPIbα-GM1] association to 9.8±1.9%, which is in the range found for the [GPIbα-GPIbα] association (9.4±2.2%). Interestingly, DANA or GlcNAc (50 μM), which blocked clustering of GPIbα, also prevented the association of GPIbα with lipid rafts. Addition of exogenous GM1 partially decreased the two types of interaction. Addition of GM3, which can interact with GlcNAc-exposing receptors,[20] completely abolished both [GPIbα-GM1] and [GPIbα-GPIbα] associations. To assess the applicability of inhibitors of GPIbα clustering to transfusion medicine, platelets were stored (for 4 h, 0°C) in the presence of DANA and GlcNAc, which were then removed by centrifugation (Figure 4C). As expected, removal of DANA left protection against GPIbα clustering intact but removal of GlcNAc started a delayed GPIbα-GPIbα association to the range found in GlcNAc-free suspensions. This property would release inhibition of cold-induced GPIbα clustering when transfused platelets enter the circulation. Chilling of platelets is accompanied by raft redistribution below the phase transition temperature of the plasma membrane (about 10°C).[12,13,21] Analysis of GPIbα associations with GPIbα and GM1 at different temperatures confirmed that both types of association started when the temperature fell to 10°C, suggesting a close dependence on raft redistribution (Figure 4D). Together, these findings show that under different conditions associations between [GPIbα-GM1] and [GPIbα-GPIbα] go hand in hand.

A second cause of cold-induced GPIbα clustering might be shedding of constituents of the $[GPIb\alpha]_2$-$[GPIb\beta]_4$-[GPV]-$[GPIX]_2$ complex, thereby removing steric hindrance for GPIbα to form clusters. During cold storage and subsequent rewarming, there was little change in GPIbα ectodomain content, as demonstrated by flow cytometry, but GPV ectodomain expression fell by 50% during chilling and by another 50% during rewarming (*Online Supplementary Figure S3A*). To account for possible affinity changes of the antibodies at lower temperature, platelets were stored at 0°C and GPIbα and GPV were measured in

immunoprecipitates of pellet and supernatant. *Online Supplementary Figure S3B* confirms that surface expression of GPV decreased during cold storage, while expression of GPIbα remained unchanged. Shedding of GPV is regulated by ADAM17 and inhibited by the broad-spectrum matrix metalloproteinase inhibitor GM6001.[22] As expected, the inhibitor prevented GPV loss during cold storage (*Online Supplementary Figure S3C*), but had no inhibitory effect on [GPIbα-GM1] and [GPIbα-GPIbα] associations (*Online Supplementary Figure S3D*).

Binding of 14-3-3ζ to the cytoplasmic tail of glycoprotein Ibα regulates clustering in lipid rafts

We demonstrated recently that cold storage induces the release of AA from the plasma membrane upon activation of $cPLA_2$ by the stress kinase P38MAPK.[9] The liberated AA then acts as a carrier for 14-3-3ζ transfer from multiple 14-3-3ζ-associated proteins to GPIbα. To investigate the contribution of 14-3-3ζ transfer to the GPIbα raft- and self-association, platelet AA content was lowered by incubation with fatty acid-free albumin (AA depletion, in short), as described in detail in an earlier report.[9] This treatment left [GPIbα-GM1] complex formation undisturbed but completely blocked formation of [GPIbα-GPIbα] complexes (Figure 5A). Conversely, when normal platelets were incubated with exogenous AA, the [GPIbα-GM1] association remained unchanged but formation of a [GPIbα-GPIbα] complex increased significantly. To confirm that AA affected GPIbα clustering by regulating the 14-3-3ζ association, [14-3-3ζ-GPIbα] complex was measured in normal and AA-depleted platelets. Cold incubation induced a 2.5-fold increase in [14-3-3ζ-GPIbα] complex, confirming earlier observations.[9] Lowering of endogenous AA completely abolished this increase. The same effect was seen in platelets treated with DANA (Figure 5B). Together these findings suggest that GPIbα must undergo translocation to lipid rafts before 14-3-3ζ can bind and induce GPIbα clusters.

