APPLICATION OF PROTEOMICS TO THE STUDY OF PROTEIN TRANSLATION IN STORED PLATELET UNITS

by

JONATHAN NOAH THON

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ABSTRACT

Platelet products have a short shelf life (5 to 7 days) owing in part to the deterioration of the quality of platelets stored at 22°C. This creates significant inventory challenges, and blood banks may suffer shortages and high wastage as a result. Proteomics offers a global quantitative approach to investigate changes occurring in stored blood products. These data sets can identify processes leading to storage-associated losses of blood component quality such as the platelet storage lesion (PSL). Changes to the platelet proteome between days 1 and 7 of storage were analysed with 3 complementary proteomic approaches with final mass spectrometric (MS) analysis: 2-dimensional (2D) gel electrophoresis/differential gel electrophoresis (DIGE), isobaric tagging for relative and absolute quantification (iTRAQ), and isotope-coded affinity tagging (ICAT). Although proteomics analyses identified many storage-associated protein changes, these varied significantly by method suggesting that a combination of protein-centric (2D gel or DIGE) and peptide-centric (iTRAQ or ICAT) approaches is necessary to acquire the most informative data.

Validation of the proteomics results by western blotting, flow cytometry, quantitative real-time polymerase chain reaction (qRT PCR) and ^{35}S-methionine incorporation confirmed that platelets are capable of synthesising biologically relevant proteins *ex vivo* throughout a 10-day storage period with particularly long-lived mRNA (half-life of approximately 2.4 days), and has provided the first evidence for one of the mechanisms of the PSL. The development of an ^{35}S-methionine assay has since shown that stored human blood platelets incorporate ^{35}S-methionine at a rate that is proportional to time and substrate concentration, and is slower for freshly drawn platelets than those stored in pooled buffy coat derived units for 10 days. More interesting still

are the observations that the overall ^{35}S-methionine incorporation rate was higher in pooled buffy coat platelet units versus freshly drawn platelets, that this rate increased upon agonist exposure in both, and that day 8 platelets showed significantly greater total protein translation than on days 2,3,7 and 10 of storage. This may be indicative of translational regulation of the platelet proteome during storage and upon activation. Translational control is a consequence of remarkable cellular specialisation and precise biochemical pathways which, in the case of platelets, may lead to storage-associated losses of blood component quality and must be understood if platelet storage times are to be extended.

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1D	1 Dimensional
2D	2 Dimensional
3D	3 Dimensional
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ASA	Acetyl-salicylic acid
ATP	Adenosine triphosphate
BC	Buffy coat
BCA	Bicinchoninic acid
Bcl-3	B cell lymphoma-3
BNDF	Brain-derived neurotrophic factor
BSS	Bernard-Soulier syndrome
CalDAG-GEF	Calcium and diacylglycerol-regulated guanine nucleotide exchange factor
CBS	Canadian Blood Services
CCL5	Chemokine (C-C motif) ligand 5
CD	Cluster of differentiation
CDS	Celera discovery systems database
CD62P	P-selectin
CGSA	Trisodium citrate dextrose saline apyrase

CHAPS	3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphonic acid
CPM	Counts per minute
CXCL7	Chemokine (C-X-C motif) ligand 7 (also β-thromboglobulin, PPBP)
DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate
DIGE	Differential in-gel electrophoresis
ECM	Extra-cellular matrix
EDTA	Ethylenediamine-tetraacetic acid
EF	Error factor
ESC	Extent of shape change
ESI	Electrospray ionisation
ETS	EDTA Tris saline
FITC	Fluorescein isothiocyanate
FT-ICR	Fourier-transformed ion cyclotron resonance
GDP	Guanosine diphosphate
GP IIb	Glycoprotein IIb (also CD 41, GP α_{IIb})
GP IIIa	Glycoprotein IIIa (also CD 61, GP β_3)
G-protein	Guanosine-binding regulatory protein
GST	Glutathione-S-transferase
GT	Glanzmann's thrombasthenia
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen

HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSD	Honestly significant difference
HSP20	Heat shock protein 20
HSR	Hypotonic shock response
ICAT	Isotope coded affinity tagging
IP_3	Inositol-1,4,5-triphosphate
IPG	Immobilised pH gradient
IPI	International protein index
ITP	Idiopathic thrombocytopenic purpura
iTRAQ	Isobaric tag for relative and absolute quantification
LAP3	Leucyl aminopeptidase 3
LC	Liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption ionisation
mRNA	Messenger RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSDB	Matrix science database
NCBI	National centre for biotechnology information
PAR	Protease-activated receptor
PAS	Platelet additive solution
PBS	Phosphate buffered saline
PC	Platelet concentrate

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pI	Isoelectric point
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLTs	Platelets
PMSF	Phenylmethanesulfonylfluoride
PRP	Platelet-rich plasma
PSI	Plexin/semaphorin/integrin
PSL	Platelet storage lesion
PTK	Protein tyrosine kinase
qRT PCR	Quantitative real-time PCR
RBCs	Red blood cells
Rho-GDI	Rho-GDP dissociation inhibitor
RIPA	RadioImmuno precipitation assay
RNA	Ribonucleic acid
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption ionisation
SILAC	Stable isotope labelling with amino acids in cell culture
SPARC	Secreted protein acidic and rich in cysteine (also osteonectin, BM-40)
SWISS-PROT	Swiss protein knowledgebase

TA-GVHD	Transfusion associated graft-versus-host disease
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TO	Thiazole orange
TRAP	Thrombin receptor agonist peptide
TrEMBL	Translated European molecular biology nucleotide sequence database
TxA_2	Thromboxane A_2
UV	Ultraviolet
VLA	Very late antigen
vWF	von Willebrand Factor

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Though I certainly take great pride in my accomplishments, I am well aware that any success that I have enjoyed has been the consequence of family and friends that have encouraged and inspired me along the way. Their love has seen me through the worst of it, and humbled by their generosity, I suppose I am most proud of the fact that I can recognize the absurdity of taking absolute ownership over one's achievements.

Two parts studious, one part lucky.

CHAPTER 1 Introduction

1.1. Platelet Storage in Canada

1.1.1. Platelet Collection Practices – 1950s to Present

The preparation of platelets as a specific type of blood product made its debut in the early 1950s, as a direct result of the development of plastic storage containers. During this period, blood processing and storage practices dictated that whole-blood units (450-500 mL), anticoagulated with acid citrate dextrose (ACD), be kept at refrigerated temperatures (1-6°C) to minimise the risk of bacterial growth and to improve red cell viability and therapeutic efficacy during storage [2]. Platelet-rich plasma (PRP) could be prepared by subjecting the blood to slow centrifugation and subsequent separation. Not unlike the practices of present day, this protocol required that the red cells and leukocytes sediment, leaving the majority of platelets in roughly 200-250 mL of supernatant plasma for transfusion into thrombocytopenic patients [2]. Early PRP preparations yielded platelet counts in the vicinity of 0.3×10^{12} cells/L such that only a very small dose of platelets could be infused per unit [2]. Survival studies conducted during this time suggested that cold-stored platelets survived only briefly in the circulation after infusion [3]. In addition, because the volume of suspension was so large with respect to the number of platelets in solution, the suspending plasma (used to produce concentrates of anti-haemophilic factors, among other things) was being wasted. While attempts were made to concentrate these platelets by high-speed centrifugation, such efforts invariably lead to irreversible platelet clumping such that these products were no longer viable for transfusion [4].

One of the most important developments in platelet storage occurred in the mid-1960s when it was discovered that platelet concentrates (PCs) from ACD PRP could be readily resuspended in a smaller volume if the PRP was first brought to room temperature (22.5°C) before high-speed centrifugation of the platelet product, and if the pellet was allowed to rest undisturbed for 30 min at room temperature before resuspension [4]. This breakthrough in platelet collection practices also led to the discovery that platelets survival was significantly improved even after several days if storage was carried out at room temperature [3], and made it possible to store PCs for longer (~3 days). In the early 1970s it became evident that agitation of the platelet product during storage significantly affected platelet quality, and that certain polyvinyl chloride (PVC) plastic containers used to store the PC demonstrated improved gas exchange properties (particularly in regards to oxygen) [5]. With the implementation of adequate platelet agitation practices and the use of the best available storage containers, platelet storage time in the early 1980s could be extended from 3 to 5 days.

Over the next 3 years, researchers began noticing dramatic decreases in PC pH during storage, and irreversible swelling and agglutination of the platelet product over these 5 days, such that these platelets were no longer viable for transfusion [5]. The serendipitous observation that this decrease in PC pH did not develop in experimental plastic containers that had an increased permeability to oxygen and carbon dioxide led to the development of second-generation storage containers in 1984, and the extension of platelet storage times from 5 to 7 days [6]. Platelets perform glycolysis during storage, the end product of which is subsequently converted into lactic acid under anaerobic conditions. Bicarbonate ions in the plasma buffer the accumulation of protons by converting them into carbon dioxide and water. Researchers observed that the pH of the storage product remained stable as long as the production of lactic

acid did not exceed the capacity of plasma bicarbonate to buffer. Given the relatively hypoxic conditions of first-generation containers, inadequate generation of ATP from the citric acid cycle led to the Pasteur effect (a decrease in the rate of carbohydrate breakdown first observed in yeast when switched from anaerobic to aerobic conditions.). Under aerobic conditions, the product of glycolysis (pyruvate) is converted to acetyl CoA that can be used in the citric acid cycle, and generates a net total of 38 molecules of ATP per 1 molecule of glucose. As the oxygen concentration decreases, pyruvate can no longer be converted of acetyl CoA, then fully oxidized to CO_2 and H_2O, and is instead converted into lactic acid, at a net yield of 2 molecules of ATP per molecule of glucose. During storage, the production of lactic acid occurred to the point of bicarbonate ion exhaustion resulting in the accumulation of protons and the accompanying decrease in PC pH. Second-generation storage containers, by comparison, were sufficiently permeable to allow the escape of carbon dioxide produced by this buffering as well as permit diffusion of oxygen into the platelet product to meet the metabolic demand of the cells within [7].

Unfortunately, it became clear just 2 years later that the bacterial contamination of platelet products during collection resulted in a significant increase (nearly four-fold) in the reported cases of clinical sepsis with the transfusion of 7-day-old platelets as compared to the 5-day storage product [8, 9]. Bacterial contamination is thought to occur as a result of venipuncture through scarred or dimpled areas of the skin that may be colonized with surface and deep bacteria and/or careless sterilisation of the skin. Due primarily to the increased risk of bacterial growth resulting in clinical sepsis, and preliminary observations that platelets exhibit a general reduction in their therapeutic efficacy and *in vitro* survival during a 7-day storage period, storage of PCs at 20-24°C was restricted to 5 days in 1986 [10, 11]. With the implementation of

mandatory bacterial testing of platelet products, the loss of cellular integrity and function (otherwise known as the platelet storage lesion) is the significant hurdle remaining at present before platelet storage time can be significantly extended once more.

1.1.2. Buffy Coat Method

Preparation of whole-blood derived platelet concentrates had remained largely unchanged in Canada until Canadian Blood Services introduced the buffy coate (BC) method to replace the PRP method in 2005 (pilot) and officially began implementation in 2008. Current platelet collection practices require that whole blood donations be rapidly cooled to room temperature on cooling trays and processed within 24 hrs of collection (versus 8 hrs for the PRP method). Initial centrifugation of whole blood is performed at high speed (3493 x g) and the plasma and red blood cells are immediately extracted, leaving what is known as the BC layer in the collection container [12]. Buffy coat preparations from four donations are pooled and diluted in plasma from one of the same four donors prior to a slow centrifugation (1258 x g) [13]. The PRP is extracted through a leukoreduction filter to produce a pooled PC, and the product is stored with gentle agitation at 22°C.

The BC method of platelet collection offers a number of advantages over the PRP method. Among these are a more consistent product, improved platelet recovery from whole blood donations, reduced leukocyte contamination, improved inventory availability due to the 24 hr production window which permits more platelets to be processed from a greater number of whole blood donations, and an increase in the amount of plasma removed from whole blood donations which permits the retention of more plasma for further fractionation [13]. Moreover,

there is some evidence to suggest that the BC method may activate platelets to a lesser extent than does the PRP method [14].

1.1.3. Platelet Storage Lesion

Platelet storage lesion is best defined as the sum of all the deleterious changes in platelet structure and function that arise from the time the blood is withdrawn from the donor to the time the platelets are transfused to the recipient. This quality of aging platelets has traditionally been quantified in terms of a decrease in platelet morphology score (percentage of platelets that are discoid versus spheroid) and extent of shape change (ESC, measure of platelet microaggregate formation), along with an increase in HSR (measure of platelet membrane loss of elasticity), platelet activation marker expression (*e.g.*, CD62) and platelet solution pH [10, 11, 15]. While platelets that are stored over a period of 7 days are still viable, these studies have suggested a general reduction in their therapeutic efficacy that is associated with morphological, biochemical and functional changes [10, 11]. Included with these are reports of the development of abnormal forms [16], loss of disc shape [17, 18], decreased mean platelet volume [19], increased volume and density heterogeneity [20], increased release of platelet alpha-granules and cytosolic proteins [21, 22], increased procoagulant activity [23], and altered glycoprotein expression [19, 22, 24-27]. In addition, *in vivo* platelet recovery and subsequent survival are reduced by at least 25 percent in autologous re-infusion studies [10, 11, 28-34]. While the symptoms of storage lesion are well characterised, the precise biochemical pathways involved have yet to be identified and are thus the focus of this dissertation.

1.2. Platelets

1.2.1 Platelet Structure

Platelets are small (2 to 3 μm), anucleate, disc-shaped, membrane-encapsulated cell fragments that are formed and released into the bloodstream primarily by bone marrow megakaryocytes [35]. In whole blood, platelet concentrations will range from 150 to 400 x 10^9 cells/L, with approximately two-thirds of platelets present in general circulation and the remaining third reversibly sequestered in the spleen [36]. Platelets have a finite lifespan in normal individuals of 9.5 ± 0.6 days [37]. Nearly 18 percent of platelet turnover is due to a fixed requirement of platelets to support vascular integrity, while the remainder is due predominantly to senescence [38]. These platelets are taken up by the mononuclear phagocytic system and accumulate in the spleen and liver where they are subsequently degraded [39, 40]. Regulation of platelet concentration is based on total platelet mass and thrombopoietin availability [41]. Also present in the lung and spleen, megakaryocytes arise from pluripotent hematopoietic stem cells, and will undergo multiple rounds of nuclear endomitosis (4 N to 128 N), organelle synthesis, and dramatic cytoplasmic maturation and expansion (48 to 100 μm) prior to pseudopod formation, pro-platelet expansion into the marrow sinus and intravascular fragmentation of individual platelet units (Figure 1) [35]. Each megakaryocyte has been estimated to release thousands of platelets [37, 42, 43]. Platelets contain a number of distinguishable structural elements including: a delimited surface membrane (procoagulant surface on which coagulation proceeds in activated platelets); invaginations of the surface membrane that form the open canalicular system (facilitates secretion from the cell); a separate, membrane-delimited dense tubular system (sequesters Ca^{2+} for rapid ion mobilisation during activation); a cytoskeletal network (rapidly

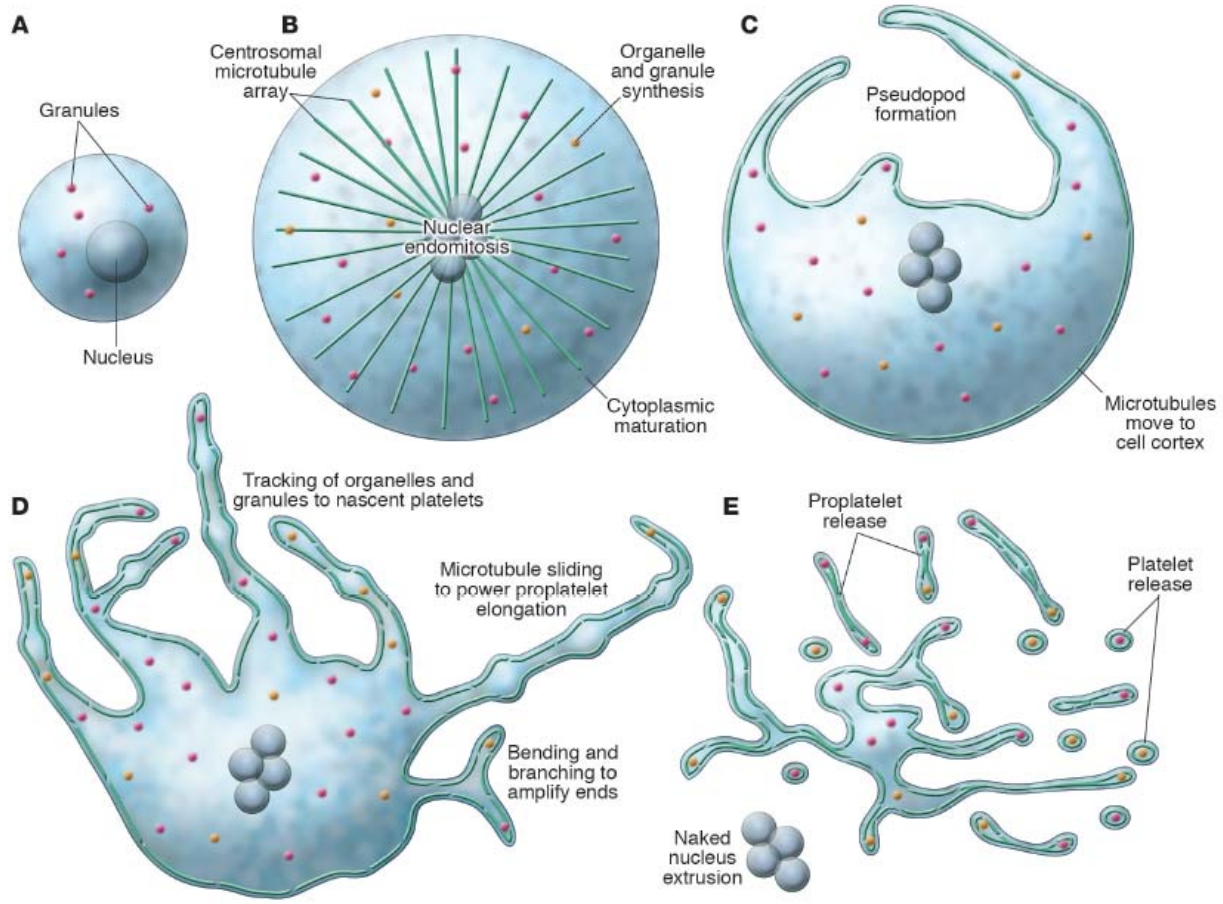


Figure 1. Megakaryocyte production of platelets.

