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A signaling pathway contributing to platelet storage lesion development: targeting PI3-kinase–dependent Rap1 activation slows storage-induced platelet deterioration

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Abstract

BACKGROUND—The term *platelet storage lesion* (PSL) describes the structural and biochemical changes in platelets (PLTs) during storage. These are typified by alterations of morphologic features and PLT metabolism leading to reduced functionality and hence reduced viability for transfusion. While the manifestations of the storage lesion are well characterized, the biochemical pathways involved in the initiation of this process are unknown.

STUDY DESIGN AND METHODS—A complementary proteomic approach has recently been applied to analyze changes in the PLT proteome during storage. By employing stringent proteomic criteria, 12 proteins were identified as significantly and consistently changing in relative concentration over a 7-day storage period. Microscopy, Western blot analysis, flow cytometry, and PLT functionality analyses were used to unravel the involvement of a subset of these 12 proteins, which are connected through integrin signaling in one potential signaling pathway underlying storage lesion development.

RESULTS—Microscopic analysis revealed changes in localization of glycoprotein IIIa, Rap1, and talin during storage. Rap1 activation was observed to correlate with expression of the PLT activation marker CD62P. PLTs incubated for 7 days with the PI3-kinase inhibitor LY294002 showed diminished Rap1 activation as well as a moderate reduction in integrin α IIB β 3 activation and release of α -granules. Furthermore, this inhibitor seemed to improve PLT integrity and quality during storage as several in vitro probes showed a deceleration of PLT activation.

CONCLUSION—These results provide the first evidence for a signaling pathway mediating PSL in which PI3-kinase–dependent Rap1 activation leads to integrin α IIB β 3 activation and PLT degranulation.

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CONFLICT OF INTEREST

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Platelet (PLT) transfusion is a life-saving medical procedure for thrombocytopenic patients. Management of the transfusion PLT inventory is challenged by the limited shelf life of 5 to 7 days. This limit is partly related to concerns about storage-related deterioration in product quality resulting in accelerated clearance from circulation and partly due to the increased risk of bacterial growth at room temperature storage.^{1,2} PLTs stored under blood bank conditions at 22 to 24°C undergo in vitro changes related to the duration of storage collectively known as the PLT storage lesion (PSL).^{3,4} This phenomenon is characterized by changes in in vitro measures of PLT function including PLT adhesion, aggregation, and PLT-dependent coagulation as well as a reduction of in vivo recovery and survival after transfusion⁵ reflecting loss of PLT integrity and function during storage.⁶ Current literature points toward a dual effect of storage on hemostatic PLT properties: appearance of activation-like processes, such as increase in surface activation markers including CD62P expression and integrin signaling, as well as reduced responsiveness toward agonists.³ As the resulting short storage period leads to constant overcollection of blood for PLTs, it is necessary to understand the molecular mechanism(s) leading to the PSL,⁴ before strategies can be developed to extend the shelf life of PLT units to minimize wastage of this resource.

Proteomics—the analysis of all proteins expressed in a biologically significant entity—has provided a new array of tools to investigate the PSL. It allows a more detailed understanding of blood elements^{7,8} and has been applied to the analysis of the proteomes of blood plasma,^{9,10} red blood cells,¹¹ and blood PLTs.^{12–18} Recently, we have applied a complementary proteomic approach to investigate changes in the human blood PLT proteome during a 7-day storage period.¹⁹ Although these analyses identified many storage-associated protein changes, they varied significantly by proteomic method suggesting that a combination of protein-centric (two-dimensional [2D] gel or fluorescence difference gel electrophoresis [DIGE]) and peptide-centric (isobaric tag for relative and absolute quantitation [iTRAQ] or isotope-coded affinity tag [ICAT]) approaches is essential to acquire adequate data.

In this study, we selected proteins based on the highest confidence in proteomic scores from the pool of proteins changing by concentration during storage identified by our previously published proteomic studies,¹⁹ resulting in 12 candidates, most of which are linked to integrin α IIb β 3 signaling. For three of them, known to be involved in PLT activation, biochemical analyses were performed to investigate their role during storage and an inhibitor study was carried out to elucidate the involvement of a signal transduction pathway underlying the PSL. These results demonstrate that the combination of proteomic data and conventional PLT biochemistry uncovered PI3-kinase–dependent Rap1 activation as one potential signaling pathway of the PSL.