Inhibition of glycoprotein Ibα clustering improves the survival of cold-stored platelets without loss of hemostatic function

To clarify how the association of cold-damaged GPIbα and [AA-14-3-3-ζ] affected the survival of platelets, we incubated murine platelets for 4 h under conditions that interfere with the change in GPIbα and the release of AA. To this end, 5-chloromethyl fluorescein diacetate (CMFDA)-labeled platelets were stored for 4 h in the absence of cold (room temperature controls), at 0°C to induce GPIbα damage and AA release, at 0°C with GPIbα protection (DANA) and AA release and at 0°C with GPIbα protection and impaired AA release (AA-depleted platelets). Recovery and survival of platelets stored at room temperature were ~83% and 80 h, respectively (Figure 6A,B). Cold storage decreased these values by about 25% and 15%. Both parameters improved upon addition of DANA. Importantly, a combination of DANA and AA depletion raised recovery and survival above levels observed for platelets stored at room temperature. Thus, optimal survival of cold-stored platelets requires both arrest of extracellular GPIbα deglycosylation and intracellular 14-3-3-ζ translocation.

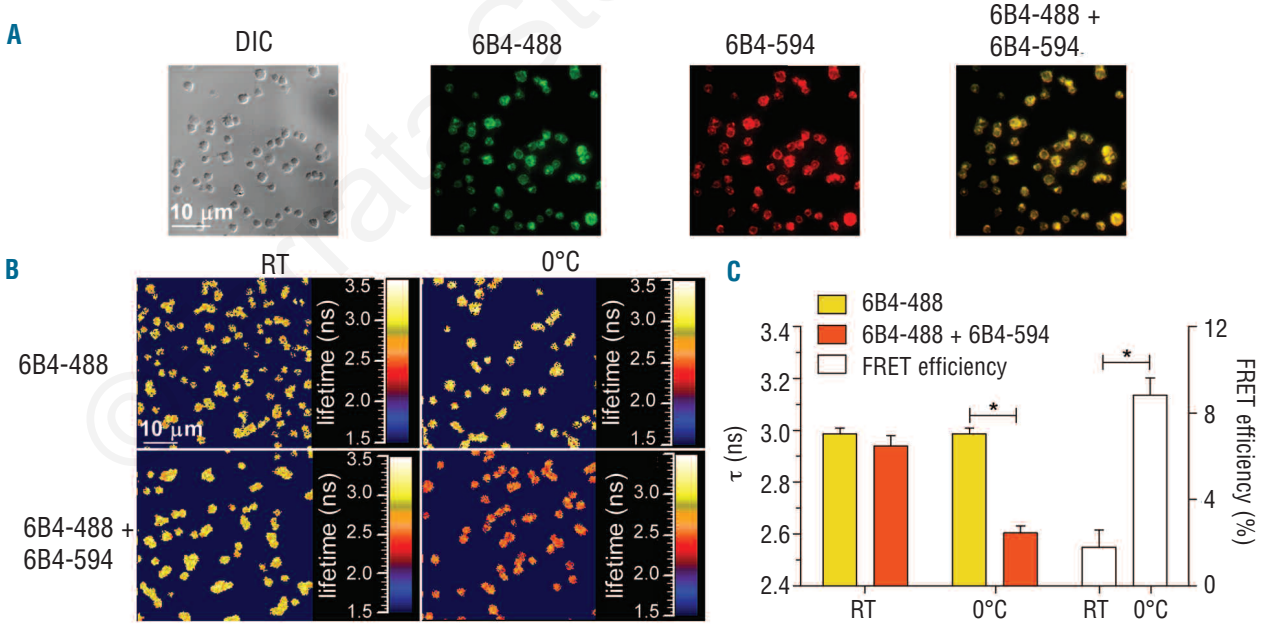


Figure 1. FRET/FLIM analysis of GPIbα distribution. Platelets were kept at room temperature (RT) or 0°C for 4h, fixed with 2% paraformaldehyde and attached to glass slides by cytospin centrifugation. (A) Platelets shown by differential interference contrast (DIC) and fluorescence microscopy. GPIbα was immunostained with 1 µg/mL 6B4-488 (donor) and 1 µg/mL 6B4-594 (acceptor) to obtain 50% GPIbα labeled with 6B4-488 and 50% with 6B4-594. (B) False color images of the donor probe fluorescence lifetimes of RT- and cold-stored platelets in nanoseconds (ns) in the absence (top panels) and presence (bottom panels) of acceptor probe. (C) Quantification of fluorescence lifetime values of donor probe in the absence (yellow bars) and presence of acceptor probe (orange bars) and corresponding mean FRET efficiencies (open bars) ± SEM (n=6).