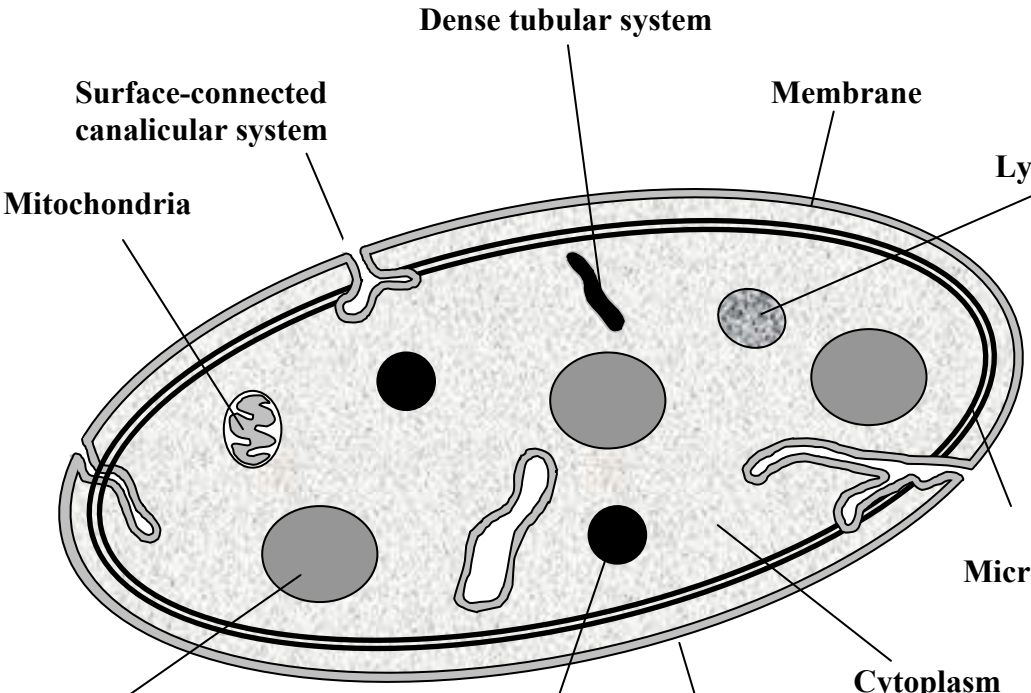
A. Immature megakaryocyte (Promegakaryoblast). *B.* Cell undergoes nuclear endomitosis, organelle synthesis, cytoplasmic maturation, expansion and centrosomal microtubule array development. *C.* Prior to the onset of proplatelet formation the centrosome disassembles and the microtubules translocate to the cell cortex. *D.* Thick pseudopods develop, elongate and amplify due to microtubule sliding, bending and branching, forming what are subsequently referred to as pro-platelets. *E.* Cellular organelles and granules are tracked into proplatelet ends, the nuclei are extruded and individual platelets are released from proplatelet ends into sinusoidal spaces where they are taken up by the circulation. This figure has been reproduced from Patel *et al.* (2005) [35].

reorganized upon platelet activation); a peripheral band of microtubules (maintain discoid shape of resting platelets); and numerous specialized organelles including α granules and dense granules (carry proteins and molecules required for haemostatic function of platelets), lysosomes (carry acid hydrolases), microperoxisomes (carry catalases), and mitochondria (provide energy to the cell) (Figure 2) [44-47].

In their resting state, platelets exist as smooth discoid cells. Negatively-charged phospholipids such as phosphatidylserine and phosphatidylinositol remain sequestered in the inner leaflet of the inactive platelet membrane which maintains the platelet surface in a non-procoagulant state. On platelet activation the cell assumes a more amorphous spherical shape with extended filopodia. Phosphatidylserine and phosphatidylinositol become expressed on the outer membrane of the platelet which produces a procoagulant surface on which coagulation may proceed. In addition to the morphological changes exhibited by the activated platelet, a number of coagulation factors and agonists become released into the surrounding media—through fusion of the α granules and dense bodies with the surface-connected canalicular system—where they are able to mediate the adhesion and aggregation response of platelets.

1.2.2 Protein Synthetic Capacity

Because they are derived from megakaryocyte progenitor cells in the bone marrow through an intricate series of remodeling events that result in the extrusion of the megakaryocyte nucleus from the mass of proplatelets (which are also released from the cell), platelets contain no nucleus of their own and therefore host no DNA [35]. Platelets do however inherit a transcriptome in the form of mRNA from their megakaryocyte progenitor cells [48, 49], and



Cytoplasm
Actin, Filamin, Talin, Myosin
Bcl-3
Lactate Dehydrogenase
Factor XIII

α-granules
Fibrinogen
Thrombospondin
Thrombosthenin
von Willebrand Factor
CXCL7
Glycoprotein IIb/IIIa
Protein S
Factor XI
IL-1β
Plasminogen Activator Inhibitor-1
Platelet-Derived Growth factor
Transforming Growth Factor β
P-selectin
α_2-Antiplasmin
High Molecular Weight Kininogen
C1-Inhibitor
Fibronectin
Vitronectin
Platelet Factor IV
Protein C
Factor V

Dense bodies
Ca^{2+}
ADP
ATP
Serotonin

Glycocalyx
Glycoprotein IIb/IIIa
Glycoprotein Ib/IX/V
Glycoprotein IV
Glycoprotein Ia/IIa
Glycoprotein Ic/IIa
Vitronectin Receptor
Thrombin Receptor
ADP Receptor
Fc Receptor

Figure 2. Platelet morphology.

Some of the organelles of a resting platelet and their contents. This figure has been adapted from Serrano (2000) [50], see also [51-54].

contain all of the necessary molecular tools and pathways necessary for protein biosynthesis from cytoplasmic mRNA, including a rough endoplasmic reticulum and polyribosomes [48, 51-61]. Platelets not only carry functional translational machinery, they have also been shown to synthesise proteins [51].

Indeed, the notion that platelets are static cytoplasmic fragments incapable of protein synthesis and with no capacity for translational regulation was first questioned in 1967 by Warshaw *et al.* [62]. They demonstrated that platelets were capable of incorporating radiolabelled amino acids into newly synthesised proteins [62]. In the early 1970s, Agam *et al.* [63, 64] published a series of papers showcasing RNA turnover in platelets which they associated with the mitochondrial system. This observation that was confirmed nearly 2 decades later by Ian Bruce and Roger Kerry using chloramphenicol and cycloheximide to block incorporation of L-[U-^{14}C] leucine into platelet trichloroacetic acid precipitable material [65]. Their work suggested that the majority of platelet protein synthesis is mitochondrial and may play a role in human platelet aggregation.

While quiescent platelets are thought to display minimal translational activity, platelet activation has been shown to lead to the rapid translation of preexisting mRNA, with the release or derivation of platelet-secreted proteins, cytokines, exosomes (vesicles), and microparticles [58]. This process, termed signal-dependent translation, uses a constitutive transcriptome and specialized pathways to alter platelet phenotype and that may have clinical relevance [51, 66, 67]. To date, a number of proteins have been shown to be synthesised by freshly drawn platelets, and include (amongst others); membrane glycoproteins (GPs) IIb, IIIa, talin, myosin, Rap1b, Bcl-3, and IL-1β [51, 52, 54, 67]. These proteins all originate from mRNAs that are abundantly expressed in platelets and have features that predict constitutive translation [51, 56, 67, 68].

Nonetheless, it is unknown whether constitutive translation is necessary to maintain threshold concentrations of these critical factors in platelets and what effect this might have on platelet storage.

1.2.3 Platelet Function

Platelets play an essential role in preserving vascular integrity and in maintaining haemostasis. Damage to the endothelial cell layer of the blood vessel wall due to mechanical injury or degenerative disease can result in the exposure of elements present at the site of the subendothelium, such as collagen IV, that may provide sites for platelet adhesion. The reversible binding of platelets to one another and to the tissue underlying the endothelium may subsequently result in the transmission of a number of intracellular signals within each cell which can cause the platelets to become activated. Platelets that have become activated will undergo a morphological change from a disc to a sphere with multiple extended pseudopods, flip the negatively-charged phospholipids phosphatidylserine and phosphatidylinositol to their outer membranes, release their granular contents, and spread to increase surface contact. In response to physiological agonists released from the platelets themselves, or generated in the plasma and on the platelet surface though the coagulation cascade, platelets will aggregate irreversibly to form what is then referred to as a fibrin clot. Circulating platelets may be recruited to the fibrin clot through a variety of cell surface interactions with vWF, fibrin, collagen (among others), which serves to seal the site of vascular damage and prevent subsequent blood loss from the region (Table 1).

Table 1. Adhesive and activating protein receptors on platelets.

This table has been adapted from Serrano (2000) [50].

	Receptor	Ligand(s)	Alternate Designation	Function
Platelet adhesive proteins	GP Ia/IIa	Collagen	$\alpha_2\beta_1$, VLA-2	Adhesion Activation
	GP Ic/IIa	Fibronectin	$\alpha_5\beta_1$, VLA-5	Adhesion Activation
	VLA-6	Laminin	$\alpha_6\beta_1$	Adhesion Activation
	GP Ib-IX-V	vWF		Adhesion (shear) Activation
	GP IV	Collagen Thrombospondin	GP IIIb, CD36	Adhesion Activation
	Vitronectin Receptor	Vitronectin Fibronectin vWF Fibrinogen Thrombospondin	$\alpha_V\beta_1$	Adhesion Activation
	GP IIb/IIIa	Fibrinogen Fibronectin Vitronectin vWF Collagen	$\alpha_{IIb}\beta_3$	Adhesion Activation Clot Retraction
G-protein coupled receptors	PAR-1, PAR-4	Thrombin		Activation
	P_2Y_1, P_2Y_{12}, P_2X_1	ATP ADP		Activation
	T_xA_2	Thromboxane A_2		Activation

1.2.4 Platelet Activation

Platelets will respond rapidly to agonists in their microenvironment through specific transmembrane receptors that are responsible for generating secondary messengers which subsequently induce protein phosphorylation and the opening of ion channels [69]. The extent of platelet activation depends on the agonist used [70]. Thrombin—a serine protease—is the most potent activator of platelets [70]. Thrombin functions by cleaving an N-terminal peptide from the G-protein coupled receptors PAR-1 (at thrombin concentrations of $\geq$ 1 nM) and PAR-4 (at thrombin concentrations of $\geq$ 30 nM), unmasking a new N-terminal sequence (SFLLRN) which is then able to dock intramolecularly within the receptor, effecting transmembrane signalling [71]. These interactions are summarized in Figure 3. Phosphorylation of the Gα subunit by PAR-1 results in the activation of membrane-associated signal generating enzymes such as phospholipase C and phospholipase A_2, which subsequently trigger the generation of second messengers IP_3, DAG, Ca^{2+} and TxA_2, respectively [72-74]. The release of intracellular Ca^{2+} from the dense tubular system, in addition to an influx of external Ca^{2+}, appear to regulate redistribution of GP IIb/IIIa and cytoskeletal reorganisation [75]. Platelet granules are brought together centrally by activated contractile proteins, such as talin, and fuse with the surface-connected canalicular system of the platelet such that their contents are released extracellularly.

Platelet agonists can induce shape change under conditions in which they do not activate PLC nor induce an increase in Ca^{2+} [76-80]. This observation suggests that an elevation of Ca^{2+} alone is not sufficient to induce platelet shape change. Indeed, thrombin and TxA_2 can induce platelet shape change in the absence of G_q-mediated PLC activation, likely through the G_{12} and G_{13} receptors [81-84]. Stimuli that are able to activate both G proteins via their respective

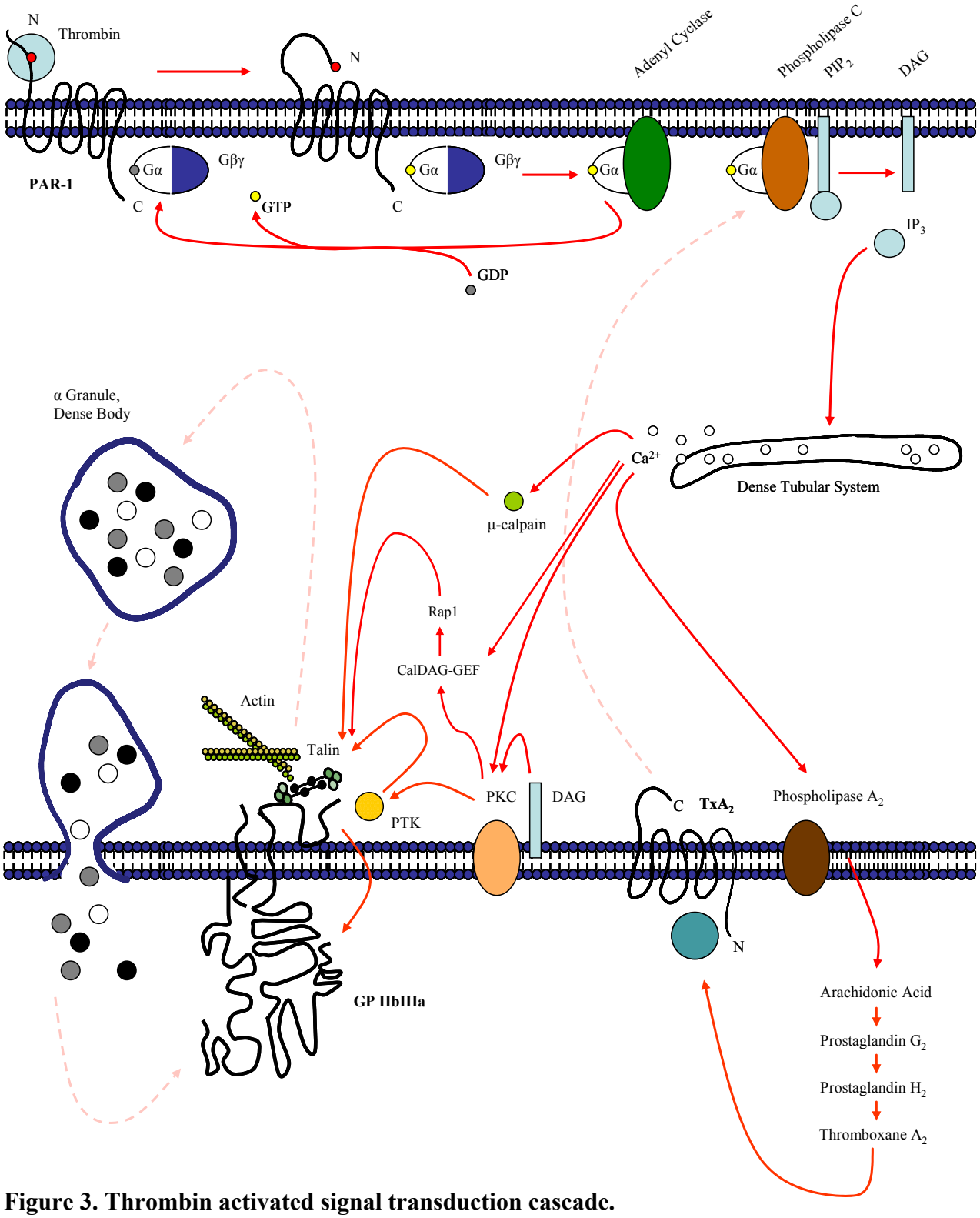


Figure 3. Thrombin activated signal transduction cascade.

Illustration of representative membrane-associated signal generating enzymes, and second messenger pathways that become triggered on platelet activation by thrombin [72-74, 85-88]. Cleavage of the N-terminal peptide from the G-protein coupled receptors PAR-1 (at thrombin concentrations of ≥ 1 nM) and PAR-4 (at thrombin concentrations of ≥ 30 nM, not shown),

unmasks the N-terminal sequence SFLLRN which is then able to dock intramolecularly within the receptor, effecting transmembrane signalling [71]. Phosphorylation of the Gα subunit by PAR-1 results in the activation of membrane-associated signal generating enzymes phospholipase C and phospholipase A_2, which subsequently trigger the generation of second messengers IP_3, DAG, Ca^{2+} and TxA_2, respectively [72-74]. The release of intracellular Ca^{2+} from the dense tubular system (in addition to an influx of external Ca^{2+}, not shown) regulate redistribution of GP IIb/IIIa and cytoskeletal reorganisation [75]. Platelet granules are brought together centrally by activated contractile proteins, such as talin, and fuse with the surface-connected canalicular system of the platelet such that their contents are released extracellularly.

receptors such as TxA_2 and thrombin preferentially use G_{13} to induce platelet shape change [87]. Conversely, stimuli such as ADP, which activates G_q-mediated signalling pathways (through the $P2Y_1$ receptor) but not signalling via G_{13}, induce platelet shape change solely via G_q [82, 84]. Activation of G_i (through the $P2Y_{12}$ ADP receptor) does not appear to be necessary for the induction of platelet-shape change, but is nevertheless involved in platelet aggregation and degranulation [87, 89].

1.2.5 Platelet Adhesive Proteins

Cell surface recognition of thrombogenic factors mediating primary haemostatic plug formation occurs primarily through the action of integral membrane proteins otherwise known as integrins. Integrins are non-covalent heterodimeric protein complexes composed exclusively of 2 distinct polypeptide chains, termed the α and β subunits (the molecular mass of which varies from about 100 000 to 140 000 Da). Each subunit is composed of an extracellular domain (approximately 23 nm), a single-pass transmembrane domain, and a short cytoplasmic tail composed of roughly 20-60 amino acids, on average [90]. Integrins are linked to the cytoskeleton via scaffolding proteins such as talin, paxillin and α-actinin at their cytoplasmic end, and are

thought to fold into an inverted V-shape, which brings the ligand-binding sites on the extracellular face of the complex in close proximity to the cell membrane [91]. To date, 18 α and 18 β subunits have been discovered—variants of which are formed by differential splicing—to produce some 24 unique integrins. Of these, GP Ia/IIa, GP Ic/IIa, and VLA-6 are particularly responsible for initial platelet binding to collagen, fibronectin, and laminin, respectively (Table 1) [92-95]. Glycoproteins IV, IIb/IIIa and $\alpha_V\beta_1$ nonetheless play important roles in subsequent platelet activation and adhesion reactions with collagen, thrombospondin, vitronectin, fibronectin, fibrinogen and vWF. Glycoprotein IIb/IIIa is particularly important in this regard as it is the most abundant integrin on the platelet surface (approximately 60 000 copies per cell) and thought to become up-regulated in platelets as a result of both thrombin exposure and prolonged storage times [96-98]. Due to their symmetry, ECM components such as fibrinogen can bridge GP IIb/IIIa receptors present on separate platelet surfaces, thereby mediating the formation of a haemostatic plug.

Although not an integrin, GP Ib-IX-V is required for early platelet adhesion to the ECM in areas of disturbed blood flow. Under conditions of high shear, otherwise globular vWF multimers become extended, exposing multiple interaction sites within the molecule that bind platelet glycoprotein Ib-alpha, and collagen [99]. This interaction enables platelets to become tethered to a collagen surface and to each other, thus permitting thrombus growth [99, 100].

1.2.6 Glycoprotein IIb/IIIa

Glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) is a noncovalently associated heterodimer formed from a 135-kDa α (GP IIb) and a 90-kDa β (GP IIIa) subunit, the major structural features of which are

highlighted in Figure 4. [97]. Each subunit contains a relatively large extracellular domain, a single-pass transmembrane domain and a short cytoplasmic tail composed of 20-60 amino acids [90]. Glycoprotein IIb/IIIa functions as a transmembrane receptor whose activation is both bidirectional and reciprocal [97]. Glycoprotein IIb/IIIa is present in roughly 500 000 to 800 000 copies per platelet—60 000 of which are expressed on the surface of the platelet in its inactive form—and is required for platelet interactions with proteins of the plasma and the ECM, most notably fibrinogen, fibrin, VWF, fibronectin, vitronectin, and collagen [97, 98, 101]. Glycoprotein IIb/IIIa equilibrates between a resting and active conformational state, the former predominating in inactive (unstimulated) platelets. Ligand binding to GP IIb/IIIa modulates receptor clustering and promotes progressively irreversible conformational changes in the protein that are transmitted to the cytoplasmic tails. Disruption of the relatively weak integrin tail-tail and tail-membrane interactions leads to chain splaying and exposure of regions in the cytoplasmic tails of the transmembrane receptor for the recruitment and/or activation of enzymes, adaptors, and effectors to form integrin-based signalling complexes [102-104]. This process has consequently been labelled ‘outside-in’ signalling. Alternatively, ‘inside-out’ signalling may occur when agonist-dependent intracellular signals stimulate the interaction of key regulatory ligands (such as talin) with integrin cytoplasmic tails (specifically the GP IIIa tail). This leads to conformational changes in the extracellular domain that result in the exposure of the ligand contact site on the surface of the receptor and a subsequent increase in the affinity of the receptor for the aforementioned ligands [102]. In addition to the conformational changes discussed above, protein-protein interactions with the GP IIb/IIIa receptor may promote the lateral mobility and clustering of integrins within the plane of the plasma membrane via multivalent ligands, ligand self-association, lateral interactions of integrins with other membrane

proteins, reversible integrin linkages to the active cytoskeleton, and homomeric interactions of the transmembrane domains which culminates in the assembly of a nascent signalling complex proximal to the cytoplasmic tail of GP IIb/IIIa and subsequent platelet activation [101, 105-110].