MATERIALS AND METHODS

Blood PLT preparation and lysis

This study was approved by the research ethics boards of Canadian Blood Services and the University of British Columbia and informed consent was obtained from all healthy volunteers before whole-blood donation. Whole-blood collection and PLT isolation were carried out by the NetCAD development laboratory of Canadian Blood Services (Vancouver,

British Columbia, Canada). Using standard operating procedures, PLT preparation was carried out 22 hours after whole-blood collection and the buffycoat fractions of blood from four individual donors were pooled for each PLT unit, defined as Day 1. Units were stored at 22°C with agitation. Sampling from storage bags was done aseptically in biosafety cabinets; washing and lysis procedures were performed as described previously.¹⁹

Proteomic methods

The analytical methods of proteomics leading to the identification of 503 individual proteins that change in concentration over a 7-day storage period were previously described in detail.¹⁹ In brief, proteins on 2D gels (pI 4-7) were subjected to quantitation with computer software, spot excision, in-gel digestion, and mass spectrometric sequence analysis of the extracted peptides before proteins were identified by MASCOT searches against current Swiss-prot databases using standard acceptance criteria (ion scores above significance threshold [$p < 0.05$], two or more peptides matching).

For ICAT analysis, samples were differentially labeled with ICAT reagents, combined, and digested and the resulting peptides were separated by cation-exchange and reverse-phase chromatography, followed by mass spectrometric peptide sequencing. Proteins were identified by spectrum matching to the International Protein Index. For relative quantitation, the ratios of Day 7 versus Day 1 peptides were normalized to the peak of the distribution to correct for skewing.

For iTRAQ experiments, samples were protease digested, differentially labeled with the iTRAQ reagents, and combined and the resulting peptides separated by cation-exchange and reverse-phase chromatography before mass spectrometric analysis. Data were searched against the Celera Discovery Systems (CDS) or Matrix Science databases (MSDB). Relative quantification of proteins was achieved by determining the ratio of the isotope tag-specific peak areas 114/116 and 115/117 Da, respectively.

Proteins listed in Table 1 fulfilled the proteomic requirement of high significance ($p < 0.01$), which discriminates statistically relevant changes from background noise, when comparing results from Day 1 versus Day 7 as well as high confidence (> 99%) in the protein identification based on the number of unique peptides using MASCOT scoring.¹⁹ This reflects the agreement of the protein change based on the consistency (reproducibility) both within repetitions of individual runs and across different approaches. For the reproducibility within the same proteomic tool, protein changes had to agree in two of four iTRAQ runs, two of three ICAT runs, eight of eleven 2D gels or proteins identified as changing by DIGE had to show at least 1.5-fold increase or decrease. Furthermore, for the relative protein change among all proteomic tools, the protein changes had to be consistent in two of the three different methods used.

Western blotting

PLT lysates or supernatant after immunoprecipitation were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gel and blotted onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada). The membrane was probed with primary antibodies against Rap1 (Santa Cruz Biotechnology, Santa Cruz, CA) or β -actin (Sigma-

Aldrich, St Louis, MO), followed by their respective secondary horseradish peroxidase-labeled antibodies (Jackson ImmunoResearch, West Grove, PA). Bands were visualized using a Western blotting detection system (ECL Plus, GE Healthcare, Chalfont St Giles, UK) and protein band intensities were determined by densitometry using the imaging analysis software (GeneTool, SynGene, Frederick, MD) on a bioimaging system (ChemiGenius2, Perkin Elmer, Waltham, MA).

Inhibitor treatment of PLTs

To analyze the effect of the PI3-kinase inhibitor, 10 mL of buffy coat PLTs was spiked with 10 $\mu\text{mol/L}$ LY294002 (Cell Signaling Technology, Danvers, MA) or with the equivalent volume of dimethyl sulfoxide as vehicle control and incubated for 7 days at room temperature under constant agitation. Samples were drawn on Days 2, 4, and 7 and subsequent flow cytometry, biochemical analysis of PLT storage, or PLT lysis was performed. For Day 7, a stored PLT control sample was incubated with 10 $\mu\text{mol/L}$ LY294002 for 45 minutes.

Flow cytometry

Fresh PLTs and PLT buffy-coat concentrates treated without or with inhibitor and sampled on Days 1, 4, and 7 of storage were incubated with CD62P-phycoerythrin (PE), CD61-PE, or Pac-1-fluorescein isothiocyanate for 30 minutes, followed by fixation with formal saline. All samples were analyzed on a flow cytometer (FACSCanto II, BD Biosciences, Mississauga, ON, Canada) with isotype controls gated at 0.2% to calculate percentage of positive values.