It is unlikely that a neuraminidase inhibitor such as DANA will interfere with the signaling properties of GPIbα and other receptors but depletion of AA stores will compromise thromboxane A_2 production and might disturb hemostatic properties. We addressed this possibility by analyzing surface P-selectin expression after *ex vivo* stimulation with PAR-4 agonist peptide. P-selectin expression in CMFDA-labeled platelets analyzed immediately after cold storage (4 h, 0°C) was similar to that observed in platelets stored at room temperature (Figure 6C; t=0). Treatment with DANA had no effect either. A second analysis of platelet reactivity 24 h after transfusion showed that P-selectin expression was preserved with and without DANA. Analysis of αIIbβ3 activation showed similar results (Figure 6D). In contrast, the combination of DANA treatment and AA depletion induced a significant fall in P-selectin expression and αIIbβ3 activation immediately after cold storage. Since DANA alone had no effect, this fall was due to the reduced AA stores. Interestingly, both responses had normalized following 24 h in the circulation. These data suggest that the recovery of AA stores after prior depletion observed *in vitro*[9] also occurs *in vivo*. Thus, inhibition of GPIbα clustering prevents the cold-induced fall in platelet survival without compromising hemostatic functions.

Discussion

The better preservation of platelet functions and the lower bacterial growth at 0°C make cold storage an attractive alternative for current procedures for platelet preservation at room temperature.[23-25] Unfortunately, the cold-inflicted changes in GPIbα collectively defined as GPIbα clustering prevents rapid introduction into transfusion medicine. The present results describe the molecular mechanism of cold-induced GPIbα clustering. First, sialic acid and galactose are removed exposing GlcNAc residues on the GPIbα ectodomain. Second, GlcNAc residues associate with the raft constituents GM1/3. Third, the adaptor protein 14-3-3ζ binds to the GPIbα cytoplasmic tail inducing a mechanism that lowers the average distance between GPIbα molecules to less than 10 nm. This subsequently leads to mitochondrial injury and phosphatidylserine exposure. They are also factors controlling platelet survival *in vivo* since inhibition of sugar loss (DANA) and inhibition of 14-3-3ζ translocation (AA depletion) improve recovery and survival of cold-stored platelets.

It has recently been shown that cold storage triggers surface up-regulation of neuraminidase-1 and β-galactosidase, which co-localize in granule-like structures under resting conditions.[17] Neuraminidase inhibition (DANA) blocks both release of sialic acid and galactose and the GPIbα-GPIbα association revealed by FRET/FLIM, indicating that sugar loss is a first step in GPIbα clustering. The removal of sialic acid and galactose induced by cold go hand in hand, indicating that loss of sialic acid residues makes galactose residues accessible to β-galactosidase. Conversely, neuraminidase blockade prevents β-galactosidase from reaching its substrate.

Loss of sialic acid/galactose exposes GlcNAc residues that associate with ganglioside GM1/3-rich areas in lipid rafts, as determined by FRET/FLIM analysis of GPIbα and GM1. This reaction is accompanied by GPIbα-GPIbα associations, as detected by the same technique. Addition of exogenous GM1, GM3 or GlcNAc inhibits GPIbα-GM1/3 associations, which is in agreement with direct