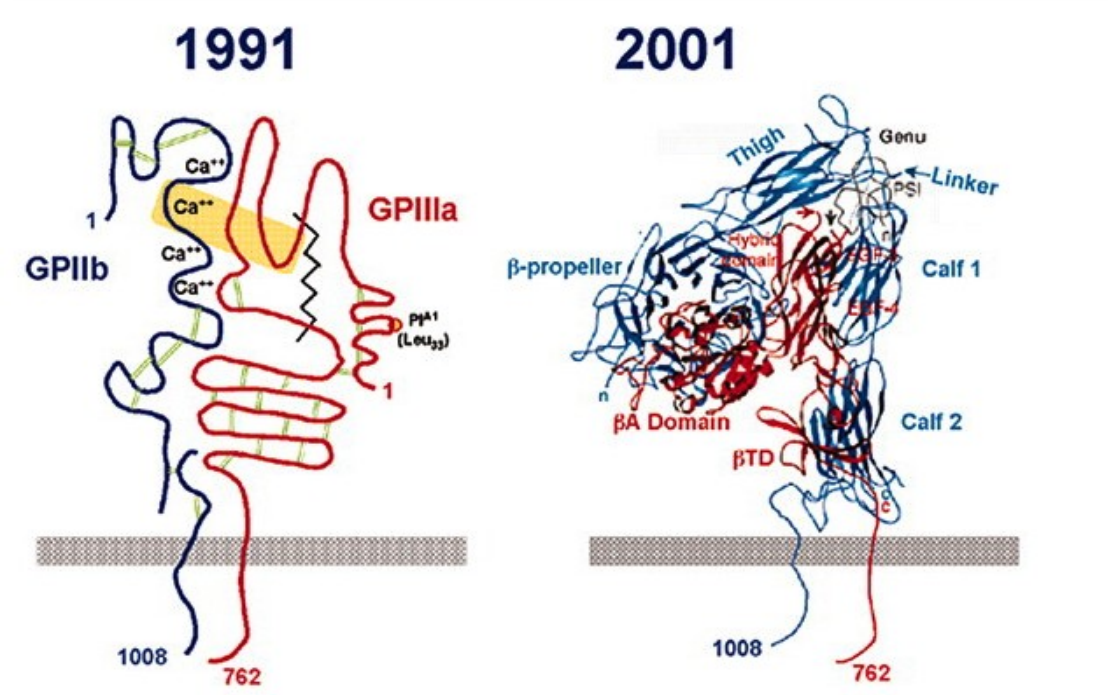


Figure 4. Structure of GP IIb/IIIa.

The GP IIb subunit is shown in blue and the GP IIIa subunit is shown in red. *Left.* Early model of the GP IIb/IIIa complex; yellow rectangle highlights major ligand contact sites. Calcium binding region, interchain and intrachain disulphide bonds have been included as well as the chymotrypsin-sensitive cleavage sites (jagged line) that remove the ligand-binding segment of GP IIIa. *Right.* Proposed 3-dimensional structure of GP IIb/IIIa based on the crystal structure of the closely related integrin $\alpha_V\beta_3$. This research was originally published in *Blood*. Shattil *et al.* 2004:104(6):1606-1615. © American Society of Hematology [97].

1.2.7 Platelet Cytoskeleton

The platelet cytoskeleton is composed of a circumferential band of microtubules and 2 actin filament-based components—a cytoplasmic actin scaffold and a membrane skeleton—that maintain platelet shape, mediate contractile events, and regulate membrane contours and stability, respectively [111]. The marginal microtubule band consists of αβ-tubulin dimers that polymerize end-to-end into protofilaments. These are subsequently bundled into hollow cylindrical filaments that coil about one another 8 to 12 times. The establishment of a marginal band of microtubules about the circumference of the platelet is thought to play a significant role in pro-platelet formation as well as helping maintain platelet shape [35]. Actin is a globular protein that polymerizes helically forming what are known as microfilaments that associate further with one another to create a 3D network within the cell called the cytoskeleton. Whereas the majority of platelet tubulin exists in a polymerized state, prior to platelet activation only 30 to 40 percent of platelet actin (which in turn comprises 20 to 30 percent of total platelet protein) is polymerized into filaments [111]. The remaining monomeric actin is believed to associate with thymosin beta 4 which prevents its incorporation into actin filaments [111]. In addition, gelsolin is thought to interact with the barbed ends of pre-existing actin filaments, further restricting actin polymerisation [111]. When platelets become activated there is a rapid increase in actin polymerisation as new filaments fill the extending filopodia and form a network at the periphery of the platelet [111]. The filamentous actin content increases to roughly 60 to 80 percent of total platelet actin, as proteins associated with the membrane skeleton are increasingly detectable in cytoskeletal fractions of the cell [112, 113]. As platelets aggregate, additional cytoskeletal reorganisations occur, and are mediated by the formation of focal adhesion complexes linking

the cytoplasmic actin scaffold and membrane skeleton. Focal adhesion sites arise from the clustering of ligand-bound adhesion receptors and subsequent assembly of cytoskeletal proteins on the cytoplasmic side of integrins connecting the extracellular matrix to the intracellular actin network; GP IIb/IIIa is one such example. On attachment to adhesive ligands in the platelet aggregate, GP IIb/IIIa will cluster and recruit cytoskeletal proteins including vinculin, talin, myosin, paxilin, α-actinin, tropomyosin and tensin to a series of intracellular attachment sites on both the integrin cytoplasmic tail and actin filaments [114]. Tension-dependent restructuring of the associated cytoskeleton through these proteins drive platelet shape change, the centralisation and release of storage granules, and clot retraction [115].

1.3. Global Quantitative Proteomic Approaches

1.3.1. Proteomics as an Analytical Screening Tool

Proteomics is the large-scale, high throughput identification and quantification of all the proteins of a system at a defined state [116, 117]. While early proteomic approaches have largely focused on protein identification [118-120], global quantitative proteomic approaches such as 2D gel electrophoresis followed by mass spectrometry (MS) analysis and stable isotope labeling methods (iTRAQ and ICAT) have provided valuable platforms for the study of relative and absolute changes in protein concentration within the cell. Current proteomic technologies can be summarized as the integration of the following four tools or steps: Protein isolation and preparation (involves homogenisation of cells or tissue followed by solubilisation of the sample with detergent and treatment with a reducing or denaturing reagent); protein separation (*eg*. 1D or 2D gel electrophoresis; high-performance liquid chromatography, HPLC; ion exchange

chromatography, affinity chromatography); MS (measures the mass-to-charge ratio of ions in the gas phase) for which many combinations of ionisation sources, mass analysers and fragmentation devices have been described (*eg*. matrix-assisted laser desorption ionisation time of flight, MALDI-TOF; surface-enhanced laser desorption ionisation, SELDI; electrospray ionisation, ESI; fourier-transformed ion cyclotron resonance, FT-ICR); and bioinformatics software (match MS data with specific protein sequences in databases) [121]. Global proteomic methods are limited, however, by their dynamic range of detection (10^2-10^4 proteins) [117], as proteins can vary widely in concentration from 10^5 in bacteria and 10^7-10^8 in human cells up to 10^{12} in plasma [122, 123]. As a result, both gel and MS methods often fail to detect low-abundance proteins and generally require the use of affinity enrichment approaches to resolve these changes [117]. Nevertheless, global quantitative proteomic approaches have proven useful for the discovery and validation of new therapeutic and diagnostic targets (biomarker discovery) [124, 125].

1.3.2. Two Dimensional Gel Electrophoresis / Differential Gel Electrophoresis

In this approach, proteins are separated by 2D electrophoresis and quantified based on the relative intensity of the protein spots of individual gels. In the first dimension isoelectric focusing is used to separate proteins according to their isoelectric points. In the second dimension, SDS-PAGE is used to separate the proteins according to their molecular weight. One potential difficulty with this approach is that gel spots corresponding between different experiments can be difficult to measure reproducibly. Differential gel electrophoresis (DIGE), by comparison, uses different fluorescent Cydyes to label 2 protein samples that are electrophoresed

together on a single 2D gel [126]. In both approaches the protein spots are subsequently excised from the gel and undergo a proteolysis step before the derived peptides can be extracted from the gel and subjected to MS analysis for identification. Although DIGE has presented a significant improvement to the quality of quantitative information that can be collected from 2D gels, this approach is still very much limited by its inability to resolve membrane proteins, excessively large or small proteins, and very acidic or basic proteins well. Moreover, 2D gel electrophoresis remains limited by its ability to resolve proteins in complex mixtures due to protein co-migration (more than one protein in a single spot) which can produce ambiguous quantitative results [127-129].

1.3.3. Isobaric Tag for Relative and Absolute Quantification

The iTRAQ methodology, unlike gel-based approaches, relies on stable isotope labeling of the different protein samples being compared. In this approach, the tryptic digest of each protein sample is incubated with a different isobaric tag containing: a reporter (114, 115 116 or 117 Da, depending on differential isotopic combinations of $^{12}C/^{13}C$ and $^{16}O/^{18}O$ in each individual reagent); a balancer (ranges in mass from 28 to 31 Da such that the combined mass of the reporter and balance groups remain 145 Da for all four reagents); and a peptide reactive group (specifically binds terminal amines, as well as the primary amines of lysine and arginine residues) [117]. As a result most of the peptides within a protein mixture are labeled and can theoretically be resolved. Once the peptides from each protein sample are labeled, the samples are mixed and relative differences in peptide concentration determined by MS/MS. During collision-induced dissociation, the reporter group is quantitatively cleaved to yield an isotope

series representing the quantity of a single peptide of known mass from each of the four possible samples. The relative intensity of these fragments is used for quantification of the individual representative peptide, which is simultaneously fragmented for sequence identification during this step [117, 130]. Because most peptides in a protein mixture are subject to iTRAQ labeling, overabundance can complicate sample analysis and lead to the preferential identification of more highly expressed proteins over others [117]. Untagged isobaric chemical noise may also confound tandem MS sequencing of the iTRAQ labeled peptides, as will the presence of protein variants that are not isobaric with the same tagged peptide from the control sample [117]. Moreover, because the first MS dimension cannot be used to pre-screen peptides for differential expression (as with ICAT), every peptide must be subject to tandem MS analysis—which can be both time-consuming and sample-intensive [117].

1.3.4. Isotope Coded Affinity Tagging

This proteomic method was first introduced in 1999 by Gygi *et al.* [131] and consists of a biotin affinity tag (for selective purification) coupled to an acid-cleavable linker region (which incorporates the stable isotopes ^{12}C or ^{13}C and permits removal of the large affinity tag prior to MS analysis), and an iodoacetamide reactive goup (specifically targets cysteinyl thiols) [132]. As with the iTRAQ label, the 2 protein samples being compared are denatured, reduced and labelled separately; the first modified with one isotopic version of the tag (^{12}C, light) whereas the second is modified with its complementary isotopic reagent (^{13}C, heavy). The protein samples are then mixed, proteolyzed to peptides, enriched by affinity chromatography of the immunobiotin tag through an avidin column, and fractionated by multi-dimensional chromatography [132].

Quantitative analysis differences in the relative expression of each peptide (expressed as ion intensity ratios between the light and heavy forms of the peptide) are resolved by MS. Subsequent analysis of the differentially expressed peptides by tandem MS enables sequencing and identification. Although ICAT is unable to resolve the sheer number of relative protein concentration changes that are otherwise identified through the use of the iTRAQ approach, by selectively purifying the cysteine-containing peptides (comprise approximately 26.6% of all tryptic peptides and demonstrate 96.1% protein coverage) [133] it is able to dramatically reduce sample complexity and enables the detection and quantification of lower-abundance proteins. Nevertheless, a peptide-selective strategy, such as ICAT, comes with its own set of disadvantages. There is always a possibility that tryptic fragments containing the cysteine residue will not ionize efficiently in the mass spectrometer or may not be recovered through the affinity chromatography step, and may lead to populations of proteins that are not represented in the ICAT analysis [117]. More significant still is the loss of peptide redundancy for a given protein, which can lead to false-positive or false-negative detection in the event of any protein variation affecting the enriched peptide or its recovery during sample preparation [117]. Lastly, as with the 2D gel electrophoresis/DIGE and iTRAQ methods, competition for sequencing between peptides tends to favour the identification of the more abundant, highly expressed proteins over others.

1.4. Application of Proteomics to the Platelet Storage Lesion

1.4.1. Proteomics and the Platelet Storage Lesion

Proteomics has gained increasing interest in haematology as a diagnostic tool—the application of which holds promise to revolutionise quality assessment and therapeutic

monitoring in transfusion medicine [124, 125, 134]. Several studies have been published on systematic in-depth analysis of the protein content of various blood products [135], such as plasma [136], red blood cells [137] as well as platelets under resting conditions [138-143] or activated by TRAP or collagen [144, 145]. To reduce the complexity of the proteomic sample, as well as improve assessment of low-abundance proteins, studies on platelet sub-proteomes—specifically the membrane [98], microparticles [146], alpha granules [147], and dense granules [148]—have been undertaken. Observations of changes in signalling proteins have since triggered the analyses of the phospho-proteome under resting [145] and activated conditions [149] as well as the determination of *N*-glycosylation sites on platelets [150].

Nevertheless, a proteomics approach yields information that must be placed in a biochemical and physiological context. Platelets play an essential role in preserving vascular integrity and in maintaining haemostasis. In the case of an injury to the endothelial cell layer of the blood vessel wall, platelets will adhere to the injury site through interactions with von Willebrand factor, aggregate with other platelets, release compounds that stimulate further aggregation, and form a loose platelet plug mediated by the formation of fibrin strands which are further crosslinked to form a fibrin net [151, 152]. At the same time, platelets become activated by the transmission of a number of intracellular signals resulting in the secretion of biologically active proteins necessary to trigger processes such as cellular chemotaxis, proliferation, and differentiation; removal of tissue debris; angiogenesis; the laying down of extracellular matrix; and the regeneration of the appropriate type of tissue [153, 154]. Because proteomics offers only a single snapshot of the platelet proteome under very specific conditions, the results of recent proteomic approaches that assess protein changes during platelet storage and specific assays that

determine platelet function under these conditions must be pooled in order to uncover a more mechanistic understanding of the storage lesion.

1.4.2. Transfusion Medicine: Limitations of Platelet Storage

Given their role in mediating haemostasis and thrombosis, it is not surprising that transfusion of platelets has become a central part of disease treatment. The first demonstration of the efficacy of platelet transfusions was described in 1910, but it was not until the development of plastic polymer platelet storage containers in the 1960s and 1970s that platelet transfusions became standard treatment for bleeding thrombocytopenic patients with bone marrow failure [155]. Studies demonstrated the benefit of prophylactic platelet transfusions to prevent bleeding as opposed to the use of platelet transfusions solely as a therapeutic strategy aimed at treating bleeding once it had occurred [156, 157]. Ever since platelet transfusions were shown to reduce mortality from haemorrhage in patients with acute leukemia in the 1950s, the use of this therapy has steadily grown to become an essential part of the treatment of cancer, haematological malignancies, marrow failure, and haematopoietic stem cell transplantation. Platelet concentrates were most frequently transfused into thrombocytopenic recipients (patients with reduced numbers of platelets or whose platelets are not fully functional) to maintain primary haemostasis either due to a specific platelet disorder or in some patients after taking medication such as ASA [158, 159]. Defects that impair function can affect platelet receptors, secretory responses, or intracellular signalling pathways; examples of qualitative platelet disorders include GT and BSS [159, 160]. The treatment of platelet disorders is primarily by transfusion of platelet concentrates when clinically necessary. Today, in Canada, a mix of more than 300 000 whole-blood-derived

and apheresis platelet products (2 million products in the USA, and 2.5 million products in Europe) are manufactured annually to meet this transfusion need.

Compared to other blood products, platelets currently have a markedly short shelf-life (5 days) owing to the deterioration of the quality of the platelets stored at 22°C. Whereas the risk of transfusion-related transmission of viral diseases has steadily decreased over the last 40 years, the risk of transmission of bacteria had remained about the same until the recent emphasis on bacterial risk reduction strategies for platelet products including the diversion of the initial 10 to 30 mL of donor blood (which contains the skin plug) as well as the culture of platelet products prior to use [161-164]. Although the aforementioned strategies result in a general reduction of bacterial risk, they do not mitigate all risk; bacterial contamination remains a significant residual transfusion risk [165, 166]. The only approach that is likely to achieve near absolute bacteriological safety is the inactivation of bacteria by pathogen reduction technologies. However, it is clear that these treatments have some effects on the platelets, and may exacerbate the development of the storage lesion [165, 167-170].

1.4.3. Platelet Storage Lesion: Monitoring *in vitro* Functionality

The PSL has traditionally been quantified by *in vitro* measures [171] resulting in a decrease in platelet morphology determined by the Kunicki morphology score and response to agonist monitored by the extent of shape change (ESC), together with an increase in hypotonic shock response (HSR); with ESC and HSR assessed by light scattering techniques. Both the morphology score and HSR are considered to have appropriate sensitivity to platelet changes during storage [172]. Flow cytometry permits the rapid analysis of large numbers of platelets

within relatively small quantities of sample assessing changes on the platelet surface such as glycoprotein expression (GP Ib, GP IIb, GP IIIa), the generation of platelet activation markers (CD40L, CD62P, CD63) and the exposure of negatively charged phospholipids as determined by annexin V binding. Monitoring of GP expression under stimulation with agonists such as ADP or thrombin revealed reduced responsiveness during storage [173]. Using clinical chemistry analysis methods, the solution pH [10, 11, 174] paralleled with determination of the blood gas pO_2 and pCO_2 as well as glucose and lactose concentration in the storage bag can be readily determined. Nevertheless, most of these measures are not currently used in hospitals for standardised quality assessment of platelets prior to transfusion, but are instead restricted to research applications. To be of clinical value, such measures should reflect one or more of the physiological functions of platelets and should be simple, practical and fit in to the transfusion laboratory setting [26].

Measurements of platelet recovery and survival following autologous transfusion of radiolabelled platelets [175] into normal volunteers have indicated a reduction of at least 25% in re-infusion studies [10, 11, 28-32]. Although these data appear to correlate very well with *in vitro* measures analysing ESC and determination of lactose production [175], we still lack a thorough understanding of how *in vitro* results in platelet concentrates predict platelet function *in vivo* following transfusion [171]. Indeed, methods of platelet preparation may alter the recovery and survival characteristics of platelets following transfusion. A recent study by Arnold *et al.* suggests that platelet viability is better preserved in TRIMA apheresis platelets (leukoreduced apheresis platelets collected on the Trima Acel automated blood collection system; Gambro BCT, Lakewood, CO) than in leukoreduced PRP platelets [176]. However, the relative clinical efficacy of these platelet products for bleeding prevention and treatment have yet to be

determined, and will need to be addressed in well designed randomised clinical trials. Comparison of platelets derived from PRP, BC or apheresis technologies has also demonstrated differences in terms of *in vitro* functional activity. This observation is believed to be related to the existence of heterogeneous subpopulations of platelets [177], although further studies are required to substantiate this hypothesis.