Rap1 activation assay

Purified recombinant glutathione *S*-transferase (GST)-tagged Rap binding domain of Ral guanine nucleotide dissociation stimulator (RalGDS) was coupled to GSH-Sepharose by incubation at 4°C for 2 hours. PLT lysates obtained from the same number of PLTs treated under the different conditions were incubated for 1 hour at 4°C with an excess of the GST-tagged Rap-binding domain of RalGDS coupled to GSH-Sepharose. Precipitated active Rap1 was washed three times with lysis buffer (1% Nonidet P-40, 50 mmol/L Tris pH 8.0, 150 mmol/L NaCl), resuspended in sample buffer (10% vol/vol glycerol, 62.5 mmol/L Tris HCl pH 6.8, 2% wt/wt SDS, 0.01 mg/mL bromophenol blue, 5% vol/vol β -mercaptoethanol), and revealed by immunoblotting with an antibody against Rap1 (Santa Cruz Biotechnology). Aliquots of the PLT lysates were used to confirm equal loading by analyzing the total Rap1 and actin concentration.

Morphology score

The morphology of PLTs was assessed by modified morphology scoring.²⁰ PLT concentrate samples were treated with an equal volume of 4% paraformaldehyde. Samples were stored at room temperature and analyzed within 1 week by light microscopy with 1000 $\times$ oil immersion lens, phase contrast 3 (Nikon, Mississauga, ON, Canada). In total, 100 PLTs were counted from each sample and categorized by shape as discoid, spiny sphere, or balloons.

The morphology score was calculated by multiplying the number of discoid PLTs by 4, spiny spheres by 2, and balloons by 1 and then adding them together.²⁰

PLT fixation and immunofluorescence

PLTs were fixed with 2% paraformaldehyde at room temperature for 45 minutes, permeabilized by adding 0.2% Triton X-100, and incubating for 30 minutes at 37°C. The PLTs were washed with phosphate-buffered saline (PBS), centrifuged at $1400 \times g$ for 3 minutes at 4°C, and resuspended in PBS. Incubation with saturating concentration of the primary antibody for 5 minutes at 37°C was followed by a washing step and the secondary Alexa Fluor 488-labeled goat anti-rabbit immunoglobulin G (Invitrogen, Burlington, ON, Canada) was added at a 1:200 dilution of PLT suspension in PBS. Samples for negative controls were prepared similarly except incubation with primary antibody was omitted. Phase contrast microscopy was carried out on a microscope (Axiophot 2, Zeiss, Peabody, MA) with differential interference contrast and fluorescence attachments. Details of immunofluorescence image acquisition and processing were as described.²¹ Briefly, three-dimensional images of labeled cells were acquired using an inverted wide-field microscope (Diaphot 200, Nikon, Tokyo, Japan) operating in epifluorescence mode with 60/1.4 oil immersion objective. After background and dark-current subtraction, the images were deconvolved using an iterative constrained algorithm and an empirically determined point spread function. Pixels are 100×100 nm and image planes were acquired every 250 nm.

In vitro measures during storage

For metabolic measurements, a PLT sample was drawn aseptically from the storage bag through the sampling port into a syringe. Analyses of glucose and lactate levels were carried out with a blood gas analyzer (GEM Premier 3000, Instrumentation Laboratory, Orangeburg, NY) using corresponding cartridges. For values exceeding the upper limit of detection of the instrument, a 1:2 dilution of the sample with PBS was prepared and reassessed.

Extent of shape change (ESC) and hypotonic shock response (HSR) assays were carried out in an aggregometer (SPA 2000, Chronolog Corp., Havertown, PA) based on light scattering assessment.^{22,23} PLT concentrations were adjusted with PLT-poor plasma obtained by centrifugation of PLT concentrate at $2060 \times g$ for 15 minutes. The ESC was calculated by the instrument by integrating the optical density reading from the PLT-poor plasma and the PLT sample without and with adenosine 5'-diphosphate (ADP) treatment (20 μ mol/L final concentration). For HSR, the PLT response to PBS (dilution response) and water (PLT swelling) was measured and recorded as a recovery at 1 and 4 minutes.