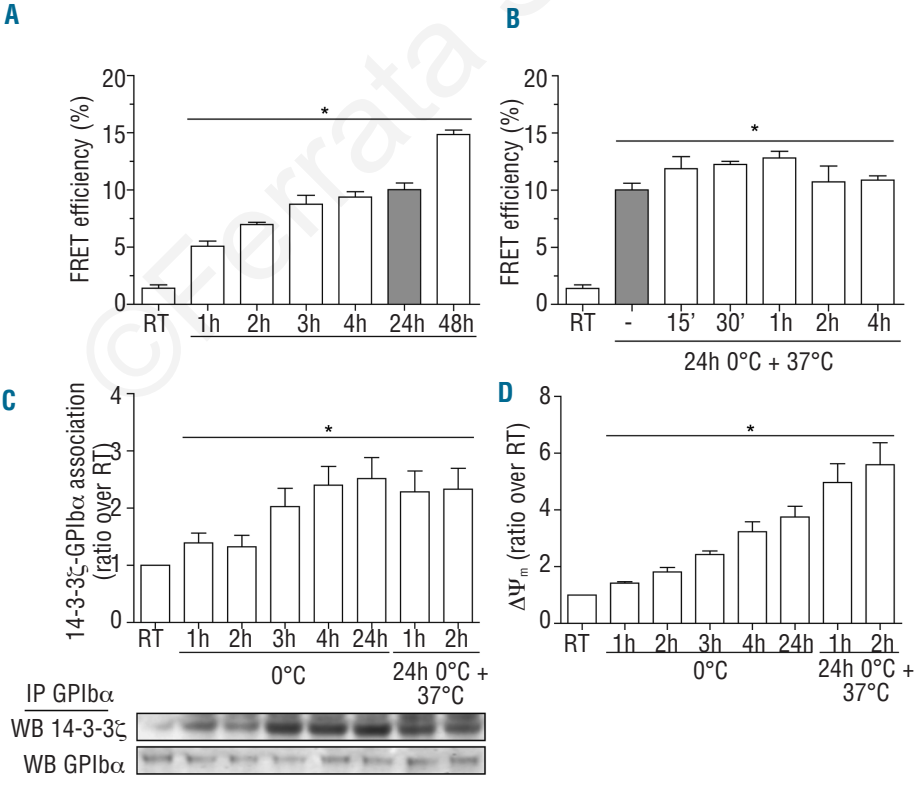


Figure 2. Cold storage induces irreversible clustering of GPIbα and downstream signaling events. (A) Platelets were incubated for indicated times at 0°C and GPIbα distribution was analyzed by FRET/FLIM. (B) Rewarming of platelets to 37°C after 24 h of cold storage did not affect the FRET efficiency, indicating that cold-induced GPIbα clustering is irreversible. (C) Formation of [14-3-3ζ-GPIbα] complex in platelets stored at room temperature (RT) and during cold storage/rewarming. (D) Cold storage leads to a collapse of the mitochondrial membrane potential (ΔΨm), indicated by the cationic dye JC-1. (C, D) Data are expressed as the ratio of treated platelets over platelets, stored at RT.

binding of GPIbα-bound GlcNAc to raft-bound GM1/3. Importantly, interference with GPIbα-GM1/3 associations also blocks GPIbα-GPIbα associations. This implies that GPIbα clustering is a direct consequence of its association with specific domains in lipid rafts. Gangliosides are glycosphingolipids with different carbohydrate chains that extend out from the cell surface and are involved in cell-cell-recognition, adhesion and signal transduction.[26] Both GM1 and GM3 concentrate in lipid rafts where they can coincide and form clusters.[27] The carbohydrate-carbohydrate interaction between GlcNAc and GM3 seems quite specific as GM1, which differs from GM3 in that it has an extra galactose and N-acetyl-galactosamine residue, only partially blocked GPIbα clustering and GM3 induced full inhibition. Earlier work showed that cold lowers the binding of an antibody directed against the GPIbα *N*-terminal flank, a change that could be prevented by GlcNAc.[6] This antibody binds to GPIbα amino acids 1-35 and the affinity change induced by cold appears to parallel the association of GPIbα residues 200-268 covered by 6B4-Fab fragments bound to the FRET/FLIM labels.

Conventional sucrose density fractionation showed earlier that 10-15% of total GPIbα is located in rafts in resting platelets, which increases 3-fold upon stimulation with VWF.[14,19] GPIbα translocation to rafts is an important step in VWF signaling since cholesterol depletion inhibits the major functions of the receptor complex, including ristocetin-induced platelet aggregation and adhesion to VWF under conditions of flow. The FRET/FLIM technique for assessing the GPIbα-GM1/3 interaction shows a 3-4% FRET efficiency at room temperature and a 4-fold increase during cold incubation, also indicating that in resting platelets only a minor part of GPIbα is bound to rafts and that this fraction increases upon stimulation. This shift occurs in the absence of VWF and represents a type of ligand-independent raft association. It might also explain why cold storage increases binding of VWF.[28]