1.4.4. Relationship of Proteins Identified as Changing in Concentration During Storage

Understanding the mechanisms which lead to the development of storage lesion has been of longstanding interest. Storage-related changes in the pattern of cytosolic and membrane proteins were first noticed in 1987 by Snyder and colleagues through the use of 2D gel electrophoresis [178]. Unfortunately, they were only able to identify 2 actin fragments as significantly accumulating in platelets during the first 7 days of storage due to limits in genome sequencing and bioinformatics at the time. The potential of proteomics as a viable tool for the identification of the PSL has since increased dramatically with the development of mass spectrometry [135], and has required the development of quantitative proteomic techniques such as DIGE, ICAT and iTRAQ (for a review see Ong, S.E. *et al.* [120]). Thiele and coworkers recently employed one such technique (DIGE) to comprehensively assess the impact of storage on the global proteome profile of therapeutic PCs [179]. Although they were unable to represent membrane proteins due to their high hydrophobicity (a frequent shortcoming of gel-based proteomic approaches), this group found that roughly 3% of the cytosolic platelet proteome displays a change in relative intensity over a storage period of 9 days [179]. Of these, septin 2,

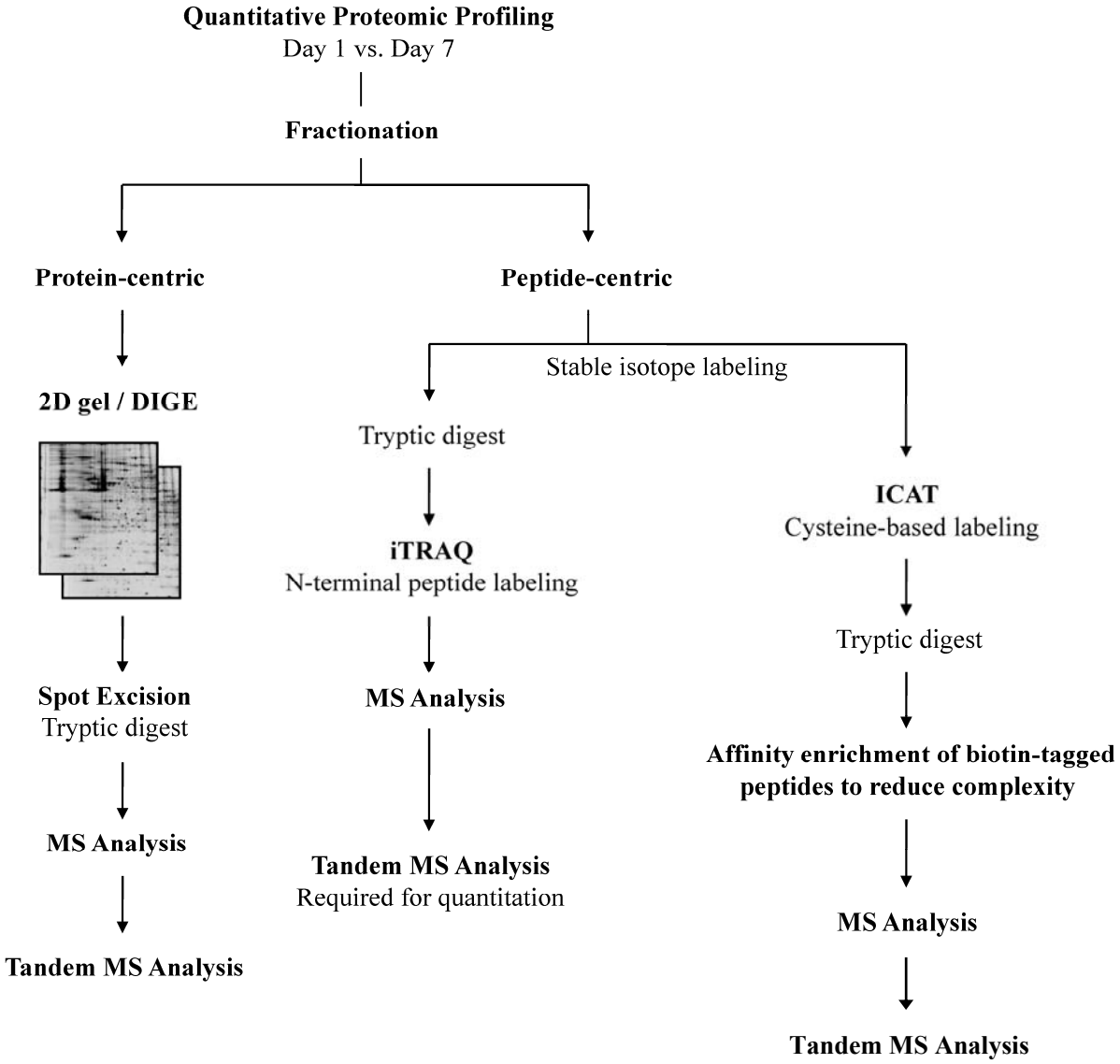
beta-actin and gelsolin were found to increase significantly in concentration during this time, and may be related to apoptosis [179-181].

In yet another gel-based study, this one focused on storage-induced changes in the PC supernatant, Glenister *et al.* identified modifications to platelet proteins tremlike transcript 1 and integrin-linked kinase, which they suggest may influence platelet-endothelium interactions [182]. Moreover, the concentration of the platelet-derived cytokines CXCL7, epidermal growth factor, platelet-derived growth factor (PDGF), brain-derived neurotrophic factor (BDNF) and CCL5 where all found to increase during a 7-day storage period—the latter 3 showing significant increases in their relative concentrations between days 5 and 7 [182]. Greening *et al.* have since performed a comparison of human membrane-cytoskeletal proteins with the plasma proteome [183]. This correlation sets the basis for the identification and classification of proteins that are selectively acquired from plasma by platelets (such as L-lactate dehydrogenase, serum albumin, fibrinogen, carbonic anhydrase, endoplasmin, and multimerin 1), from those that are endogenous to platelets (such as actin, actinin, filamin, tropomyosin, thrombospondin 1, platelet basic protein, platelet factor 4, and stomatin) and are potentially released into the circulation or made available for concentrated and focal release at vascular sites of injury.

The observations made in these 2 manuscripts [179, 182] were corroborated in a complementary proteomic study of my own design which addressed the relative differences among DIGE, ICAT and iTRAQ in the analysis of the PSL (Figure 5), and further identified platelet proteins α-actinin 1, ARP2/3 complex 16 kD subunit, cofilin, GP IIb alpha chain precursor, myosin heavy chain, Rap1, talin 1, 14-3-3 protein ζ/δ (also identified by Thiele *et al.*), thrombospondin 1, and the tubulin beta 5 chain, as changing significantly in relative concentration over a 7-day storage period [1]. Integrin-linked kinase and CXCL7 also showed

Figure 5. A schematic of the experimental setup and workflow for a complementary proteomic assessment of changes occurring in a platelet unit during storage.

Abbreviations: 2D gel, 2-dimensional gel-electrophoresis; DIGE, differential in-gel electrophoresis; iTRAQ, isobaric tag for relative and absolute quantification; ICAT, isotope coded affinity tags; MS, mass spectrometry.



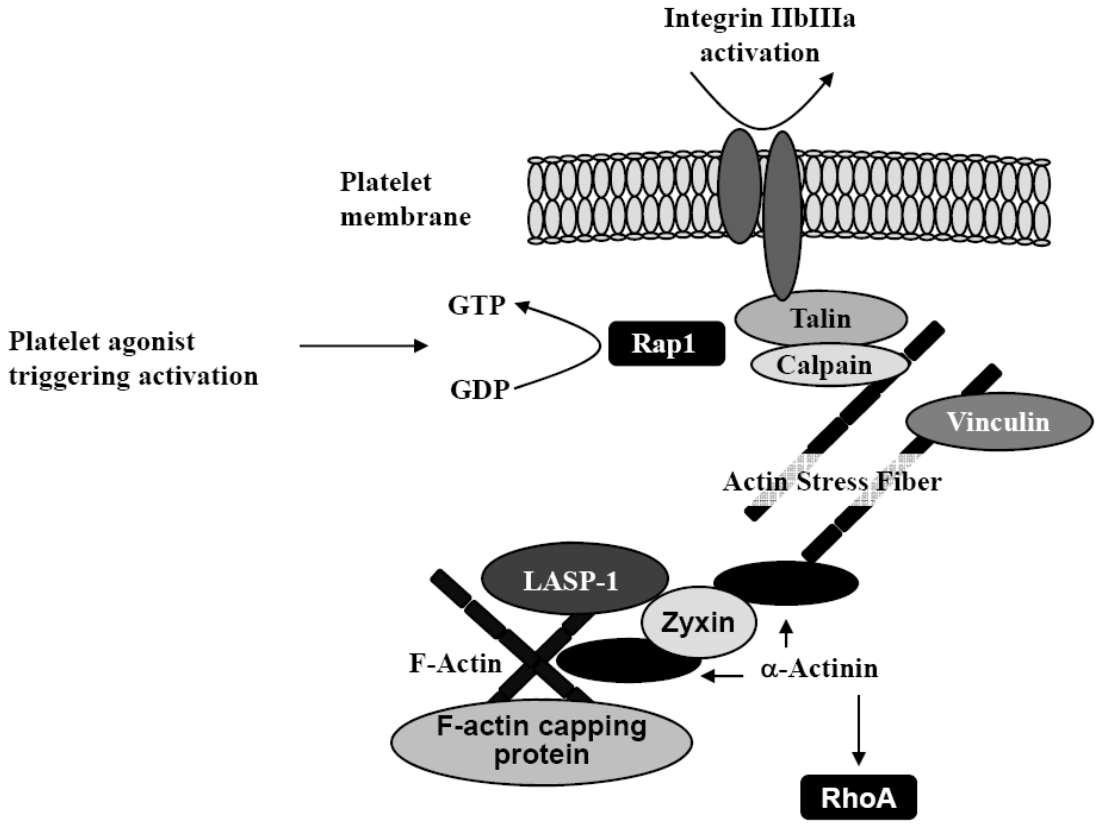
significant changes in their relative concentrations during storage, however both presented lower levels in platelets on day 7 versus day 1. It is therefore likely that these cytokines are released by platelets into the surrounding plasma during storage and so account for their elevated levels in the PC supernatant [182]. Further analysis of the integrin GP IIb/IIIa complex established that GP IIIa increases in both relative concentration and surface expression within the first 7 days of storage and demonstrated that platelets are capable of translating both GP IIb and GP IIIa over a period of 10 days [96].

It is interesting to observe how all of these proteins are related. Actin and tubulin are both components of the cytoskeleton and interact directly with actinin, ARP2/3, cofilin, myosin, talin, proteins 14-3-3, Rap1 (some of which are involved in cell signalling via GP IIb/IIIa, Figure 6), and thrombospondin (which has been shown to act on a subpopulation of platelets in response to simultaneous activation with collagen and thrombin [184] to induce the formation of focal adhesions [185]). Septin 2 is a member of an evolutionarily conserved family of GTP-binding proteins [186] that interact with the actin cytoskeleton and have been implicated in cytokinesis, cellular morphogenesis, and vesicle trafficking [187, 188]. Fibrinogen, pleckstrin, and the 78-kD glucose-dependent protein were also identified as changing in relative concentration during storage in both studies and, along with the aforementioned list, support reports of platelet activation during storage [189]. Moreover, the increase in relative concentration of GPs IIb/IIIa—along with a remarkable number of proteins known to participate in integrin signalling—indicates a possible link between this pathway and that of the storage lesion.

Unfortunately, despite significant strides in the field of platelet proteomics, variation in individual protein concentration (donor-donor variability) continues to represent an important limiting factor in the study of the PSL. This observation is complicated by clear differences in

Figure 6. Model of the integrin signalling pathway mediated by the GP IIb/IIIa.

All proteins displayed are identified as changing during platelet storage by proteomic approaches leading to the potential involvement of this pathway to the storage lesion. Abbreviations: GDP, guanosine diphosphate; GTP, guanosine triphosphate; LASP-1, LIM and SH3 domain protein 1.



the types and number of proteins identified by the different proteomics tools that are currently available [190]. As a result, careful attention must be paid to determining which technology yields the most appropriate information [190, 191]. A combination of both protein- and peptide-centric approaches should generally be considered when analysing the platelet proteome, as using any single proteomics method to study platelet storage changes may give insufficient information [1]. High-abundance proteins such as actin can also represent an important limiting factor in the study of platelet proteomes because of the wide range of protein levels within a cell (which can vary by up to 8 orders of magnitude). Due to competition for sequencing between peptides, which favour those derived from highly expressed proteins, proteomic tools such as 2D gels and iTRAQ often fall short for often interesting but lower-abundance proteins (eg. kinases, receptors). To overcome this problem, many pre-fractionation (protein purification) techniques have been proposed over the years to help facilitate protein identification, and should be considered [192]. The most common is the use of multiple affinity columns, which help concentrate the protein, and improve the likelihood that sequence information will be obtained. Lastly, it is important to keep in mind the extent to which we can rely on the proteomic information collected. In this regard, researchers should adhere to standards proposed by the International Society of Thrombosis and Haemostasis [193]. This requires that investigators minimise the degree of platelet activation during blood collection and cell isolation to limit activation-dependent changes in post-translation modification; stop experimental reactions as quickly as possible to minimise post-activation changes in proteins, notably degradation; ensure that the degree of contamination of the platelet sample with other cell types and plasma is kept to an absolute minimum; remain conscious of factors that might influence the uptake of proteins and protein binding by platelets (*e.g.*, buffer), and which may alter the composition of the

platelet proteome; recognise that there is a potential for error in peptide sequencing that is governed by the criteria used for acceptance of the predicted sequence and the potential of splice variants, which cannot be predicted from genomic databases; and appreciate that there is a potential for error in searching protein databases such as NCBI, SWISS-PROT and TrEMBL and that the predictive value of sequenced peptides does not take into account factors such as alternative splicing, polymorphisms and post-translational modifications.

1.5. Rationale and Objectives

Platelet storage and availability for the purposes of transfusion are currently restricted by a markedly short shelf-life of 5 to 7 days owing to an increased risk of bacterial growth and storage-related deterioration (the PSL). With the development and implementation of bacterial detection and pathogen inactivation strategies, the only remaining issue is the quality of platelets during the extended storage. While the manifestations of the storage lesion have been well studied using a variety of *in vitro* measures, the precise biochemical pathways involved in the initiation and progression of this process have yet to be identified. Proteomics has emerged as a powerful tool to identify and monitor changes during platelet storage, and in combination with biochemical and physiological studies facilitates the development of a sophisticated mechanistic view. This thesis aims to resolve some of the processes leading to storage-associated losses of blood component quality resulting from the PSL by posing the following hypotheses (underlying points denote specific aims designed to test each hypothesis):

Hypothesis 1:

Complementary proteomics approaches can be employed to identify relative changes in human blood platelet protein concentration during unit storage.

a. Analysis of protein changes in the human blood platelet proteome during storage by 2D gel/DIGE, iTRAQ and ICAT proteomic approaches.
b. Comparison of the proteomic approaches employed with regard to protein identification and agreement among approaches.
c. Identification of protein markers of the PSL by stringent significance criteria and validation of results.

Hypothesis 2:

Human blood platelets contain particularly long-lived mRNA and are capable of synthesising biologically relevant proteins *ex vivo* throughout a 10-day storage period.

a. Analysis of protein mRNA expression during storage by northern blot hybridisation and qRT PCR amplification.
b. Development of a ^{35}S-methionine incorporation assay by which to measure protein translation in human blood platelets.
c. Comparison of ^{35}S-methionine incorporation rates in freshly drawn and stored human blood platelets in the presence and absence of agonist.

CHAPTER 2 Materials and Methods

2.1. Blood Platelet Preparation and Storage Conditions

Ethical approval for this study was granted by the University of British Columbia Clinical Ethics Board and informed consent granted by the donors (see *APPENDIX 3*). Donors that had taken medications other than birth control pills or vitamins within 72 hrs of donation were excluded from the study. Whole blood collection and platelet isolation were carried out by Canadian Blood Services (Vancouver, Canada) using their standard operating procedures and the Pall Leukotrap® RC-PL (PRP), MacoPharma Leucoflex® LQT (BC), Gambro BCT Trima Accel® (Apheresis) or Haemonetics MCS+® (Apheresis) systems. The unit was aseptically sampled before and after storage (days 1 and 7, respectively; *see APPENDIX 1*). Platelets were sedimented (500 x g, 10 min) and washed twice gently in CGS buffer (10 mM trisodium citrate, 30 mM dextrose, 120 mM NaCl, pH 6.5) supplemented with 1 U/mL apyrase to further clear the sample of residual leukocytes. The platelets were resuspended in ETS buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4) and a cell count obtained (Advia 120 Haematology System, Bayer, Leverkusen, Germany). Leukocyte enumeration was done using the Leukosure kit (Beckman Coulter, Fullerton, CA) on an EPICS® XL-MCL flow cytometer (Beckman Coulter) and yielded leukocyte counts well below 2.5 x 10^{-6} percent of the platelet count (< 0.5 cells/µL). The platelets were sedimented and the pellet was solubilised over ice in 50 mM Tris, 0.2% sodium dodecyl sulphate (SDS), 4 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to a protein concentration of approximately 5 mg/mL. The solubilised platelet sample was incubated at 100°C for 10 min to fully denature the protein, and subsequently placed on ice. Finally, an equal volume of 9.5 M urea was added and the sample was incubated on ice with intermittent

vortexing for 1 hr. Samples were centrifuged (12000 x g, 10 min, 4°C) to remove insoluble cellular debris, and the supernatants comprising the whole cell lysate were stored frozen.

2.2. Two-Dimensional Gel Electrophoresis

Platelets were sampled on days 1 and 7 of storage as outlined above. Washed samples were lysed in a buffer containing 7 M urea, 2 M thiourea, 65 mM 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulphonic acid (CHAPS), 2 mM fresh tributylphosphine and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), then stored at -80°C. Protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL). For the quantitative analysis using the DIGE technique, labelling with CyDye reagents was carried out according to the manufacturer's protocol (GE Healthcare, Chalfont St. Giles, UK). To 2 mg of unlabelled or 50 µg of DIGE-labelled platelet lysate, ampholytes appropriate for each pI range strip (4-7 or 3-10) were added at a final concentration of 1.5% (v/v), and samples were prepared as described [142]. For the second dimension, the strips were placed on top of a 12% SDS-PAGE gel using Mark-12 (Invitrogen, Burlington, ON) as a protein marker. The proteins were separated with 50 V for 1 h followed by voltage adjustments until 1800 volt hrs were reached. Following electrophoresis, gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid, stained with Sypro Ruby (Gibco, Langley, OK), and scanned at the wavelength specific for the fluorescent labels.

2.3. Validation of the Normalisation of Samples to Total Protein Concentration

To assess data reliability, it was necessary to determine the extent of within-sample variability that exists in platelets that have been stored for 7 days. Equal amounts of total protein sampled from stored platelets on days 1 and 7 were loaded onto immobilised pH gradient (IPG) strips with a pI range of 3-10 or 4-7, and separated by 2D gel electrophoresis as outlined previously (Figure 7). The spot intensities of each gel were quantified following staining and compared between the 2 sampling days. A 7% and 27% variation in overall intensity of the protein spots was noted for pI ranges of 3-10 and 4-7, respectively, over a 7-day storage period. The relatively small change in overall protein spot intensity detected for pI range 3-10 supports the practice of normalising the platelet samples for total protein loaded as a basis for comparative analysis by 2D gel electrophoresis. Considering that only a subset of proteins subjected to protein quantification are amenable to 2D gel analysis, the minor discrepancies observed are likely due to quantitative changes of hydrophobic, acidic, and basic proteins or loss of small fragments upon protein degradation that are not detectable in the gels. While a larger variation in overall protein spot intensity was observed when the pI 4-7 strips were used, the majority of protein changes occurred within this narrow pI range and the resulting gels showed significantly better resolution of the protein spots; a conclusion also reached by Thiele *et al.* [179]. As such, all subsequent analyses were carried out with a pI range of 4-7 for a more detailed assessment of protein changes.