Statistical analysis

Data are reported as the mean $\pm$ 1 standard deviation (SD) of the number of observations. A two-way analysis of variance with replication was performed to deduce significant interactions between samples without and with inhibitor treatment, and a p value of less than 0.05 was considered significant.

RESULTS

Stringent proteomic criteria to identify potential protein markers of the PSL

Many lines of evidence reveal that the PSL involves several aspects of PLT function;²⁴⁻²⁶ however, the underlying mechanisms are still elusive. Recently, we applied a complementary proteomic approach to account for the majority of conceivable protein changes occurring during PLT storage, including altered protein concentration and protein posttranslational modifications.¹⁹ As a result, hundreds of proteins were identified as changing between Day 1 and Day 7 of PLT storage providing an overview of the diversity of the storage lesion. To narrow down these 503 protein changes identified with high proteomic confidence and significance¹⁹ even further, we applied in this study an additional proteomic criterion: consistency (see Material and Methods). Twelve proteins were identified that fulfilled the requirement of consistency as well as the significance and confidence criteria (Table 1), representing a focused set of proteins from PLT lysates comprising potential protein markers for PSL.

Assessment of protein localization by fluorescence microscopy

Screening the existing literature for evidence of their function in PLTs revealed that some of these 12 identified proteins have been linked to PLT activation through integrin signaling.^{27,28} To determine whether the proteins identified as changing are not only potential markers of the PSL, but also functionally involved in the processes leading to PSL, a more detailed investigation into their functional state was launched. Specifically, experiments were designed to compare PLT changes induced by exposure to known agonists (i.e., ADP) with those observed in the PSL. Glycoprotein (GP)IIIa,²⁹ which forms a heterodimer with GPIIb for a functional complex GPIIb/IIIa, which is known in integrin nomenclature as α IIb β 3, the GTPase Rap1, and the cytoskeletal protein talin were chosen to investigate the potential changes in the localization of these proteins during storage compared to freshly drawn resting and ADP-activated PLTs.³⁰ Immunofluorescence in PLTs requires dedicated protocols and state-of-the-art equipment due to the small size of PLTs (2-3 μ m). To address this issue, wide-field epifluorescence microscopy was employed, as it has been shown to work well with cells smaller than 30 μ m.²¹ This technique reveals both topographic and dynamic information by acquiring Z-stack images of the sample and has been used for real-time in vivo imaging of PLTs during thrombus formation.^{31,32} Our images showed a well-ordered array of fluorescent spots, indicating that all three proteins are well distributed in discrete clusters throughout the PLT (Fig. 1). Fluorescence microscopy revealed an even distribution of Rap1 throughout the PLT in its resting state as well as on Day 1 of storage. After either 7 days of storage or ADP-induced activation of freshly drawn PLTs, punctate relocalization of Rap1 to the PLT membrane was detectable, albeit the extent of relocalization was higher after activation than that on Day 7 of storage. Similar distribution patterns were also observed for GPIIIa and talin during storage and after ADP induction (Fig. 1) providing evidence for protein relocalization during storage based on this qualitative trend.

Correlation between Rap1 and PLT activation

To validate the changes observed by immunofluorescence, alternative methods were employed to quantify the extent of activation in stored PLTs. Flow cytometric analysis of CD62P (P-selectin) surface expression of ADP-activated and stored PLTs (Fig. 2A)—commonly used to assess the activation state of PLTs³³—was conducted. Resting fresh and Day 1 stored PLT samples showed similar low levels of CD62P surface expression while those of ADP-activated fresh PLTs were much higher and exceeded the elevated levels of PLTs stored for 7 days.

Since the work of others has established a relationship between Rap1, affinity modulation of GPIIb/IIIa and the actin cytoskeleton,³⁴ we determined the activation status of Rap1 during storage compared to ADP-mediated activation. In pull-down experiments, active Rap1 was purified using a fusion protein consisting of GST and the Rap1-binding domain of RalGDS, which specifically recognizes the active GTP-bound form of Rap1. The amount of active Rap1 detected on a Western blot against Rap1 on Day 1 of storage was almost identical to the resting control, but increased by Day 7 of storage (Fig. 2B). However, the degree of activation in Rap1 resulting from PLT storage was lower than that of the ADP-activated PLT control. Comparison of the CD62P expression data with that of Rap1 activation levels revealed a significant correlation ($p = 0.007$) between the two probes using a Pearson correlation test.