A final step in cold-induced GPIbα clustering is the binding of 14-3-3ζ adaptor protein to the cytosolic tail. This reaction is restricted to raft-bound GPIbα since blockade of raft association with DANA inhibits both the GPIbα-GPIbα and 14-3-3ζ-GPIbα associations. Both associations are lower in AA-depleted platelets than in control platelets. Cold storage activates the stress kinase P38MAPK, which together with cPLA2 releases AA from membrane phospholipids. Lipid rafts are enriched in AA and might be the source of the released AA.[29] Released AA then accumulates due to poor COX-1 activity at low temperature.[9] AA binds directly to 14-3-3ζ, induces 14-3-3ζ multimerization and releases the protein from binding partners.[30] One of these binding partners is ADF/cofilin, a protein that destabilizes actin filaments when dephosphorylated upon release from 14-3-3ζ.[31] In muscle cells, the 14-3-3ζ association regulates clustering of acetylcholine receptor.[32] A similar process may contribute to clustering of GPIbα. The cytoplasmic tail of GPIbα has multiple binding sites for 14-3-3ζ.[33-35] The domain of amino acids 551-564 contains the binding site for filamin A (amino acid ser559),[36] which anchors GPIbα to the membrane skeleton under resting conditions. The functional activity of 14-3-3ζ depends on its dimerization[37] and it has been indicated that a single 14-3-3ζ dimer can disrupt this interaction by competitive binding, thereby releasing GPIbα.[35] In a next step, 14-3-3ζ dimer/multimer might bind two or more GPIbα molecules, creating a 'crosslink platform' that facilitates clustering.

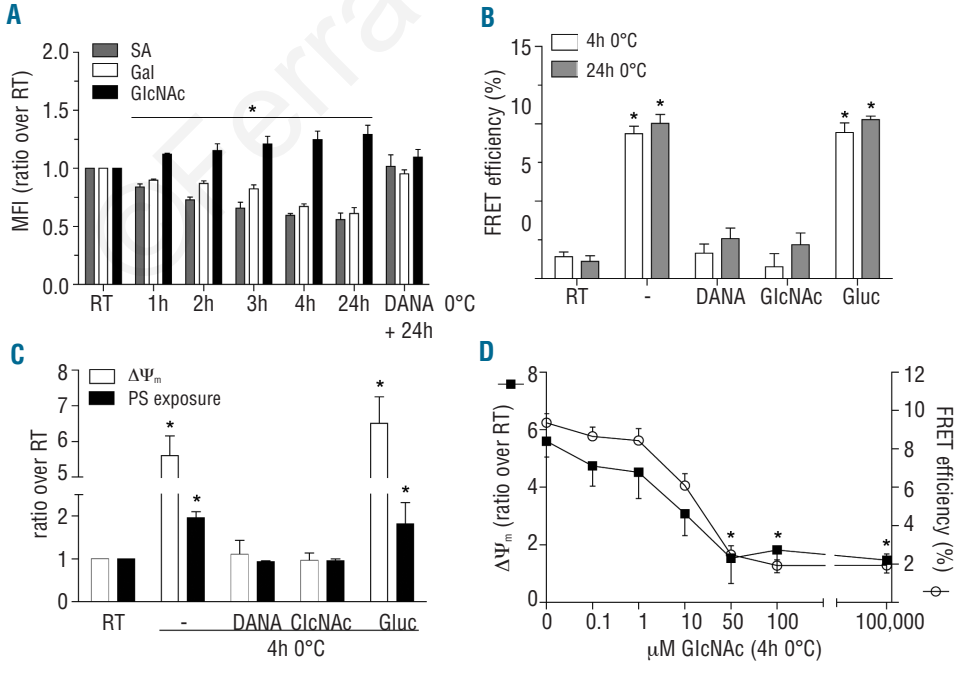


Figure 3. Deglycosylation triggers cold-induced GPIbα clustering leading to apoptosis events. (A) FACS analysis of sialic acid (SA), galactose (Gal) and GlcNAc exposure, as detected with SNA, RCA-1 and sWGA FITC-labeled lectins, respectively. Lectin binding to platelets stored at room temperature (RT) and cold-incubated platelets is shown at indicated times, in the absence and presence of the neuraminidase inhibitor DANA (200 μM). Data are presented as the ratio of mean fluorescence intensity (MFI) of treated platelets over RT platelets. (B) GPIbα distribution measured by FRET/FLIM of platelets stored for 4 h (open bars) or 24 h (gray bars) at 0°C pre-incubated with or without DANA (200 μM), GlcNAc (100 mM), or glucose (Gluc; 100 mM). (C) The mitochondrial membrane potential ($\Delta\Psi_m$; open bars) and phosphatidylserine (PS) exposure (black bars) in the conditions of the experiment shown in Figure 3B. (D) GlcNAc dose-dependently inhibits GPIbα redistribution and mitochondrial depolarization. Cold-stored platelets (4 h) were preincubated with indicated concentrations of GlcNAc and $\Delta\Psi_m$ (black squares) and GPIbα clustering (open circles) was measured. (C,D) Data are expressed as the ratio of treated platelets over RT platelets.