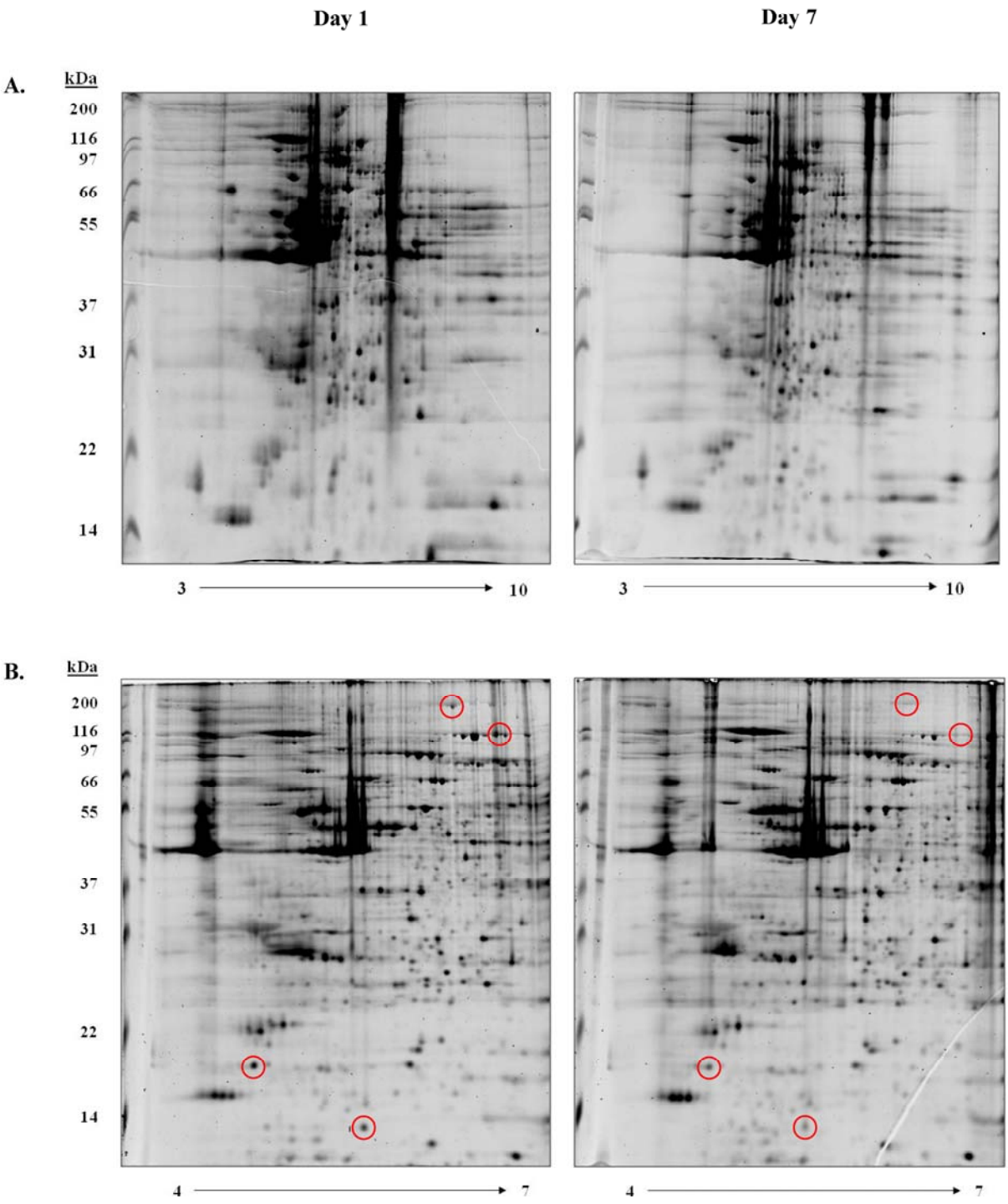


Figure 7. Representative 2D gel analyses of the blood platelet proteome during storage.

A pair of Sypro Ruby-stained gels shows protein separation in the pI range 3-10 (*A*) and 4-7 (*B*) for day 1 and day 7 samples of a single platelet unit. The majority of protein changes during storage were observed within the pI 4-7 range, and had significantly better resolution of the protein spots when resolved on the pI 4-7 range strip than on the pI 3-10 range strip. Circles represent examples of spot intensity changes during storage.

Protein quantities per spot were determined using ProFinder 2D software (Perkin Elmer, Boston, MA). Protein spots were excised and digested in-gel with trypsin as described [194]. Following peptide extraction, mass spectrometric analysis was carried out using a 4700 Proteomics Analyzer or a QSTAR XL (both Applied Biosystems, Foster City, CA) before proteins were identified by MASCOT searches against current Swiss-prot databases [195]. For standard 2D gel electrophoresis, protein spots were required to change in at least 8 of 11 gels in order to compensate for differences in staining and migration. Proteins identified as changing by DIGE had to show at least 1.5-fold increase or decrease to be included in the data set.

2.4. Isotope Coded Affinity Tagging Analysis

Of 3 different pairs of frozen samples from days 1 and 7 of storage, termed ICAT I, II and III, ICAT I and II were analysed at the University of Victoria, Genome BC Proteomics Centre (Victoria, Canada) and the third sample was sent to the Institute for Systems Biology (Seattle, USA). Samples were differentially labelled with the day 1 and day 7 ICAT reagents, combined, digested with trypsin, and the resulting peptides were separated by cation exchange chromatography. Identification and relative quantification of the peptides were carried out on a QSTAR Pulsar mass spectrometer (Applied Biosystems, Foster City, CA) or an LTQ linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). In both cases samples were loaded into the mass spectrometer by electron spray ionisation (ESI). Possible protein identities were obtained by matching peptides to the International Protein Index (IPI, http://www.ebi.ac.uk/IPI/IPIhelp.htmL). The ratios of the day 7 peptides versus the day 1 peptides were normalised to 1 for the peak of the distribution to correct for any skew in the data.

A significant change in concentration ($p < 0.01$) and a high confidence in identification ($\geq 99\%$) were the inclusion criteria for proteins identified by this approach to be included in the data set.

2.5. Isobaric Tag for Relative and Absolute Quantification Analysis

Four different pairs of frozen samples from days 1 and 7 of storage, termed iTRAQ I, II, III and IV, were sent for iTRAQ analysis to the University of Victoria, Genome BC Proteomics Centre. Samples were digested with trypsin, differentially labelled with the iTRAQ reagents, combined, and the resulting peptides were separated by cation exchange and reverse phase chromatography; analysis was performed with a QSTAR Pulsar mass spectrometer. Data analysis for the iTRAQ experiments was performed with Pro QUANT version 1.0 and displayed in a Pro Group Report (version 1.0.5) provided by the University of Victoria Genome British Columbia Proteomics Centre. iTRAQ sample IV was processed under identical conditions and analysed twice to account for within-sample differences; iTRAQ runs IV(a) and (IV(b). iTRAQ I was searched against the Celera Discovery Systems database (CDS), iTRAQs II and III were searched against the Matrix Science database (MSDB), and iTRAQs IV(a) and IV(b) (replicates of the same sample) were searched against both MSDB and IPI. The tolerance set for peptide identification in Pro QUANT searches was 0.20 Da for both mass spectrometry (MS) and tandem MS (MS/MS) analyses. Relative quantification of proteins was performed on the MS/MS scans and was calculated using the ratio of the areas under the isotope tag-specific peaks at 114 and 116 Da for iTRAQ I, and 115 and 117 Da for iTRAQs II, III, IV(a) and IV(b). A significant change in concentration ($p < 0.01$) and a high confidence in identification ($\geq 99\%$) were the inclusion criteria for proteins identified by this approach. A comprehensive table of all proteins

identified as changing significantly in concentration ($p \leq 0.01$) and over a 7-day storage period in human platelets, as determined by 2D gel, DIGE, iTRAQ and ICAT has been posted online (*see APPENDIX 2,* http://www.blood.ca/CentreApps/Internet/UW_V502_MainEngine .nsf/page/Research_Data?OpenDocument).

2.6. Immunoblotting

To confirm protein changes detected by proteomics analysis, immunoblotting was performed on a representative range of protein types. Platelet lysates of day 1 and day 7 of storage were run in triplicate on a SDS-PAGE gel and blotted onto a nitrocellulose membrane. The membrane was probed with primary antibodies against superoxide dismutase, septin 2, Rap1 and zyxin (Santa Cruz Biotechnology, Santa Cruz, CA), Rho-GDP dissociation inhibitor, tubulin-beta, beta-actin (Sigma, St. Louis, MO) and 14-3-3 (Abcam), followed by their respective secondary horseradish peroxidase-labelled antibodies (Jackson Immunoresearch, West Grove, PA). Protein bands were visualised using the ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont, UK) and their respective intensities were measured on a ChemiGenius2 bio-imaging system (Perkin Elmer, Waltham, MA). To monitor total GP IIIa increase during storage, platelet lysates were normalised to cell count and the protein band intensities normalised against day 1 values. Relative intensity readings were taken within the quantitative linear range of detection for all samples.

2.7. Flow Cytometry

For analysis of changes in GP IIb/IIIa and GP Ib-alpha/IX/V surface expression during storage, platelet samples were collected on days 1, 5, 7, and 10. The ADIAflo^{TM} platelet GP IIb/IIIa and GP Ib-alpha/IX/V Occupancy kits (American Diagnostica Inc., Stamford, CT) were used for platelet membrane glycoprotein quantification by flow cytometry, and included mouse IgG antibodies specific for GP IIIa bound to the GP IIb subunit, GP Ib-alpha, GP IX and GP V. Briefly, the total number of GP IIb/IIIa, Ib-alpha, IX, and V surface receptors were determined by converting the fluorescence intensity generated from the bound polyclonal antibody anti-mouse IgG-FITC stain in each separate sample into the corresponding number of sites per platelet, based on a calibrated bead standard curve. Using a standard acquisition procedure described by the manufacturer, platelets were isolated from other whole blood cells by their characteristic forward (FS) and side scattering (SS) as they pass through the flow cytometer, and their mean fluorescent intensity per platelet calculated after subtraction of a negative isotypic control (mouse monoclonal antibody, IgG) measurement. Analysis of each platelet unit was performed in triplicate. Samples were normalised for cell count. Data were subject to one-way analysis of variance (ANOVA) for four independent samples and Tukey honestly significantly different (HSD) analysis.

2.8. Leukocyte Enumeration

Leukocyte counts were acquired with the LeukoSure™ Enumeration kit (Beckman Coulter, Fullerton, CA) as per the manufacturer's instructions. Briefly, the platelet sample is lysed and permeabilized using the LeukoSure Lyse Reagent to eliminate RBCs and PLTs, and prepare the cells for subsequent addition of the LeukoSure Stain Reagent which contains propidium iodide and RNAse. In the absence of RNA, propidium iodide binds only to double stranded DNA so that nucleated cells in the sample emit fluorescence in proportion to their DNA content, which is subsequently measured by flow cytometry. Since mature platelets and red blood cells do not contain DNA, the stained cells represent the leukocyte component of the platelet sample. The enumeration method depends upon mixing a known volume and concentration of Leukosure Fluorospheres with an identical volume of sample to be tested. After analysis the absolute count of the specimen is calculated thus representing the absolute number of leukocytes in the specimen. Samples were run on an EPICS® XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA) and analysis performed on at least 3 different platelet units in triplicate.

2.9. RNA Purification

Total RNA was isolated using the TRIzol Reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions. The isolated platelets were lysed in TRI Reagent by pipetting. Lysates were vortexed vigorously and the RNA was isolated by phenol/chloroform extraction followed by isopropanol, and air-dried. RNA pellets were resuspended in 10 mL

diethyl pyrocarbonate treated water (DEPC-H_2O) and heated in a 57°C water bath (Forma Scientific, Marietta, OH) for 5 min to solubilise RNA. The RNA content was determined by measuring optical densities in a ND-1000 UV/vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA was stored at -80°C. Samples were normalised for cell counts.

2.10. Northern Blotting

Platelet RNA was run in triplicate on a 1.0% agarose gel and blotted onto a nylon membrane. The membrane was incubated with γ ^{32}P-labelled DNA probe (GCT CTG GGC GAC TGT GCT) at 37°C overnight. Bands were visualised by autoradiography overnight against a storage phosphor screen (Amersham Biosciences, Little Chalfont, UK). A Typhoon 8600 variable mode imager (Molecular Dynamics, Sunnyvale, CA) was used to collect the resultant image.

2.11. Reverse Transcription

First-strand cDNA synthesis was performed using the Superscript II Reverse Transcription System (Invitrogen, Burlington, ON) according to the manufacturer's instructions. Two hundred units of Superscript II reverse transcriptase and 0.5 μg oligo$(dT)_{12\text{-}18}$ serving as primers were used to transcribe 1.0 μg of total RNA per 20 μL of the reaction mixture.

2.12. Polymerase Chain Reaction Amplification

Samples were prepared using the Qiagen Long Range PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Polymerase chain reaction amplification was performed in a PTC-200 Peltier thermal cycler (MJ Research, Miami, FL) under the following conditions:

94°C → 2 min

94°C → 30 sec

55°C to 45°C → 30 sec

68°C → 2 min 30 sec

94°C → 30 sec
45°C → 30 sec
68°C → 2 min 30 sec
} 35x

68°C → 2 min

Primers for PCR amplification of GP IIIa (Long Range PCR 1 and 2) and the gamma chain of T cell antigen receptor-associated T3 complex (CD3G) mRNA were selected manually using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) so that the product of transcription spanned exons 1 to 15 and 1 to 16 of the GP IIIa mRNA transcript (platelet), and a 243 bp region of the CD3G mRNA transcript (control), respectively. The GP IIIa and CD3G

nucleotide sequences were retrieved from the gene bank (NM000212, NM_000073), and the probes were constructed by meeting the following requirements: Approximately 18-27 bp in length, T_m roughly 60°C, at least 50% G/C content, no runs of more than 3 G/Cs at the 3' end, 3' ends must not be complimentary and primers must not be self-complementary. The primers were synthesised by Integrated DNA Technologies Inc. and are listed below:

GP IIIa – Long Range PCR 1 (2 340 bp product)

Upper Primer: 18-mer 5' GCT CTG GGC GAC TGT GCT 3'

Lower Primer: 18-mer 5' TAA GTG CCC CGG TAC GTG 3'

GP IIIa – Long Range PCR 2 (4 341 bp product)

Upper Primer: 20-mer 5' GAC AAG GGC TCT GGA GAC AG 3'

Lower Primer: 20-mer 5' CAC CTG TTC CCT GCC ACT AT 3'

CD3G (243 bp product)

Upper Primer: 20-mer 5' CTT GTG ATG CAG AAG CCA AA 3'

Lower Primer: 20-mer 5' TGC TGA CGA TTT CAG CAA AG 3'

Samples were electrophoresed on a 1.0 or 2.0% agarose gel containing 10 µg ethidium bromide and visualized on the ChemiGenius2 bio-imaging system transilluminator (Perkin Elmer, Waltham, MA).

2.13. Real-Time PCR Amplification

Samples were prepared using the Syber Green Jumpstart Taq ready mix (Sigma, St. Louis, MO) according to the manufacturer's instructions. Ten µL Syber Green and 1 µM of the forward and reverse primers were used to transcribe 5 µL of the template cDNA per 15 µL of the reaction mixture. Samples were run alongside a cDNA dilution ladder and a 'no template' control. Real-time PCR amplification was performed in a DNA Engine Opticon 2 continuous fluorescence detector (MJ Research, Miami, FL) under the following reaction conditions:

50°C → 2 min

95°C → 10 min

95°C → 15 sec
60°C → 30 sec
72°C → 30 sec
} 40x

72°C → 10 min

Primers for PCR amplification of GP IIIa were selected manually using the PrimerSelect Software 5.00 (DNA-STAR Inc., Madison, WI), and designed such that they spanned 5 short (50-200 bp) equidistant regions along the entire mRNA transcript.. The GP IIIa nucleotide sequence was retrieved from the gene bank (NM000212), and the probes were constructed by meeting the following requirements: Approximately 20-24 bp in length, T_a roughly 62°C, product length no larger than 200 bp, at least 50% G/C content, no runs of more than 3 G/Cs at

the 3’ end, 3’ ends must not be complimentary and primers must not be self-complementary. The primers were synthesised by Integrated DNA Technologies Inc. and are listed below:

qRT PCR 1 (195 bp product)

Upper Primer: 22-mer 5’ GCG TAG GAG GGC CCA ACA TCT G 3’

Lower Primer: 24-mer 5’ CTC GGG CCT CAC TCA CTG GGA ACT 3’

qRT PCR 2 (63 bp product)

Upper Primer: 20-mer 5’ CCT CCA GCT CAT TGT TGA TG 3’

Lower Primer: 20-mer 5’ TCA CGC ACT TCC AGC TCT AC 3’

qRT PCR 3 (50 bp product)

Upper Primer: 20-mer 5’ CCG TGA CGA GAT TGA GTC AG 3’

Lower Primer: 20-mer 5’ CAT CCT TGC CAG TGT CCT TA 3’

qRT PCR 4 (58 bp product)

Upper Primer: 20-mer 5’ GTT CTC TCG CAA GGG AAG TC 3’

Lower Primer: 20-mer 5’ ATC CTC ACT CCC AAC AGG TC 3’

qRT PCR 5 (56 bp product)

Upper Primer: 20-mer 5’ GTG GAA GCT CAT GCC TGT AA 3’

Lower Primer: 20-mer 5’ GAG CCA CCA CAC CTG TCT TA 3’

Plates were read at 83°C using the Opticon Monitor version 3 software package (MJ Research, Miami, FL), and sample values were normalised for cell counts. Quantification was performed using a relative standard curve that was calculated from the slope of the best linear fit of the logarithm of the dilution factor of cDNA, plotted versus the measured increase in the PCR amplification cycle at which the reporter dye fluorescence passed the selected baseline (C_T). To confirm the presence of a single protein band samples were run on a 2.0% agarose gel containing 10 μg ethidium bromide and visualized on the ChemiGenius2 bio-imaging system transilluminator (Perkin Elmer, Waltham, MA).

2.14. ^{35}S-methionine Incorporation into Platelet Protein

Platelet samples were collected on days 1, 5, 7, and 10, and subsequently washed as previously described, save for the following changes: samples were normalised for cell counts and incubated 1:24 (v/v) with Easy Tag Express Protein Labelling Mix (32.7 MBq ^{35}S-methionine) for 30 min. Platelets were sedimented (500 x g, 10 min) and the pellets solubilised over ice in RIPA lysis buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 μg/mL PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 2 μg/mL aprotinin) for 10 min by repeated passage through a 21-gauge needle.

2.15. GP IIIa Immunoprecipitation

Platelet GP IIIa immunoprecipitation was carried out as outlined on the Santa Cruz Biotechnology website (http://www.scbt.com/protocol_2.php). Platelet lysates were pre-cleared with 1.0 μg IgG_1 mouse anti-human beta-actin control (Sigma, St. Louis, MO), together with suspended (25% v/v) Protein G-plus agarose conjugate (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitation was performed by adding 1.0 μg IgG_1 mouse anti-human GP IIIa (Santa Cruz Biotechnology, Santa Cruz, CA) to the pre-cleared platelet lysate, followed by the aforementioned agarose conjugate suspension, and were subsequently left to incubate at 4 ºC on a rocker platform for 1 hr prior to collection.

2.16. Autoradiography

Glycoprotein IIIa samples were run in triplicate on a SDS-PAGE (7.5% stacking, 4% separating) gel. The samples were fixed (10% acetic acid, 25% isopropanol) for 30 min and then incubated in Amplify Signal (NAMP 100, Amersham Pharmacia Biotech, Piscataway, NJ) for an additional 30 min prior to gel drying (Gel Dryer Model 583, Bio-Rad Laboratories, Hercules, CA). Autoradiography was performed overnight against a storage phosphor screen (Amersham Biosciences, Little Chalfont, UK). A Typhoon 8600 variable mode imager (Molecular Dynamics, Sunnyvale, CA) was used to collect the resultant image. Protein band intensities were quantified with ImageQuant version 5.2 (Molecular Dynamics, Sunnyvale, CA) and normalised against day 1 values.

2.17. Liquid Chromatography Tandem Mass Spectrometry Analysis

Protein band excision protocol for liquid chromatography (LC) tandem mass spectrometry (MS/MS) was adapted from Wilm, M. [196]. The extracted peptide samples were run on an API QSTAR Pulsar mass spectrometer and searched against the SwissProt database for human proteins. A cut-off of 95% confidence was established for each analysis.

2.18. Quantification of ^{35}S-methionine Incorporation into Platelet Protein

Samples were normalised for cell count at physiological platelet concentrations (1.5×10^8 platelets/mL) and EasyTag™ Express protein labelling mix (Perkin Elmer, Waltham, MA) was added to each at the various listed concentrations (47 to 752 pmol/mL). Samples were then divided into 200 µL aliquots and incubated at RT and with gentle agitation for increasing periods of time. Platelet samples that were suspended in a boiling water bath (95°C) for the duration of the assay or exposed to 0.6 J ultra-violet (UV) light (254 nm wavelength) prior to the addition of the ^{35}S-methionine protein labelling mix served as negative controls. Translation was arrested by exposure of the platelet sample to 0.6 J UV light. Platelets were sedimented as before, and washed once in an equal volume of ETS before being solubilised over ice in RIPA lysis buffer (50 mmol/L HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 20 µg/mL PMSF, 1µg/mL leupeptin, 1µg/mL pepstatin A, and 2 µg/mL aprotinin) and stored at -80°C. Proteins were precipitated and washed using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA). Protein concentrations were determined by running samples on a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA) alongside a molecular weight standard.