Storage-dependent effect of Rap1 activation

As shown above, Rap1 is activated during PLT storage and other studies indicate that the activation of Rap1 occurs in a PI3-kinase-dependent manner.^{35,36} To test whether inhibiting PI3-kinase-mediated signaling influences activation of Rap1 and integrin α IIB β 3, we incubated PLTs with the PI3-kinase inhibitor LY294002 at a final concentration of 10 μ mol/L for 7 days in PLT storage bags. Western blot analysis of samples drawn on Days 2, 4, and 7 of storage revealed a reduced amount of activated Rap1 in the presence of the PI3-kinase inhibitor compared to the untreated control until Day 4 (Fig. 3, top row). Furthermore, the inhibitor had no effect on the total amount of Rap1 (Fig. 3, middle row), suggesting that it is influencing solely the activation status of Rap1. To confirm equal loading of total protein in the Western blot analysis, aliquots of PLT lysates were also blotted for β -actin, which we have previously shown not to change in concentration^{19,29} consistent with earlier studies that during PLT storage the amount of actin was maintained at levels present in fresh PLTs.³⁷ Changes observed in the DIGE experiment are most likely due to alterations in the actin isoform ratio over the storage period compared to the negligible change in the iTRAQ values (Table 1). The sample on Day 7 showed no effect of LY294002; however, the control PLTs that had been stored for 7 days without the inhibitor revealed a reduction of activated Rap1 when treated with the inhibitor after the 7-day storage period (Fig. 3, far right column, +*), suggesting that the inhibitor added to the bag on Day 1 had lost its effectiveness during storage at least by Day 7.

Since Rap1 activation is linked to GPIIb/IIIa activation, we also analyzed the effect of LY294002 on the PLT activation marker CD62P (Fig. 4A), the activation status of GPIIb/IIIa accessed by the specific antibody Pac-1 (Fig. 4B), and the surface expression of CD61

(GPIIIa; Fig. 4C) during storage. While the expression of GPIIIa was not significantly affected by the PI3-kinase inhibitor, PLT activation and integrin α IIb β 3 activation were significantly reduced with probability *p* values of less than 0.05 until Day 4 of storage, in accordance with the observation for the levels of activated Rap1 as mentioned above (Fig. 3).

Influence of the inhibitor LY294002 on in vitro PLT storage measures

Since the PI3-kinase inhibitor seemed to reduce PLT activation, we assessed the linkage of this observation to PLT functionality during storage using in vitro measurements. The metabolic activity was assessed by determination of glucose and lactate levels on different days of storage. The untreated control bag showed values consistent with those published before;³⁸ however, LY294002 inclusion seemed to slow down the glucose metabolism as reflected by reduced glucose consumption and consequently lower lactate production (Fig. 5).

In addition, ESC and HSR assays were carried out to determine the responsiveness of PLTs treated in the absence and the presence of the PI3-kinase inhibitor to activation with agonists (ADP) or stress (hypotonic shock). The extent of ADP-induced shape change signaling via PI3-kinase for the LY294002-treated samples was significantly higher until Day 4 (Fig. 5), which was consistent with the observation of the expression of the PLT activation marker CD62P (Fig. 4A). However, this was not the case for the Day 7 sample consistent with the loss of the LY294002 effect over time supporting the observation of the storage-dependent measurement described above (Figs. 3 and 4). To achieve a visual assessment of the status of PLT activation during storage in the absence and presence of the inhibitor, PLT morphology (Fig. 5, inset) was assessed by modified Kunicki scoring (Fig. 5). The morphology score decreased in a storage time-dependent manner as previously reported.^{38,39} The presence of the inhibitor improved the score due to a higher proportion of discoid PLTs in the sample. Finally, HSR seemed not to be influenced by the inhibitor at all.

DISCUSSION

Since the PSL results in reduced quality of PLTs for transfusion, it is crucial to understand the underlying mechanisms to intervene in this process. Proteomics offers an excellent tool to investigate changes in proteomes over time, which led us to apply a complementary proteomic approach to identify protein changes during PLT storage.¹⁹ By applying stringent proteomic criteria based on confidence of the protein identification and significance of the protein change as well as consistency within data sets and between proteomic tools, we identified 12 proteins as the most consistent changes in our data set; these are listed in Table 1 along with their proteomic characteristics. Several protein changes could be confirmed by Western blot analysis.¹⁹ The majority of the proteins in Table 1 are involved in cytoskeletal reorganization either as binding proteins to the actin filaments or as regulatory proteins for actin polymerization.^{12,40-43} Some proteins are already known to change in association with the PSL such as actin,⁴⁴ actinin,⁴⁵ thrombospondin,⁴⁶ and GPIIIa.³⁰ As suggested in several studies, a major morphologic characteristic of the PSL is the change in the PLT shape,³⁴ which is linked to rearrangements in the cytoskeleton and subsequent distribution of GPs to

the plasma membrane. Changes in the localization of GPIIIa were confirmed by immunofluorescence microscopy in both ADP-activated and stored PLTs.