AA-depletion disturbs the GPIbα-GPIbα interaction, but leaves the GPIbα-GM1/3 interaction intact. This interesting discrepancy separates GPIbα binding to rafts (AA-independent) from the [GPIbα-GPIbα] association (AA-dependent). Cooling of platelets leads to the coalescence of small rafts into larger patches, which then become signaling platforms.[11-13] This is particularly evident when the temperature decreases below ~10°C and membrane phospholipids change from the liquid-crystalline phase into the gel phase. We showed earlier that the P38MAPK-mediated AA-release starts below this temperature and the start of GPIbα-GPIbα associations below 10°C might well result from the AA-mediated attachment of 14-3-3ζ to the GPIbα cytosolic tail. However, GPIbα binding to GM1/3 also starts when the temperature falls below 10°C. Since this reaction is AA-independent, a direct effect of the phase transition on GPIbα binding to rafts is evident. Together, these data imply that the phase transition of the plasma membrane contributes to GPIbα clustering at two levels: first, by stimulating GPIbα binding to rafts and second, by inducing GPIbα binding to [AA-14-3-3ζ].

One of the protein complexes that is disturbed by the cold-induced AA accumulation is [14-3-3ζ-phosphoBad], which upon release of 14-3-3ζ is dephosphorylated and becomes an activator of pro-apoptotic Bax, mitochondrial damage, caspase-9 and phosphatidylserine exposure.[9]