Eight hundred μL of each sample was then added to 5 mL Cytoscint scintillation cocktail (Thermo Fisher Scientific, Waltham, MA) and run on a TRI-CARB 2100TR liquid scintillation analyser (Perkin Elmer, Waltham, MA) to determine scintillation count. Radioactivity of the samples was never less than twice the background level. Counting time was selected to yield at least 10 000 counts so that the standard error of the count was less than 1%.

To calculate the amount of ^{35}S-methionine incorporated into the protein, the samples were run alongside a ^{35}S-methionine serial dilution and values for counts per minute (CPM) were converted to pmol ^{35}S-methionine incorporated per $1x10^9$ platelets. Each incubation was carried out with at least 3 different buffy coat platelet units in duplicate and normalised against its 20 min time point. Platelet units were tested for microbial growth at the end of the storage period by the BacT/ALERT 3D system (Biomérieux Industry, Hazelwood, MO).

2.19. Effect of Agonist Exposure on Protein Translation in Platelets

Platelets were sampled from fresh blood donations and pooled buffy coat platelet units on day 6 of storage as previously described. Samples were normalised for cell count at physiological platelet concentrations (1.5×10^8 platelets/mL) and EasyTag™ Express protein labelling mix (Perkin Elmer, Waltham, MA) was added to each in final concentration of 752 pmol/mL. Adenosine diphosphate-activated platelets were incubated with 10 μM ADP for 10 min prior to ^{35}S-methionine addition. As a negative control, platelet samples were exposed to 0.6 J UV light (254 nm) prior to the addition of the ^{35}S-methionine protein labelling mix. Translation was arrested by exposure of the platelet sample to 0.6 J UV light at 20, 40, 80, 120 and 160 min

time points. The amount of ^{35}S-methionine incorporation per $1x10^9$ platelets was quantified by scintillation count and normalised against the 20 min time point.

2.20. Effect of Storage on Protein Translation Rates in Platelets

Platelets were collected from fresh blood and sampled from pooled buffy coat platelet units on days 2 to 10 of storage as previously described. Samples were normalised for cell count at physiological platelet concentrations (1.5×10^8 platelets/mL) and EasyTag^{TM} Express protein labelling mix (Perkin Elmer, Waltham, MA) was added to each at a concentration of 376 pmol/mL. Translation was permitted to proceed for 20 and 80 min for each sample, and arrested by exposure of the platelet sample to 0.6 J UV light. The amount of ^{35}S-methioinine incorporation per $1x10^9$ platelets was quantified by scintillation count and normalised against the 20 min time point to obtain a rate (per hr) value. Data were subject to one-way ANOVA for 3 independent samples and Tukey HSD analysis.

CHAPTER 3 Comprehensive Proteomic Analysis of Protein Changes During Platelet Storage Requires Complementary Proteomic Approaches

Thon J.N., Schubert P., Duguay M., Serrano K., Lin S., Kast J., Devine D.V. Comprehensive proteomic analysis of protein changes during platelet storage requires complementary proteomic approaches. Transfusion, 2008. 48(3): p. 425-435.

Proteomics methods may be used to analyse protein changes occurring in stored blood components for the purposes of identifying processes leading to storage-associated losses in quality, such as the PSL. Although many proteins are better resolved and/or represented by one proteomics method over another, the optimal strategy to perform proteomic analyses on stored platelet units, such as to obtain the most informative data sets, has remained largely undefined. This study addresses relative differences among proteomics approaches to the analysis of the PSL. Changes to the platelet proteome between days 1 and 7 of storage were analysed with 3 complementary proteomic approaches with final mass spectrometry analysis: 2D gel electrophoresis/DIGE, iTRAQ, and ICAT. Observed changes in concentration during storage of selected proteins were confirmed by immunoblotting.

3.1. Analysis of Within-Sample and Between-Sample Variability by 2D Gel Electrophoresis

Before comparing the proteomes of different platelet samples over a period of 7 days, 5 replicates of a single platelet sample were analysed by 2D gel electrophoresis to gauge the effect

of within-sample variability by this approach for the 2 sampling days (1 and 7). Each of the 2D gel pairs contained > 95% of the total number of protein spots identified in all 5 replicate pairs. Conversely, between-sample comparative analysis of the platelet proteome for a total of 11 different platelet samples yielded 85% agreement amongst the replicate pairs, indicating that the majority of the variation was due to actual differences among the samples themselves rather than variability in runs within the proteomics approach.

3.2. Analysis of Protein Changes in the Blood Platelet Proteome

A total of 977 different protein spots were detected among 11 sample replicates; 575 were successfully identified by MS, indicating protein abundance sufficiently high for accurate resolution of protein spots by MASCOT analysis, and correspond to 93 different proteins. Of these, only 9 proteins had a detectable change in concentration in at least 8 of the samples, 6 of which were later attributed to charge distribution of isoforms causing a shift in the position of the protein on the gel (*see APPENDIX 2*). Differential gel electrophoresis analysis was employed to compensate for differences in protein staining and migration observed for standard 2D gels, and to provide a more quantitative assessment of these changes. Of the 27 proteins identified as changing by this approach, 19 showed an at least 1.5-fold increase or decrease in concentration during storage (*see APPENDIX 2*). When combined, a total of 17 unique proteins demonstrated agreement in the direction of change in both protein-centric approaches (2D gel/DIGE).

Four different samples were prepared for iTRAQ analysis and led to the identification of 355 proteins; 299 for which the direction of change in protein concentration could be resolved (*see APPENDIX 2*). In order to account for within-sample and between-sample differences,

iTRAQ sample IV was processed under identical conditions and analysed twice; iTRAQ runs IV(a) and IV(b). Of the 228 proteins identified in iTRAQ sample IV alone, 69 proteins agreed in the direction of concentration change between the 2 sample runs, while 119 proteins increased or decreased in concentration in only 1 of 2 replicates. The remaining 56 proteins identified in iTRAQ sample IV could not be resolved conclusively as increasing or decreasing in relative concentration during storage as the direction of the protein change disagreed between the 2 replicate runs. Likewise, 3 different samples were subjected to ICAT labelling and analysis, which led to the identification of 139 proteins (*see APPENDIX 2*). Of these, 127 proteins agreed in the direction of their concentration change.

3.3. Comparison of Proteomic Approaches with Protein Identification and Agreement

In total, 503 uniquely identified proteins showed differential expression in response to platelet storage. By method, 93 proteins were identified by 2D gel/DIGE, 355 by iTRAQ, and 139 by ICAT. Comparative analysis of 2D gel/DIGE, iTRAQ, ICAT indicated that only 5 proteins were common to all 3 proteomic approaches employed (Figure 8*A*). In addition, 27 proteins were accessible to ICAT and iTRAQ, but not 2D gel analysis; 44 proteins were shared by the 2D gel and iTRAQ approach, but not the ICAT approach; while only 3 proteins were detected by 2D gel electrophoresis and ICAT, but not iTRAQ. Of those remaining, 279 proteins were only identified by iTRAQ, 104 were only identified by ICAT and 41 were only identified by 2D gels.

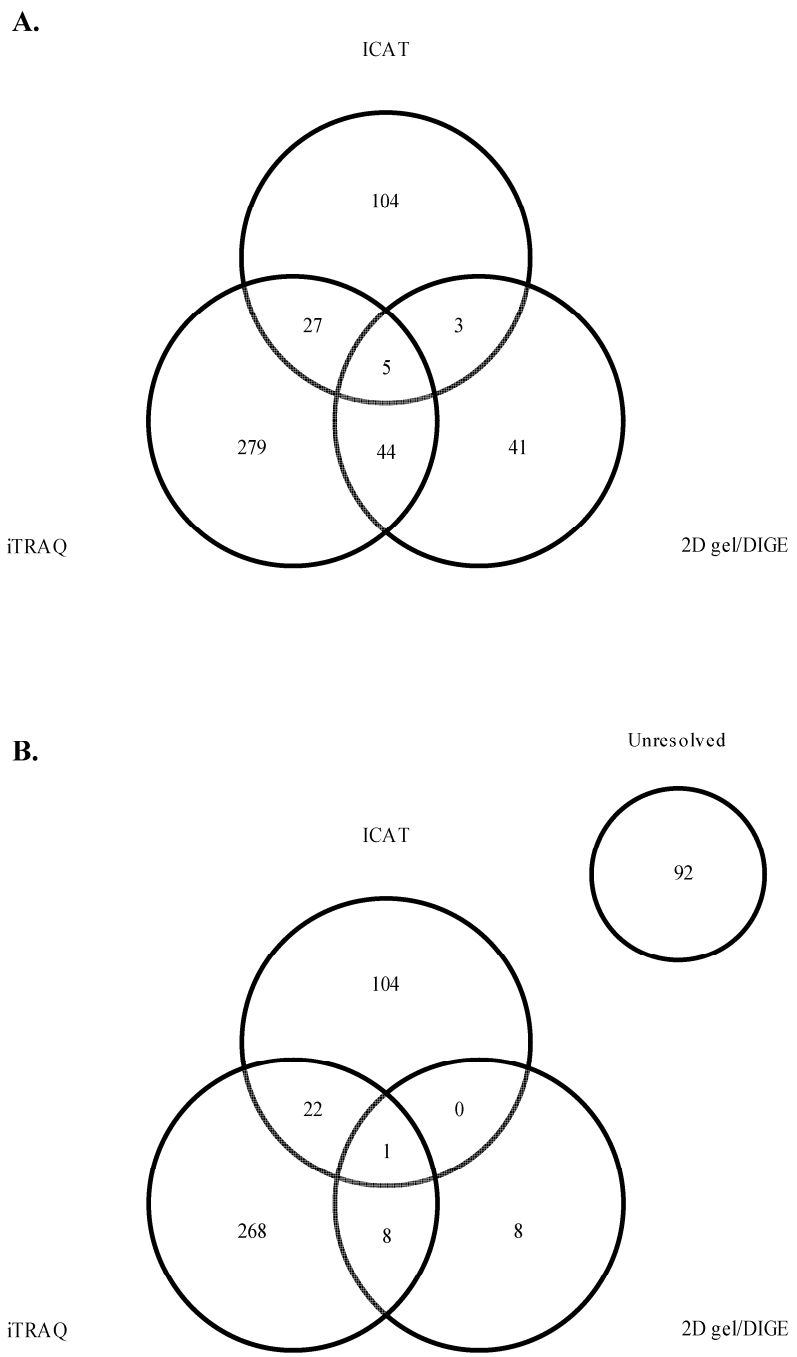


Figure 8. Agreement in protein identification (*A*) and concentration change (*B*) by 2D gel/DIGE, iTRAQ and ICAT.

(*A*) Venn diagram illustrating the agreement among 3 different proteomics approaches in the individual proteins listed in *APPENDIX 2*. (*B*) Venn diagram illustrating the agreement among 3 different proteomics approaches when identifying the relative change in protein concentration during a 7-day storage period, for proteins listed in *APPENDIX 2*.

Comparative analysis of the direction of concentration change over a 7-day storage period for those proteins identified by 2D gel/DIGE, iTRAQ and ICAT revealed only 1 protein whose change in concentration was common to all 3 proteomic approaches employed (Figure 8*B*). A further 22 proteins showed a consistent change in concentration between ICAT and iTRAQ; 8 changed consistently in the 2D gel/DIGE and iTRAQ approaches; whereas there was no agreement in the direction of concentration change for the 3 proteins identified solely by both 2D gel electrophoresis and ICAT. Two hundred sixty-eight, 104 and 8 proteins were detected as changing by only the iTRAQ, ICAT or 2D gel/DIGE approaches; with an additional 92 proteins classified as unresolved because they indicated changes by at least 1 method, but failed to agree in the direction of that change between replicates of that approach, or amongst the other proteomic approaches in which that protein changed.

In order to confirm these changes, specific protein levels were assessed in the samples (Figure 9). Immunoblot analysis of superoxide dismutase, Rho-GDI, septin 2 and zyxin revealed a significant increase in protein concentration over a 7-day storage period relative to the beta-actin loading control which agreed with the protein changes obtained through our proteomic screen, further validating our results.

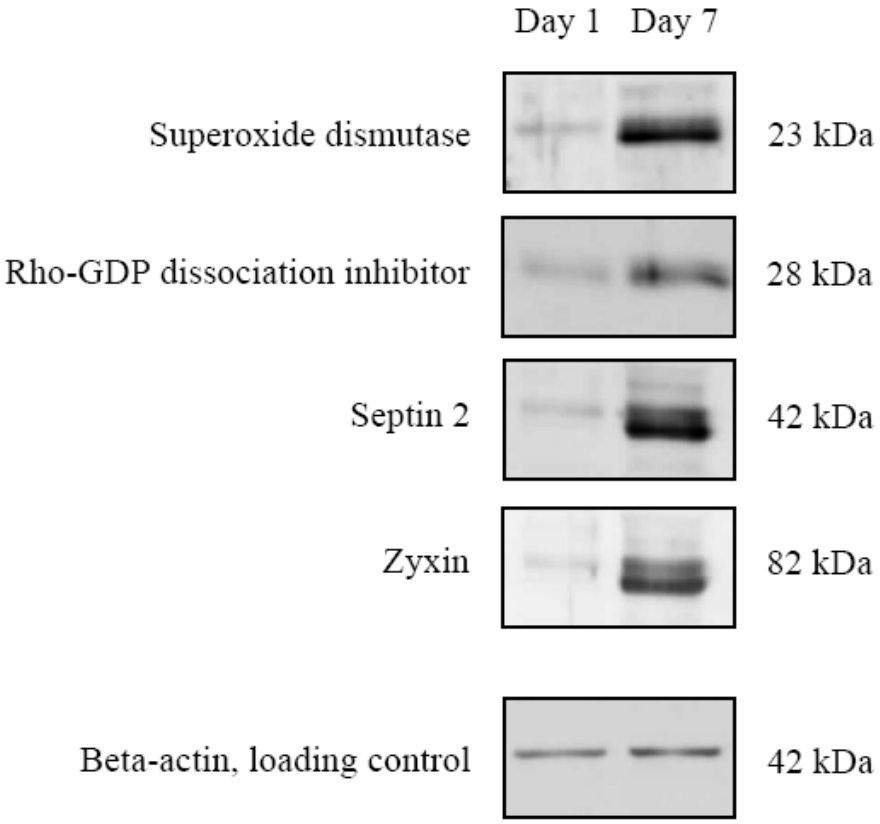


Figure 9. Immunoblot analysis of selected proteins identified as changing during platelet storage.

Representative immunoblots of superoxide dismutase, Rho-GDP dissociation inhibitor (Rho-GDI), septin 2, and zyxin demonstrating a marked increase in protein concentration during platelet storage relative to the beta-actin loading control. Samples were run in triplicate. Isotype controls showed no reactivity.

3.4. Strategies for Data Analysis

Proteins were classified by sub-cellular localisation using Swiss-Prot and GO databases to assess their compartmentalisation in platelets (Figure 10*A*). Interestingly, the majority of proteins identified by 2D gel electrophoresis and DIGE analysis were localised to either the cytoplasmic (42 proteins) or cytoskeleton (12 proteins) and organelle (11 proteins) fractions, whereas significantly fewer proteins were of membrane, extracellular, and nuclear origin (5, 6, and 4 proteins). Two proteins were suspected contaminants from other cell types (haemoglobin, and HSP20), and 11 proteins remained unclassified. Analysis of protein localisation in platelets by peptide-centric approaches showed similar sub-cellular distributions. Proteins expressed primarily in the cytoplasm (96 and 32) were most commonly identified by iTRAQ and ICAT, respectively. These were followed by almost equal numbers of proteins associated with membrane (55 and 20), organelle (46 and 16), cytoskeletal (36 and 7), and extracellular (25 and 15) fractions. An additional 10 proteins, identified by both iTRAQ and ICAT, were classified as nuclear, with the remainder (3 proteins) possibly due to sample contamination (haemoglobin, LAP3 protein, and full-length cDNA 5-PRIME end of clone CS0DF026YA16 of fetal brain of Homo sapiens, HSPC300, sperm-associated antigen 1). Eighty-four and 36 proteins whose sub-cellular localisation could not be determined were identified by both iTRAQ and ICAT approaches, respectively. It should be noted that since platelets can both adsorb and endocytose proteins, and the entire platelet proteome has not been completely elucidated, it is possible that proteins identified as suspected contaminants by our proteomic screen may be of platelet origin. Indeed, Gnatenko *et al.* reported that platelets contain RNA for haemoglobin which may account for its appearance in our proteomic screen [48]. Cellular function was used as an alternative classification criterion by which to compare the different

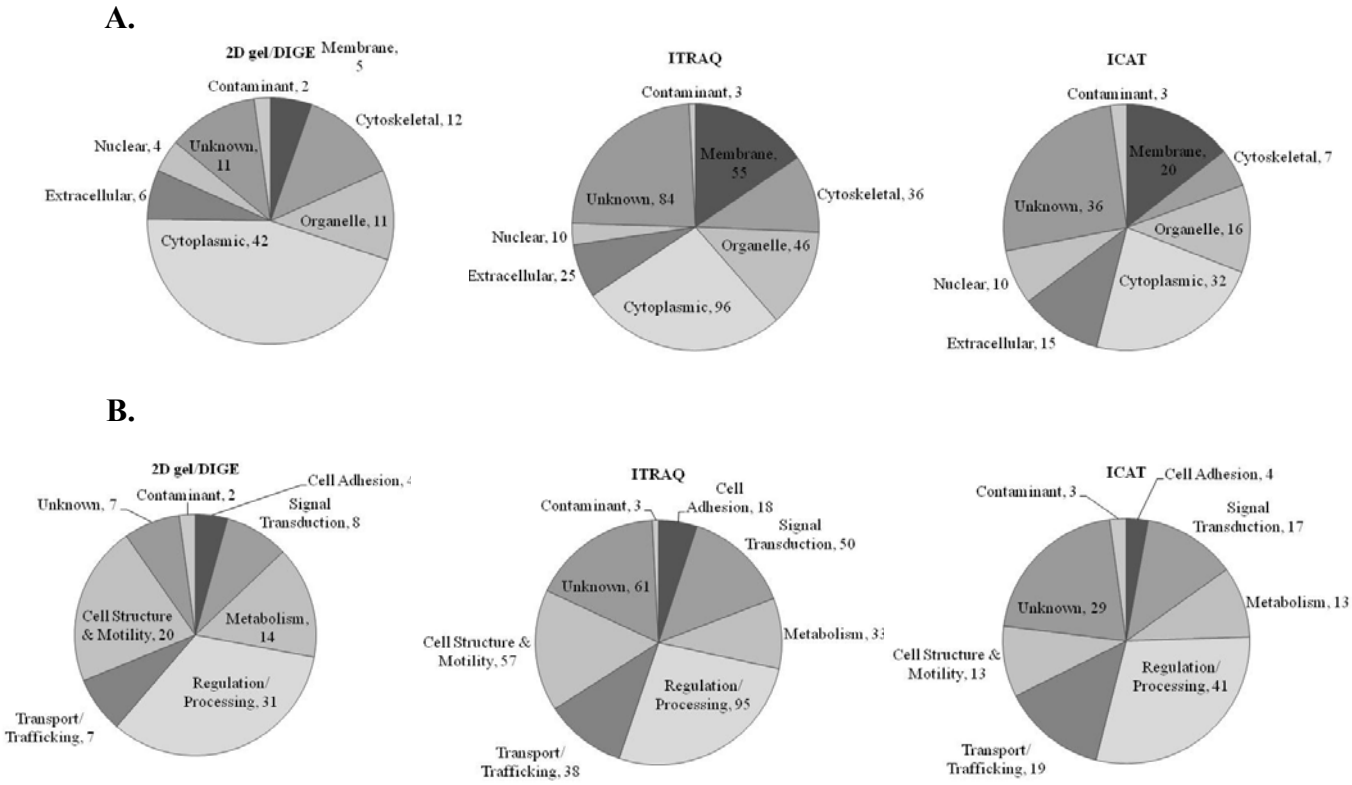


Figure 10. Pie charts illustrating (*A*) sub-cellular localisation and (*B*) cellular function of proteins identified by 2D gel/DIGE, iTRAQ and ICAT.