To evaluate the extent of activation during storage we compared stored PLTs on Days 1 and 7 to resting and ADP-activated PLTs prepared from freshly drawn blood. Flow cytometric analysis of CD62P (P-selectin) surface expression in both ADP-activated PLTs and stored PLT units suggests that the extent of PLT activation during the 7-day storage period is less severe than that induced by ADP. Further analysis of the proteins that changed significantly in stored PLTs revealed that the small GTPase Rap1 became activated during storage, but to a lesser extent than in ADP-activated PLTs (Fig. 2B). Rap1 relocates to the membrane during activation of fresh PLTs,²⁸ an observation that was extended to stored PLTs in our immunofluorescence imaging studies (Fig. 1). In PLTs, Rap1 modulates GPIIb/IIIa affinity, most likely through effects on the actin cytoskeleton.³⁴ Talin, an adaptor protein that links *GPIIb/IIIa* to the actin cytoskeleton and also increased in relative concentration during storage, underwent a similar localization pattern to that of Rap1. These observations, together with those independently obtained from the morphology changes and flow cytometry, reveal moderate PLT activation during storage in agreement with observations published before.³

Strikingly, the activation and relocalization of these proteins during PLT activation are consistent with a recently published model for agonist-induced inside-out signaling via GPIIb/IIIa.²⁸ In this model, ADP activation of the PLT is propagated through a Rap guanine nucleotide exchange factor or protein kinase C, triggering activation of Rap1 and its translocation to the membrane. At the membrane, Rap1 interacts with RIAM, an Ena/VASP profilin ligand, which leads to the recruitment of talin to the membrane and the subsequent formation of an “integrin activation complex.” Our immunofluorescence images show localization patterns consistent with this model, and increased Rap1 activation supports its involvement in this process. This constellation of findings suggests that the activation of stored PLTs through this pathway is a likely mechanism that contributes to the PSL.

Several signaling pathways in PLTs lead to Rap1 activation.^{35,36} Epinephrine binds to α_{2A} adrenergic receptors, which activate Rap1 via a G_z -dependent pathway that is at least partially dependent on PI3-kinase. ADP stimulates both G_{i2} - and G_q -coupled receptors, leading to two pathways of Rap1 activation, a calcium-dependent pathway downstream of G_q and a G_i -dependent step that appears to be dependent on the activation of the PI3-kinase. On the other hand, thrombin binds to Par-1 and/or Par-3 and activates G_i and $G_{12/13}$ or G_q and $G_{12/13}$, respectively. This signal transduction mechanism results in the release of ADP from dense granules and acts as an autocrine signal on the ADP receptors P2Y1 and/or P2Y12. To test whether the mechanism leading to the storage lesion followed a PI3-kinase-dependent signaling pathway, we stored PLTs with and without the PI3-kinase inhibitor LY294002 for 7 days. This compound exhibits broad substrate specificity, and a recent study revealed a new subset of possible targets whose inhibition could affect various cellular processes, particularly metabolism, transcription, or protein trafficking.⁴⁷ However, the concentration used in our study has been shown to inhibit all PI3-kinase isoforms equally without acting on other kinases such as protein kinase C, protein kinase A, or mitogen-

activated protein kinases.⁴⁸ We are therefore confident that the majority of the effects we observe are a result of PI3-kinase inhibition, but cannot fully rule out minor side effects.