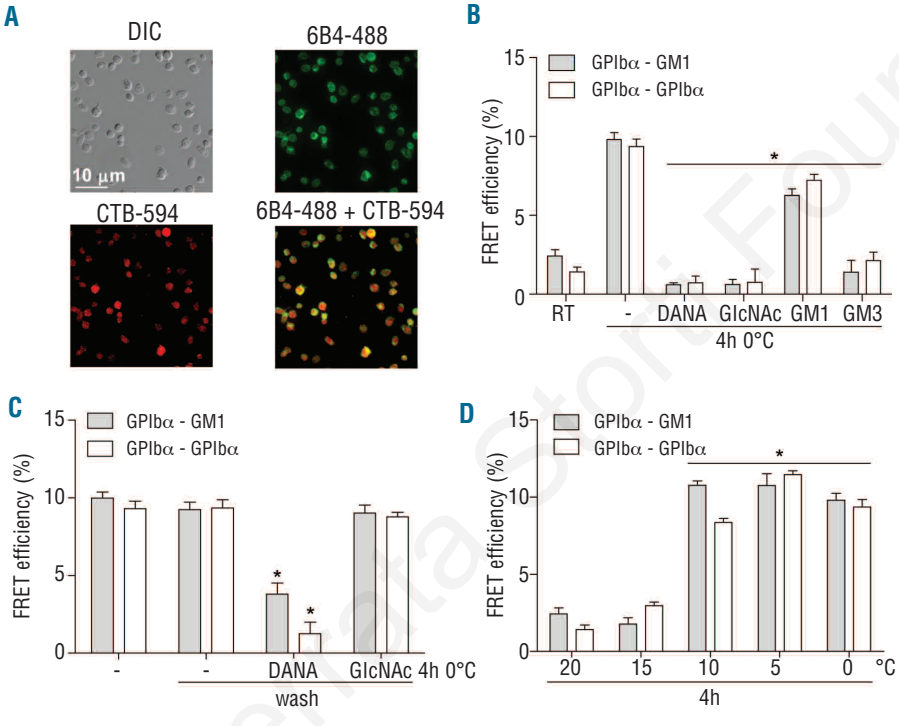


Figure 4. Cold-induced deglycosylation triggers GPIbα to cluster in lipid rafts. (A) Platelets shown by differential interference contrast (DIC) and immunostaining of GPIbα with 6B4-488 (donor probe, 1 μg/mL), labeling of raft-specific GM1 ganglioside with CTB-594 (acceptor probe; 2 μg/mL) and dual staining of donor and acceptor probe. (B-C-D) FRET/FLIM analysis of [GPIbα-GM1] (gray bars) and [GPIbα-GPIbα] associations (open bars) of platelets stored for 4 h at 0°C. (B) Cold-incubation induces the [GPIbα-GM1] association. Cold-induced redistribution of GPIbα is blocked by pre-incubation with 200 μM DANA, 50 μM GlcNAc or GM3, and partially by GM1 (50 μM). Data statistically compared to those of platelets stored at 0°C. (C) The effect of removal of DANA or GlcNAc after 4 h of cold storage on GPIbα redistribution. Platelets were stored at 0°C for 4h in the absence and presence of DANA and GlcNAc. Both agents were subsequently removed by centrifugation (wash; at 0°C) with protection of PGI_2 and again GPIbα distribution was measured. Data statistically compared to those for 0°C platelets. (D) Cooling of platelets triggers GPIbα redistribution. A temperature fall to 10°C and below triggers GPIbα clustering. Data statistically compared to those for platelets stored at room temperature (RT).

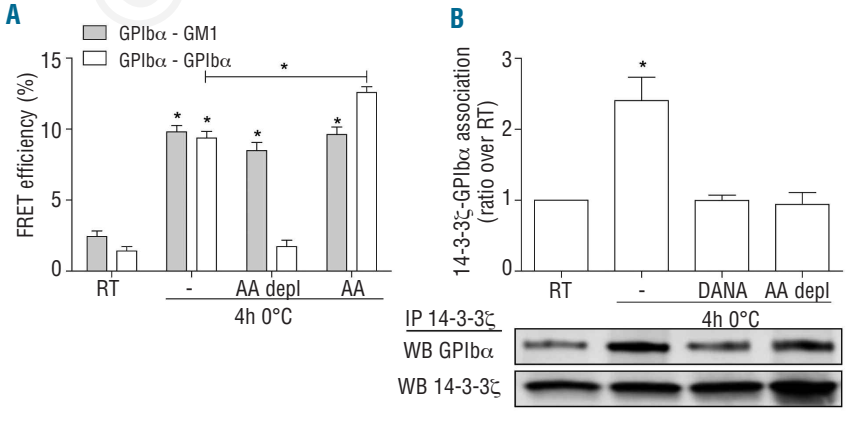
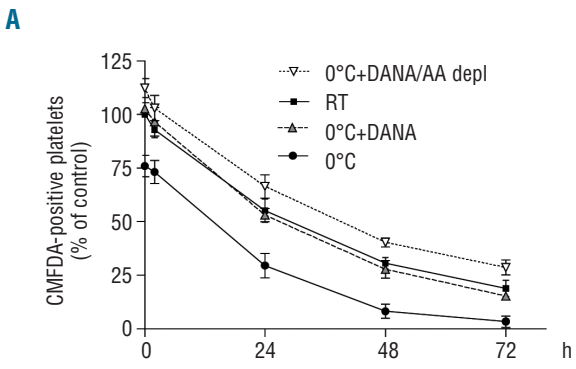


Figure 5. Arachidonic acid (AA) transfers 14-3-3ζ to the cytoplasmic tail of GPIbα, leading to clustering in lipid rafts. (A) GPIbα co-localization with GM1 (gray bars) and clustering (open bars) of cold-stored platelets after AA depletion and addition. AA content of cold-incubated platelets was lowered by incubation with fatty acid free-BSA (AA depl), leading to a decrease in [GPIbα-GPIbα]. Addition of 10 μM exogenous AA increased cold-induced [GPIbα-GPIbα]. (B) The effect of DANA and AA depletion on cold-induced [14-3-3ζ-GPIbα] complex formation. Immunoprecipitations show that addition of 200 μM DANA or AA depletion inhibits 14-3-3ζ binding to GPIbα. Data are expressed as the ratio of treated platelets over room temperature (RT) platelets.