Proteins listed in *APPENDIX 2***Error! Reference source not found.** were classified by sub-cellular localisation (*A*) and cellular function (*B*) using Swiss-Prot and GO database terms. These represent structure controlled vocabularies that describe gene products in a species-independent manner, and are used by collaborating databases to facilitate uniform queries across them.

proteomic approaches employed and to further validate our approach (Figure 10*B*). Of the 93 proteins identified by 2D gel electrophoresis and DIGE, 31 proteins are involved in regulation and processing. The majority of the remaining proteins are involved in cell structure and motility (20 proteins), and metabolism (14 proteins), whereas 8, 4, and 7 proteins have functions relating to signal transduction, cell adhesion, and transport and trafficking, respectively. For 7 proteins, no known function could be assigned; these were subsequently classified as "unknown." As with the localisation results, the functional distribution of proteins identified by iTRAQ and ICAT was very similar. The majority of proteins identified are involved in regulation and processing (95 and 41 for iTRAQ and ICAT, respectively); followed closely by cell structure and motility (57 and 13 proteins), signal transduction (50 and 17 proteins), and transport and trafficking (38 and 19 proteins). Eighteen and 4 proteins have roles in cell adhesion, while 61 and 29 proteins identified by each proteomic approach (iTRAQ, ICAT), respectively, could not be assigned a known function.

3.5. Stringent Proteomic Criteria to Identify Potential Protein Markers of the PSL

Many lines of evidence reveal that the PSL affects several aspects of platelet function [197-199]; however, the underlying mechanisms are still elusive. *APPENDIX 2* provides an overview of the diversity of the storage lesion. While the proteins included in this table fulfilled the proteomic requirement of high significance with a probability factor $p \leq 0.01$ when comparing results from day 1 vs. day 7 as well as high confidence (≥99%) in the protein identification based on the number of unique peptides [1], the relative changes in protein concentration during storage (as identified by MS) remain small.

To narrow down these protein changes identified with high proteomic confidence and significance, I applied an additional proteomic criterion: consistency. This reflects the agreement of the protein change based on the 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 2 out of 4 iTRAQ runs, 2 out of 3 ICAT runs, 8 out of 11 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 2 out of the 3 different methods used. Twelve proteins were identified that fulfilled the requirement of consistency on top of the significance and confidence criteria (Table 2), representing a reduced set of proteins from platelet lysates comprising potential protein markers for PSL.

3.6. Discussion

This study reports a comprehensive analysis of protein concentration changes in platelets over a 7-day storage period. Differential protein analysis was performed using 2D gel electrophoresis, DIGE, iTRAQ and ICAT techniques, and is the first direct comparison among these 4 proteomic methods representing 2 discrete approaches with respect to their quantitative reproducibility and specificity of protein identification in platelets.

Isobaric tag for relative and absolute quantification (iTRAQ, the most inclusive of these methods), utilises isobaric tags containing both a reporter and a balancer group; for a detailed review see Schneider [117]. This means that competing untagged isobaric peptides do not interfere with quantification as they do in ICAT. The iTRAQ method is designed to isotopically

Table 2. Proteins significantly changing during platelet storage.

Proteins listed by protein function were selected by applying stringent proteomic criteria to protein changes during platelet storage identified by more than one proteomic approach. Changes determined by 2D gel and DIGE are displayed by comparison of protein patterns of day 1 and day 7 samples as characterised by visible shift, increase (↑) or decrease (↓) in intensity of protein spot (2D gel and DIGE). Proteins were identified by mass spectrometry[1] or by comparison with other 2D gels[2] and validated by western blot analysis[3]. For iTRAQ and ICAT, the ratio of labelled peptides at day 1 and 7 was determined, with a ratio of 1.00 indicating no change, ratios > 1.00 indicating relative accumulation of protein in the platelet and ratios < 1.00 indicating relative depletion of protein in the platelet. The number in brackets represents the number of unique peptides (#) used for protein identification.

Protein Function	Protein Name	2D gel	DIGE	iTRAQ	ICAT
Cell Adhesion	Thrombospondin-1		↓ 0.47[2]	0.94 (8)	
Cell Structure and Motility	Actin, cytoplasmic 2; Actin, cytoplasmic 1	shift	↑ 1.80[1] (3)	1.10 (10) 1.04 (17) 1.08 (11)	
	Alpha-actinin 1		↑ 1.78[2]	1.03 (9) 0.93 (20) 1.29 (29) 1.04 (32) 1.02 (26)	
	ARP2/3 complex 16 kD subunit		↓ 0.39[1] (2)	0.93 (3)	
	Cofilin, non-muscle isoform	shift		0.86 (4) 0.94 (4)	
	Myosin heavy chain, nonmuscle type A	shift		1.15 (32) 1.13 (52) 1.07 (60) 0.99 (47) 1.09 (8)	10.9 (8)
	Septin 2 [3]			1.03 (5) 1.02 (4)	8.33 (2)
	Tubulin beta-5 chain		↓ 0.39[1] (3)	0.90 (4)	
Signal Transduction	14-3-3 protein zeta/delta[3]	↑ shift		1.32 (10) 1.07 (11) 1.03 (8)	
	Platelet glycoprotein IIb alpha chain precursor			1.10 (6) 1.10 (9) 1.17 (4) 1.03 (5)	1.02 (5)
	Ras-related protein Rap-1[3]	↑		1.30 (6) 1.00 (7) 1.02 (6)	
	Talin 1			1.28 (68) 1.03 (68) 1.01 (66)	1.52 (12)

encode virtually all of the peptides from a protein digest and generally yields a higher number of protein identifications (355 proteins) than either 2D gel/DIGE or ICAT methods (93 and 139 proteins, respectively; Figure 8) [200]. However, due to the very large number of labelled peptides that result from complex systems such as the platelet proteome, and because the first MS dimension cannot be used to pre-screen peptides for differential expression before tandem MS identity determination, changes in only the most abundant peptides are detected. Use of the ICAT method can reduce the complexity of peptide mixtures by [131, 201] selectively targeting cysteine residues with an isotopic iminobiotin tag such that only peptides that contain labelled cysteine are analysed; this may select for certain lower abundance proteins not easily captured by iTRAQ. This explains why 77% of proteins identified by ICAT were not identified by iTRAQ (Figure 8).

There was considerable overlap amongst proteins identified by the different proteomic approaches [138, 142, 190, 202], particularly with respect to the iTRAQ method, which identified 23% and 53% of the proteins identified by ICAT and 2D gel/DIGE, respectively (Figure 8). Conversely, there was little overlap in the number of proteins identified by 2D gel/DIGE and ICAT but not iTRAQ (3 proteins) perhaps due to the loss of labelled cysteine-containing peptides in ICAT and an under-representation of membrane proteins by 2D gel/DIGE. A total of 5 proteins were identified by all 3 proteomics approaches, with an additional 44%, 79%, and 75% of proteins identified solely by 2D gel/DIGE, iTRAQ, and ICAT, respectively. These observations are consistent with what is expected through the use of either one of these proteomic strategies [49, 119, 138, 142, 190, 202]. Further, they reveal that the protein-centric 2D gel/DIGE approach is largely complementary to the peptide-centric iTRAQ approach, and suggest that at least one peptide-centric and a protein-centric approach must be employed to

improve proteome coverage, with the use of all 3 leading to an additional increase in coverage. Such detailed comparisons were not performed for the protein changes, as they might be misleading due to the lack of change originating in failed identification or unchanged amounts.

While the study of Thiele *et al.* was unable to represent membrane proteins due to their high hydrophobicity (indeed, none of these proteins were identified as changing significantly by our own DIGE analysis), it does identify a number of proteins also found by our proteomic screens, including beta-actin, septin 2, and gelsolin, which those authors suggest might be suitable markers for monitoring platelet concentrate alterations on a routine basis [179]. Interestingly, subsequent immunoblotting of beta-actin, a major component of the cytoskeleton, revealed no significant change in its total amount (Figure 9). Because platelets continue to be metabolically active at room temperature it was not surprising that proteins involved in maintaining glucose catabolism should also show a relative change in concentration during platelet storage [203]. Indeed, we observed increases in relative protein concentration for a number of proteins involved in glucose metabolism, such as glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, and hexokinase [204]. In addition, platelets undergo morphological changes from a discoid (resting) shape to spherical (activated) shape during storage [17, 18]. Cytoskeletal rearrangement is required to release the contents of α-granules upon platelet activation [205]. Our study revealed a significant decrease in the relative concentration of α-granule proteins, including thrombospondin (identified by iTRAQ and ICAT), and fibrinogen (identified by DIGE, iTRAQ and ICAT) [206]. As some of the characteristics of the PSL—such as the change in expression of platelet membrane receptors, change of metabolism in the platelet, cytoskeletal reorganisation, and degranulation [207, 208]—are shared with platelet activation, we compared the results of this study to the proteomic

analysis of TRAP-activated fresh platelets [144]. Strikingly, many proteins such as 14-3-3, fibrinogen, pleckstrin, and the 78-kD glucose-dependent protein changed in both studies, supporting earlier reports of platelet activation during storage and emphasising the significance of complementary proteomic approaches in the identification of novel potential markers for the onset of the PSL [189].

Since the PSL results in reduced quality of platelets for transfusion, it is crucial to understand the underlying mechanisms in order to intervene in this process. Proteomics offers an excellent tool to investigate changes in proteomes which led us to apply a complementary proteomic approach to identify protein changes during platelet storage [1]. By applying stringent proteomic criteria based on confidence of the protein identification, 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 **3**2 along with their proteomic characteristics. The majority of the proteins in Table **3**2 are involved in cytoskeletal reorganisation either as binding proteins to the actin filaments or regulatory proteins for actin polymerisation [182, 209-212]. Some proteins are known to be linked to the PSL such as actin [213], actinin [214], thrombospondin [215] and GP IIIa [216]. As suggested in several studies, a major morphological characteristic of the PSL is the change in the platelet shape [209], which is linked to rearrangements in the cytoskeleton and subsequent distribution of glycoproteins to the plasma membrane; thus, the inclusion of these proteins in Table 2 serves to reinforce this previous work.

In addition to providing a comparative evaluation of the changes in platelet protein concentration during storage by 3 protein analysis methods, this study is also the first to apply iTRAQ and ICAT to the study of platelet storage. A multifaceted response of human blood

platelets to storage was observed, characterised by changes in proteins involved in cell adhesion, signal transduction, metabolism, regulation and processing, transport and trafficking, and cell structure and motility. Other work to date has focused primarily on the cataloguing of platelet proteins and in the alterations to platelet proteins that occur when fresh cells are activated by physiological agonists such as thrombin [49, 124, 138, 139, 141, 142, 144, 202, 210, 217]. Thiele *et al.* have recently reported the application of DIGE to analyse changes in the platelet proteome during storage of platelet concentrates [179]. Although these widely used 2D gel electrophoresis-based methods are capable of quantitative and reproducible comparisons of resolved protein spots, they suffer from difficulties in resolving proteins that are hydrophobic, basic (pI > 9), very large or very small, or of particularly low abundance [218, 219]. As expected, 2D gel and DIGE approaches together identified substantially fewer proteins associated with the membrane fraction than did either the iTRAQ or ICAT approaches alone, suggesting that the peptide-centric approaches more adequately represent this subset of the proteome. Thus despite the benefit of the technique for some applications, 2D gel electrophoresis, even when combined with DIGE, is not sufficient on its own to determine changes in the platelet proteome with storage. Gel-free proteomics methods, such as iTRAQ and ICAT, that analyse complex peptide mixtures using liquid chromatography followed by MS, can alleviate many of the shortcomings that are intrinsic to protein-centric proteomic screens—particularly with respect to low-abundance molecules, due to their higher dynamic range [130, 190, 219].

Although various disagreements in the proteins identified between the efforts of Thiele *et al.* and my own [1, 179] might be due to the technologies employed, different protocols (laboratory-to-laboratory variation) [220], effects of undiscovered changes in post-translational modifications or the lack of specific protein detection due to low abundance, a direct comparison

of our results nevertheless reveals excellent agreement in observed protein changes for talin, tubulin and thrombospondin. Furthermore, platelets undergo changes in shape during storage and other signs of activation. This is supported by the observation of increasing expression of the platelet activation marker CD62P on the surface, however, to a moderate level compared to agonist activation [221]. The recent proteomic approaches reveal that some features monitored during storage were also observed in a study analysing the changes in the platelet proteome during activation [220]. Proteins such as fibrinogen, 78-kD glucose-regulated protein and 14-3-3 ζ/δ change in agreement with and foster the activation hypothesis of early investigations [207]. Moreover, the decrease in thrombospondin, SPARC (osteonectin), and pleckstrin that is observed during storage may be explained by their release from the platelet. Most recently, a proteomic study of proteins released during storage has supported this explanation [182], providing further evidence of platelet activation during storage. These examples demonstrate the complementarity of different proteomic approaches to achieve a complete working model. On this basis it is now possible to design biochemical and physiological experiments to understand the meaning of these proteomic findings. These first proteomic studies on the storage lesion also enable one to correlate *in vitro* measures used to assess platelet functionality with proteomics results, with some examples listed in Table 3. As mentioned above, the increased expression of the activation marker CD62P is in agreement with signs of activation during storage when compared to studies from activated platelets. The increased expression of GP IIIa is explained by translation of GP IIIa during storage [96] and changes in shape change are consistent with alteration of actin isoforms (unpublished data); changes in the metabolic activity are linked to changes in mitochondrial proteins. In order to confirm these links and improve our understanding of how *in vitro* measures relate to platelet viability and function *in vivo*, future work should include platelet

Table 3. Correlation of platelet quality *in vitro* measures with proteomic results.

Platelet function	**Impact of storage on *in vitro* measures**	**Information gained from proteomic approaches**
Activation	Increase in CD62P expression due to alpha granule release	Some proteins found changing during storage agree with changes observed in proteomic studies analysing platelet activation [144]; examples include thrombospondin, clusterin and cyclophilin A which decrease in concentration and are known to be released from the platelet
Morphology	Shape change from discoid to spheroid monitored by extent of shape change (ESC)	Appearance of actin isoforms as well as changes in actin binding proteins, *e.g.*, cofilin, gelsolin or proteins of the ARP2/3 complex most likely involved in cytoskeletal rearrangement [1]
Glycoprotein expression	Increased expression of CD41 and CD61	Several proteins increase in their amount most likely due to protein synthesis as shown for GP IIIa [96]
Metabolic activity	Increase in pO_2 and lactate; decrease in pCO_2 and glucose	Changes in metabolic pathway proteins such as pyruvate kinases and acyl-protein thioesterase [1]
Signalling	Slight decrease in vWF binding	Amounts of subunits of the GP Ib/IX/V complexes remain almost constant; Proteins involved in the signalling pathway mediated by this pathway such as 14-3-3 ζ/δ, and filamin are observed to change spot positions most likely due to alterations in post-translational modifications [1]
	Reduced calcium ion flux	Decrease in the protein amount of calmodulin and changes in its associated protein caldesmon [1]
	Increase in GP IIb/IIIa activation monitored by binding of the antibody Pac-1	Proteins involved in the GP IIb/IIIa pathway are observed to change spot positions due to alterations of post-translational modifications [1]
Adhesion	Decrease in fibrinogen binding	Reduced protein amounts of different fibrinogen chains [1]
Coagulation	Increase in phosphatidyl-serine exposure monitored by annexin-V binding	Changes in coagulation factors V and XII [1]

fractionation and proteomic analysis of the membrane, cytosol, cytoskeleton and the different platelet granules during storage and upon platelet activation. Signalling, adhesion, and coagulation factors will need to be examined closely by both proteomic and classical *in vitro* measures and related to metabolic and translational changes that have been observed in platelets during storage.

Proteomics is an evolving science and future improvement in instrumentation sensitivity, labelling chemistry, and chromatography is needed to enable routine quantification of proteins/peptides by mass spectrometry. In addition, the choice of proteomics technologies must be guided by the question being posed. Some proteins or peptides may be well resolved and/or represented in one method, but not in another [190], and so careful attention should be placed in determining which technology yields the most appropriate information. Nevertheless, a combination of protein- and peptide-centric approaches should be considered, as using any single proteomics method to study changes may give insufficient information. The comparative analyses of protein changes in stored platelets demonstrate the value of combining complementary protein- and peptide-centric approaches in the investigation of the PSL. This study represents the most comprehensive analysis of the protein changes that occur during platelet storage to date. The proteins that were identified in this analysis as reproducibly changing over the storage period will be an important resource for subsequent, more detailed analyses and biochemical studies, and represent an important step toward designing targeted interventions that can extend the storage of platelets beyond 7 days.

CHAPTER 4 Translation of Glycoprotein IIIa in Stored Human Platelets

Thon J.N., Devine D.V. Translation of glycoprotein IIIa in stored blood platelets. Transfusion, 2007. 47(12): p. 2260-2270.

Although platelets contain no nucleus of their own, they inherit a transcriptome in the form of mRNA from their megakaryocyte progenitor cells [48, 49], contain all of the necessary molecular tools and pathways for protein biosynthesis [48, 51-61], and have been shown to synthesise proteins [51]. The study of GP IIb/IIIa translation in stored platelet units was informed by previous proteomics analyses which indicated an increase in relative GP IIb and IIIa concentration during storage. Glycoproteins IIb and IIIa (the major platelet glycoprotein) comprise a single noncovalently associated heterodimer on the platelet surface (GP IIb/IIIa, the major platelet integrin) which functions as a transmembrane receptor to bind proteins of the plasma and the ECM [97, 98, 101]. In addition to there being a rich literature available for both these proteins, the GP IIb/IIIa receptor is required for interactions mediating platelet activation—characteristics of which are expressed during platelet storage. Glycoprotein IIb/IIIa is known to interact with proteins talin, myosin, Rap1b, and actin, which were also identified through the proteomics screen, and may be related to platelet activation via a single signalling pathway [102-104]. Platelet GP IIIa concentration and GP IIb/IIIa surface expression was examined by western blot and flow cytometry over a storage period of 10 days. Relative GP IIIa mRNA concentration during platelet unit storage was determined by northern blot and qRT PCR, and protein synthesis was confirmed by ^{35}S-methionine labelling.

4.1. Assessment of GP IIb/IIIa Concentration and Surface Expression During Storage

Whole blood collection and platelet isolation was carried out by Canadian Blood Services under standard conditions and from separate donors. Platelet rich plasma (PRP) units were sampled on days 1, 5, 7, and 10. The cells were lysed and the protein extract was subsequently loaded onto a SDS-PAGE gel in triplicate. Samples were normalised for cell count in order to account for the relative stability of different proteins, and probed with antibodies specific for GP IIIa and beta-actin (loading control). Band intensities were quantified and normalised using day 1 values. Western blot analysis of GP IIIa revealed a 2-fold increase in concentration at day 7 of storage, and a roughly 4-fold increase by day 10 (Figure 11*A,B*). The change in protein concentration during storage was subject to one-way analysis of variance (ANOVA) for 4 independent samples and Tukey honestly significantly difference (HSD) analysis, and was found to change significantly during storage. Beta-actin did not change significantly in concentration over the 10-day storage period when normalised for cell count. Given its abundance in platelets, a standard curve of actin was used to validate that the lack of change in the relative concentration of beta-actin during storage was not due to the intensity of the chemiluminescent signal being outside the quantitative linear range of detection for the assay (Figure 11*C*).