Reduced Rap1 activation could be detected which was independent of the increase in Rap1 protein concentration during storage (Fig. 3). Furthermore, moderate reduced activation of GPIIbIIIa led to the conclusion that Rap1 seems to regulate—at least in part—the final step in integrin activation. This reduction is not due to a change of GPIIbIIIa expression on the surface. More strikingly, the LY294002 treatment resulted in a reduction of PLT activation/degranulation as monitored by CD62P surface expression. This result was confirmed by morphology analyses based on Kunicki morphology scores. Furthermore, PLTs stored with the inhibitor exhibited an improved responsiveness to ADP as monitored by ESC assays, which demonstrates maintained PLT functionality. On the other hand, the inhibitor seems to have little or no effect on PLT membrane integrity since no significant changes in the HSR could be detected. Finally, along with the improved PLT functionality, LY294002 influences the metabolic activity by decelerating the rate of glucose consumption, hence leading to a decrease in lactate production. This observation is of high interest for PLT storage, since lactate levels of the storage solution are very critically monitored due to their effect on pH and are buffered with diverse additive solutions.

Taken together, the results of this study demonstrate that the identification of proteins contributing to the activation of PLTs during storage supports a model involving PI3-kinase-dependent activation of Rap1 most likely through a G-protein-coupled receptor mediating activation of GPIIbIIIa via an integrin signaling pathway. These findings identified Rap1 as a novel marker of the PSL, with the level of Rap1 activation correlating with the expression of CD62P on the PLT surface. However, since there are questions about the safety profile of inhibitors in transfusion medicine, the emphasis in this study is solely on their application to unravel potential mechanisms, not as preventive agents. As such, it also demonstrates the power of proteomic approaches to further our understanding of complex processes such as the PSL when combined with subsequent targeted biochemical experiments.

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ABBREVIATIONS

2D	two-dimensional
DIGE	fluorescence difference gel electrophoresis
ESC	extent of shape change
GP	glycoprotein
GST	glutathione <i>S</i> -transferase
HSR	hypotonic shock response
ICAT	isotope-coded affinity tag
iTRAQ	isobaric tag for relative and absolute quantitation
PSL	platelet storage lesion
RalGDS	Ral guanine nucleotide dissociation stimulator

Fig. 1.

Protein localization of Rap1, talin, and GPIIIa. PLT-rich plasma from freshly drawn PLTs was either left untreated (resting PLTs) or activated with 10 $\mu\text{mol/L}$ ADP for 30 minutes. Samples from stored PLT concentrates were taken on Days 1 and 7 of storage. The localization of the respective proteins is visualized by type-specific secondary antibodies labeled with Alexa Fluor 488 in a white-field epifluorescence microscope. A representative image (marked by an arrow) with a threefold magnification is displayed on top of each sample.

Fig. 2.

PLT and Rap1 activation during storage. (A) Fresh PRP (■) was left untreated (resting PLTs) or activated with 10 μ mol/L ADP and labeled with CD62P-PE, as were PLT concentrates (□) sampled on Days 1 and 7 of storage. The graph shows the mean of at least four separate samples $\pm$ 1 SD. (B) The activated form of Rap1 was purified using glutathione-Sepharose beads coated with GST-Rap1 binding domain of RalGDS. Bar graph (top) represents the amount of activated Rap1 in the pull-down assay determined by quantification of the intensity of the bands on Western blots against Rap1. The data represent the mean $\pm$ 1 SD of four individual experiments and values are relative to the resting sample, which was set to 1. A representative blot is shown (below). An aliquot of the lysates taken prior to the pull-down assay was analyzed for the total Rap1 amount, with actin as a loading control. This analysis was repeated on at least four different donors of fresh PLTs or at least four different PLT units.

Fig. 3.

Influence of LY294002 on the amount of activated and total Rap1 during storage of PLTs. Pairs of untreated and treated supernatants after pull-down (top panel) or an aliquot of the total lysate (bottom panel), respectively, were loaded from Storage Days 1, 4, and 7 without and with the inhibitor. The last lane (+*) represents data from control PLTs stored for 7 days and then treated with fresh 10 $\mu\text{mol/L}$ LY294002 for 45 minutes. Representative Western blots from experiments performed five times with similar results are shown. Band intensities were determined by densitometry and values normalized to the Day 1 sample for active and total Rap1, respectively.

Fig. 4.