B

	Recovery (%)	Survival (h)
RT	82.5±3.4	81.3±2.4
0°C	62.7±3.4*	64.9±2.9*
0°C+DANA	84.8±1.9[*]	76.7±1.3[*]
0°C+DANA/AA depl	92.7±3[*]	89.3±1.9*[*]

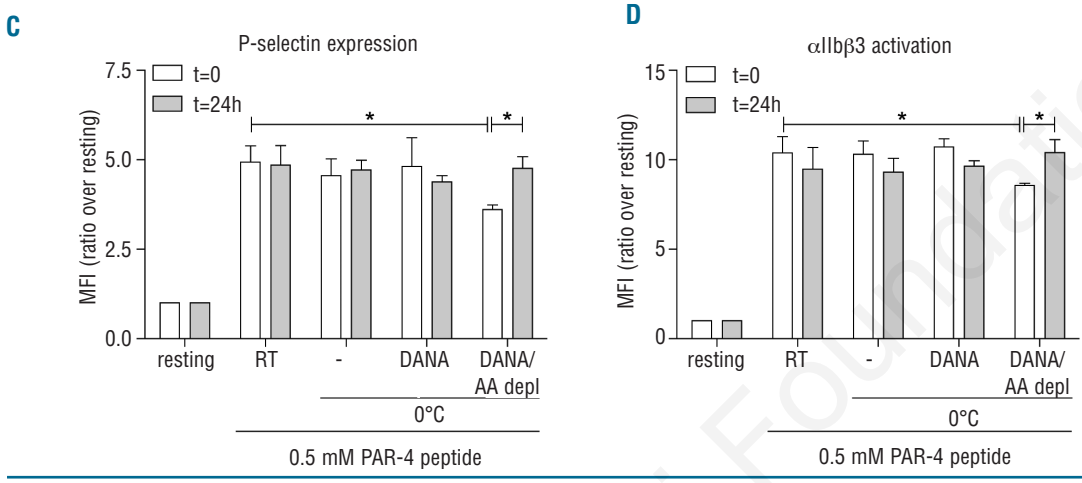


Figure 6. Inhibition of GPIbα redistribution improves the survival of cold-stored platelets without affecting their hemostatic function. (A) survival of murine platelets after cold storage. Mouse platelets labeled with CMFDA were kept at room temperature (RT) or at 0°C (4 h) in the absence or presence of 200 μM DANA or depleted of AA in combination with DANA treatment. The platelets were then washed with protection by PGI_2 and injected into recipient mice. Blood was collected at 2 min, 2, 24, 48 and 72 h after injection. Recovery of RT platelets at 2 min was set at 100%; other data are expressed as a percentage of this value. (B) Recoveries and survival times of CMFDA-labeled platelets (means±SEM of four mice in each group). Data were statistically compared to those of platelets stored at RT (*) or at 0°C ([*]). (C,D) Flow cytometric analysis of (C) surface P-selectin expression and (D) activation of integrin αIIbβ3 upon *ex vivo* platelet stimulation with 0.5 mM PAR-4 agonist peptide. The platelets were analyzed immediately after cold storage (t=0) and 24 h post-transfusion (t=24h). Data refer to CMFDA-labeled platelets and are expressed as a ratio of MFI of stimulated over resting platelets.

These reactions are inhibited completely by arrest of GPIbα deglycosylation (DANA), raft association (GlcNAc) and 14-3-3ζ binding (AA depletion) and, therefore, critically depend on the clustering of GPIbα molecules. Since this type of apoptosis induction occurs in the absence of VWF, it represents a form of ligand-independent signaling inflicted by cold.

To investigate whether interference with GPIbα clustering changed the survival of cold-stored platelets, murine platelets were stored at room temperature (controls) and 0°C in the presence of DANA without and with prior depletion of AA. DANA treatment improved platelet recovery by 35% and the combination with AA-depletion by almost 50%, thereby surpassing the recovery observed with platelets stored at room temperature. The effect of these treatments on platelet survival was smaller, albeit statistically significant, and again demonstrated that the combined treatments completely prevented the platelet disturbance inflicted by cold. Treatment with DANA alone left P-selectin expression and αIIbβ3 activation induced by PAR-4 agonist peptide undisturbed. In contrast, both responses were reduced following preparation of AA-depleted platelets, probably reflecting a shortage of thromboxane A_2 production, which is known to support these functions. Interestingly, both defects had disappeared 24 h after transfusion, raising the possibility that AA-depleted platelets accumulate extracellular AA and restore their responsiveness in the circulation.

In conclusion, inhibition of GPIbα clustering by DANA and AA depletion provides a simple means to prevent the damage that compromises the recovery and survival of cold-stored platelets.

Authorship and Disclosures

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