Influence of the PI3-kinase inhibitor LY294002 on the expression of selected surface proteins. Determinations of (A) CD62P, (B) active state of α IIb β 3 monitored by Pac-1 binding, and (C) CD61 on different days of PLT storage were made in the absence (■) or presence (▒) of the PI3-kinase inhibitor LY294002. The right bar (7*) indicates addition of 10 μ mol/L LY294002 to the previously untreated control sample on Day 7 (light gray). The results represent the mean $\pm$ 1 SD of four independent samples (n = 4). *p < 0.05 and **p < 0.01 comparing results with versus without the inhibitor LY294002.

	day 1	day 2		day 4		day 7		day 7*
LY294002	-	-	+	-	+	-	+	+
Glucose (mmol/L)	17.1 ± 0.3	16.4 ± 0.4	17.0 ± 0.8	15.2 ± 0.5	16.2 ± 0.2*	11.5 ± 0.4	12.1 ± 0.6	n.d.
Lactate (mmol/L)	7.3 ± 0.1	8.7 ± 0.2	8.1 ± 0.2	10.1 ± 0.3	9.2 ± 0.2*	13.4 ± 0.3	12.6 ± 0.1	n.d.
ESC (%)	21.8 ± 0.5	23.5 ± 0.6	26.7 ± 0.3*	22.0 ± 0.3	27.8 ± 0.4*	19.5 ± 0.6	21.5 ± 0.4	n.d.
HSR (%)	55.3 ± 5.5	60.9 ± 4.9	58.2 ± 4.1	57.2 ± 4.8	58.1 ± 3.1	47.6 ± 3.4	46.9 ± 4.0	n.d.
Morphology
Morphology score	315 ± 12	275 ± 18	289 ± 8	270 ± 11	285 ± 7	255 ± 11	259 ± 18	271 ± 14

Fig. 5.

Influence of the PI3-kinase inhibitor LY294002 on PLT quality over time of storage. In vitro measures were determined on Day 1 (control) and on Days 2, 4, and 7 during storage (n = 5). Metabolic activity was monitored by assessing glucose and lactate levels. PLT integrity was assessed using the ESC and HSR as well as morphology using a modification of the Kunicki morphology score. On Day 7, 10 µmol/L fresh inhibitor was added 45 minutes before the morphologic analysis (day 7*). n.d. = not determined. The results represent the mean ± 1 SD of four independent samples (n = 4). *p < 0.05 comparing results with versus without the inhibitor LY294002.

TABLE 1

Proteins significantly changing during PLT storage*

Protein function	Protein name	2D gel	DIGE	iTRAQ	ICAT
Cell adhesion	thrombospondin-1		↓ 0.47	0.94 (8)	
Cell structure and motility	Actin, cytoplasmic 2; actin, cytoplasmic 1 *	Shift	↑ 1.80 (3)	1.10 (10)	
				1.04 (17)	
	α-Actinin 1		↑ 1.78	1.08 (11)	
				1.03 (9)	
				0.93 (20)	
				1.29 (29)	
				1.04 (32)	
				1.02 (26)	
	ARP2/3 complex 16-kDa subunit		↓ 0.39 (2)	0.93 (3)	
	Cofilin, nonmuscle isoform	Shift		0.86 (4)	
				0.94 (4)	
	Myosin heavy chain, non-muscle type A	Shift		1.15 (32)	10.9 (8)
				1.13 (52)	
				1.07 (60)	
				0.99 (47)	
	Septin 2 *			1.09 (8)	
				1.03 (5)	8.33 (2)
				1.02 (4)	
	Tubulin β-5 chain *		↓ 0.39 (3)	0.90 (4)	
Signal transduction	14-3-3 protein ζ/δ *	↑ shift		1.32 (10)	
				1.07 (11)	
				1.03 (8)	
	PLT GPIIb α chain precursor			1.10 (6)	1.02 (5)
				1.10 (9)	
				1.17 (4)	
				1.03 (5)	

Protein function	Protein name	2D gel	DIGE	iTRAQ	ICAT
	Ras-related protein Rap-1 *	↓		1.30 (6) 1.00 (7) 1.02 (6) 1.28 (68) 1.03 (68) 1.01 (66)	1.52 (12)
	Talin 1				

* Proteins listed by protein function were selected by applying stringent proteomic criteria to protein changes during PLT storage. Changes determined by 2D gel and DIGE are displayed by comparison of protein patterns of Day 1 and Day 7 samples as characterized by visible shift and increase (↑) or decrease (↓) in intensity of protein spots (2D gel and DIGE). Proteins were identified by mass spectrometry and some are validated by Western blot analysis (*). For iTRAQ and ICAT, the ratio of labeled peptides on Days 1 and 7 was determined, with a ratio of 1.00 indicating no change, ratios of greater than 1.00 indicating relative accumulation of protein in the PLT, and ratios of less than 1.00 indicating relative depletion of protein in the PLT. The number in parentheses represents the number of unique peptides used for protein identification.