In order to determine the surface expression of GP IIb/IIIa during storage, platelets were sampled on days 1, 5, 7 and 10, and incubated with fluorescein isothiocyanate (FITC)-conjugated antibodies specific for the integrin complex. Figure 12 illustrates a representative histogram of platelet membrane glycoprotein quantification via flow cytometric analysis and demonstrates a Gaussian distribution in the surface expression of GP IIb/IIIa about the mean fluorescent intensity for the sample. Platelet samples were normalised for cell count and subject to one-way

ANOVA for 4 independent samples and Tukey HSD analysis. Surface expression of GP IIb/IIIa appeared to increase by about 10 000 receptors per platelet on day 7 of storage, and 25 000 receptors per platelet on day 10 ($p < 0.01$) (Figure 13). Glycoprotein IX did not appear to show any significant change in surface expression over storage whereas GP Ib-alpha and V both decreased during storage. These observations confirm earlier reports of increased surface expression of GP IIb/IIIa in stored platelets [14, 25, 27]. Glycoprotein Ib-alpha/IX/IV levels are consistent with those described in the literature [25, 26, 222, 223].

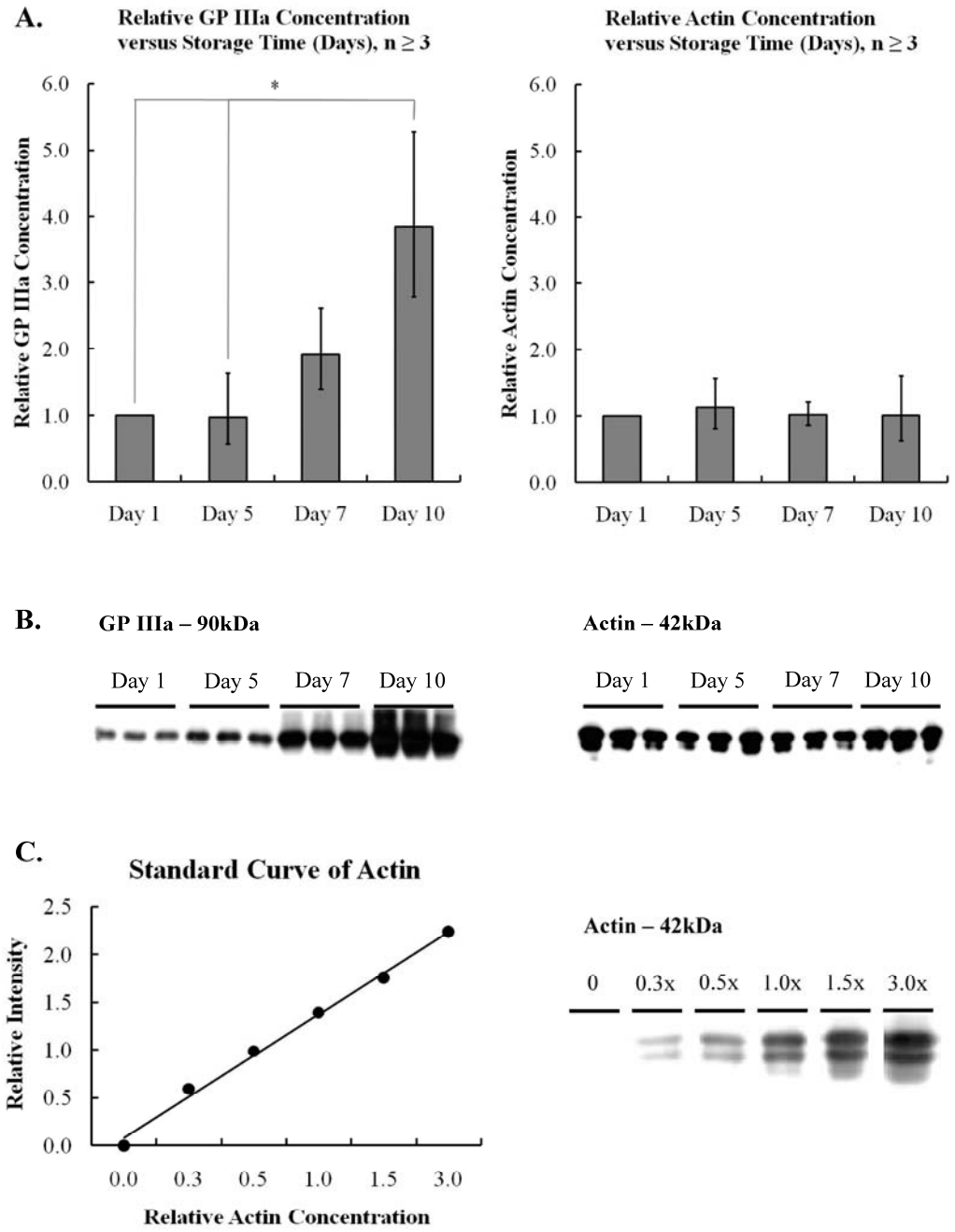


Figure 11. Western blot analysis of GP IIIa and beta-actin during storage.

Platelet units were sampled on days 1, 5, 7 and 10 of storage. *(A)* Quantification of GP IIIa and beta-actin western blots. Samples were normalised for cell counts. Error bars represent ± 1 standard deviation about the mean band intensity. Data were subject to one-way analysis of variance (ANOVA) for 4 independent samples and Tukey honestly significantly different (HSD) analysis. *(B)* Representative western blot analysis of GP IIIa and beta-actin outlining changes in relative protein concentration over storage time. Glycoprotein IIIa and beta-actin western blots showed similar expression profiles when normalised for total protein concentration (data not shown). (*C*) Standard curve for western blot analysis of actin. Relative intensity readings were taken within the quantitative linear range of detection for all samples.

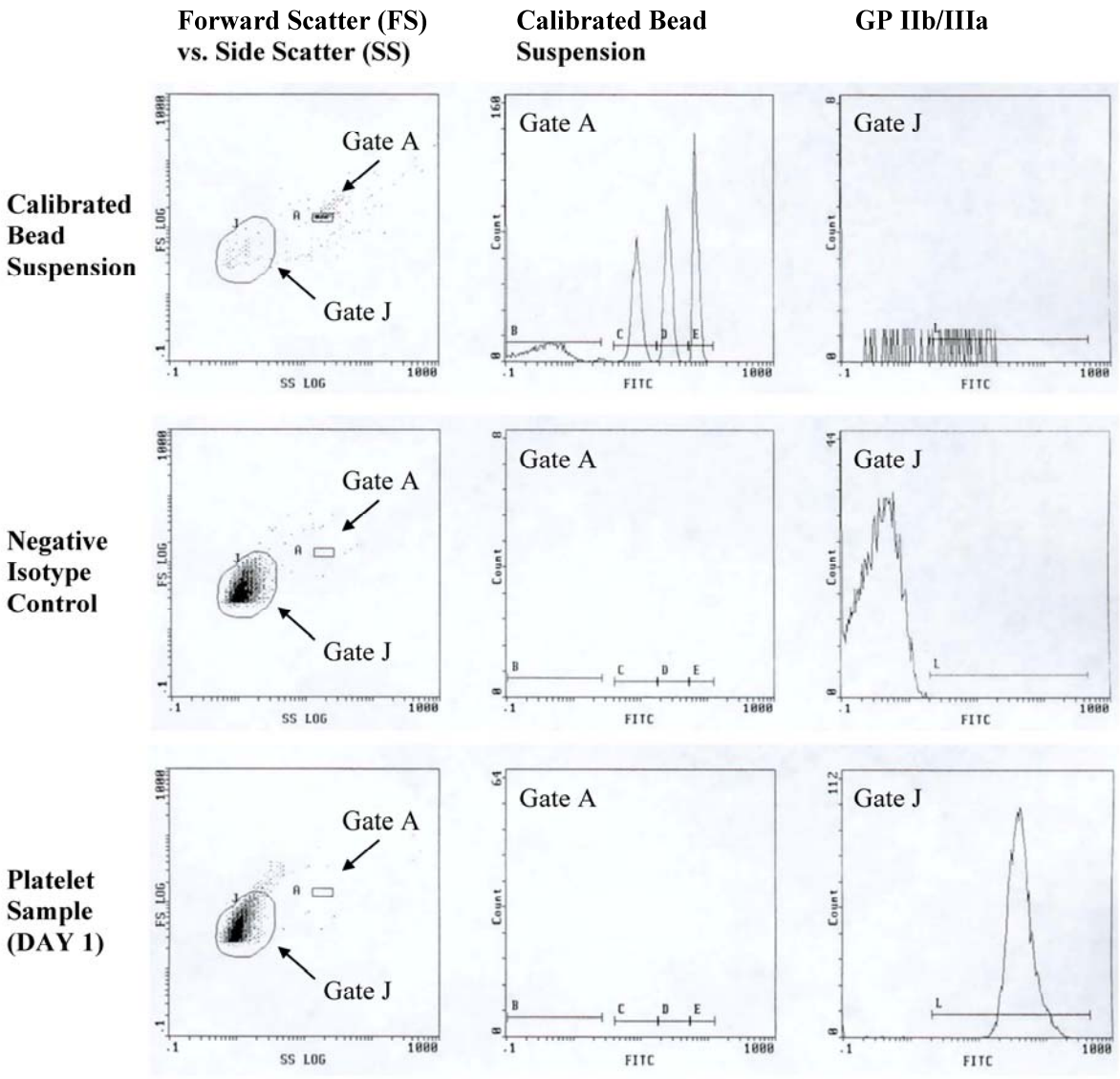


Figure 12. Representative histogram of platelet membrane glycoprotein quantification via flow cytometric analysis.

Platelet units were sampled on days 1, 5, 7 and 10 of storage. Membrane GP IIb/IIIa was labelled with FITC-conjugated antibodies specific for the integrin complex (ADIAflow^{TM} platelet GP IIb/IIIa Occupancy Kit, American Diagnostica) and measured on an EPICS$^{®}$ XL-MCL flow cytometer (Coulter). Analysis of each platelet unit was performed in triplicate. Samples were normalised for cell count and the number of total GP IIb/IIIa receptors were determined by converting the fluorescence intensity generated from the bound polyclonal antibody anti-mouse IgG-FITC stain in the sample into the corresponding number of sites per platelet, based on a calibrated bead (coated with a known quantities of mouse immunoglobulins IgG) standard curve. Using a standard acquisition procedure described by the manufacturer, platelets were isolated from other whole blood cells by their characteristic forward (FS) and side scattering (SS) as they pass through the flow cytometer, and their mean fluorescent intensity per platelet calculated after subtraction of a negative isotypic control (mouse monoclonal antibody, IgG) measurement.

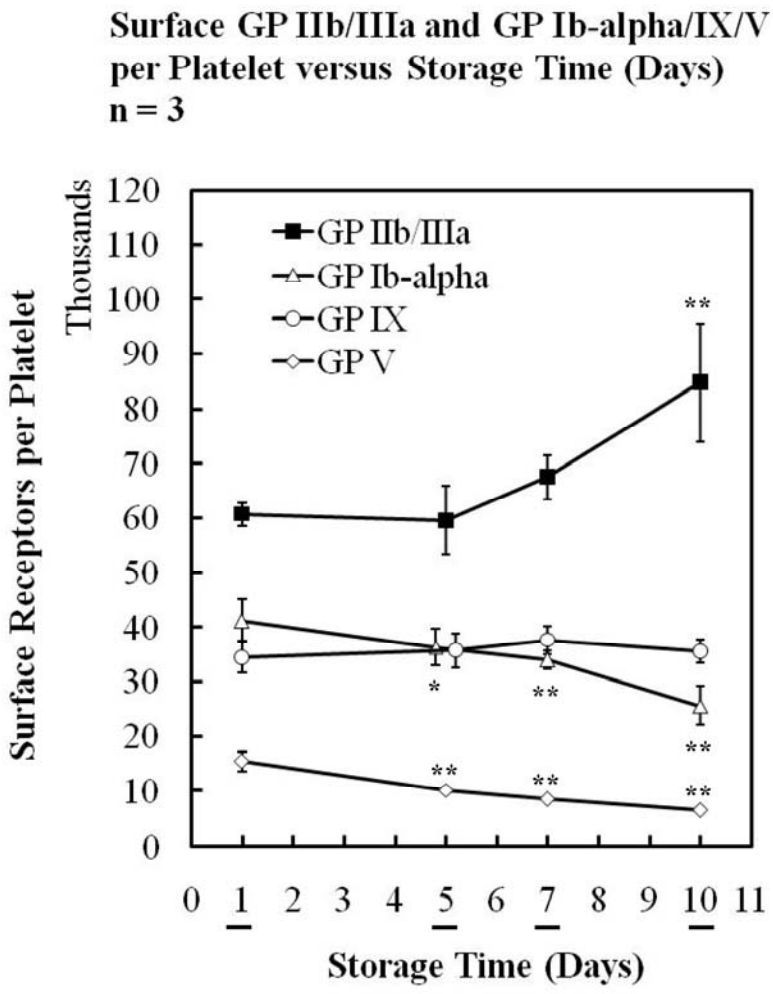


Figure 13. Flow cytometry of GP IIb/IIIa and GP Ib-alpha/IX/V surface expression during storage.

Platelet units were sampled on days 1, 5, 7 and 10 of storage. Membrane GP IIb/IIIa and GP Ib-alpha/IX/V were labelled with FITC-conjugated antibodies specific for the integrin complex (ADIAflow^{TM} platelet GP IIb/IIIa and GP Ib-alpha/IX/V Occupancy Kit, American Diagnostica) and measured on an EPICS$^{®}$ XL-MCL flow cytometer (Coulter). Analysis of each platelet unit was performed in triplicate. Samples were normalised for cell count. Error bars represent ± 1 standard deviation about the mean fluorescence intensity. Data were subject to one-way analysis of variance (ANOVA) for 4 independent samples and Tukey honestly significantly different (HSD) analysis.

4.2. Northern Blot Hybridisation and PCR Amplification of GP IIIa from Stored Platelet Units

For GP IIIa to be constitutively synthesised in platelet units throughout a 10-day storage period, GP IIIa mRNA must be preserved. Since GP IIIa is present in leukocytes as well as platelets, pooled buffy coat platelet units were used to minimise the risk of leukocyte contamination in washed platelet samples. Total RNA was isolated by phenol/chloroform extraction from 3 different pooled buffy coat platelet concentrates sampled on days 1, 5, 7, and 10 of storage. Samples were normalised for cell count prior to northern blot hybridisation and probed with a γ ^{32}P-labelled DNA primer specific for the 3' end of GP IIIa mRNA (Figure 15*A*; *Top*). The resultant bands were visualised by autoradiography overnight against a phosphor screen and revealed the presence of full length GP IIIa (4 894 bp) throughout the 10-day storage period. Platelet RNA was then reverse transcribed to cDNA. PCR primers were designed such that they flanked exons 1 to 15 and 1 to 16 of the GP IIIa transcript (Figure 14, Long Range PCR 1 and 2, respectively). Amplification of mRNA derived cDNA by PCR confirms the presence of intact GP IIIa mRNA product 2 340 bp and 4 341 bp in size (Figure 15*A*; *Bottom* and 16*B*). This represents the transcript from which translation can ensue. Leukocytes are a major source of contamination in platelet preparations and, unlike erythrocytes, actively transcribe GP IIIa mRNA [49, 224]. In order to ensure that there was no leukocyte contamination of the processed samples, pooled buffy coat platelet concentrates were washed and leukocyte counts were taken on each sampling day. Leukocytes comprised less than 2.5×10^{-6}% of the platelet count (< 0.5 cells/μL) prior to RNA isolation. The presence of a 243 bp fragment of leukocyte-specific CD3G mRNA was used as a marker of leukocyte mRNA (Figure 15*C*). In order to confirm that contaminating DNA from other cell types were not present in the samples and that GP IIIa

mRNA remained intact during storage, primers were constructed such that they flanked introns 1, 2, 8, and 12 of genomic GP IIIa and spanned short (50-200 bp) regions equidistant from one another along the full length of the GP IIIa transcript (Figure 14, qRT PCR 1 through 5). Melting curves for the products of the PCR amplification (Figure 16*A*) and subsequent analysis of the samples by gel electrophoresis (Figure 16*B*) reveal the presence of a single cDNA product of expected size from the specific amplification of GP IIIa for each of the listed primer pairs.

Glycoprotein IIIa cDNA was amplified by qRT PCR to determine the relative concentration of the mRNA during platelet storage. Glycoprotein IIIa was present in stored buffy coat platelet concentrates throughout the 12-day storage period and had a half-life of roughly 2.4 days (Figure 17*A* and 18). Baseline leukocyte controls were run in order to account for any background signal generated from the presence of contaminating leukocytes in the platelet preparations. Samples were manufactured such that they contained the highest number of leukocytes found in a standard platelet RNA preparation (6×10^3 cells), and run in triplicate as previously described. Real-time PCR amplification of leukocyte cDNA revealed no significant contribution to the relative concentration of GP IIIa calculated from platelet preparations containing this number of contaminating leukocytes (Figure 17*B*). Amplification of any contaminating DNA that may have been present in the total RNA preparation was avoided by using primers flanking introns 1, 2 (qRT PCR 1), 8 (qRT PCR 2), and 12 (qRT PCR 3).

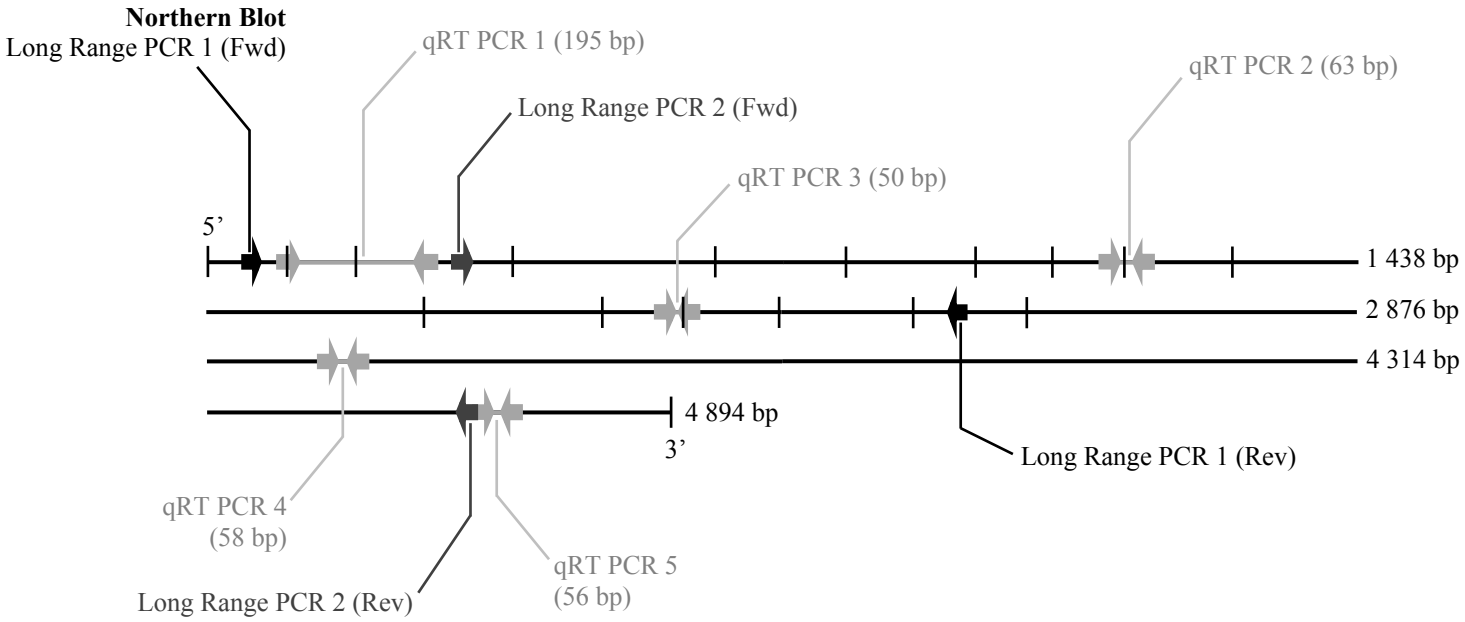


Figure 14. A schematic representation of GP IIIa mRNA highlighting the regions of primer annealing and subsequent products of northern blotting and PCR amplification.

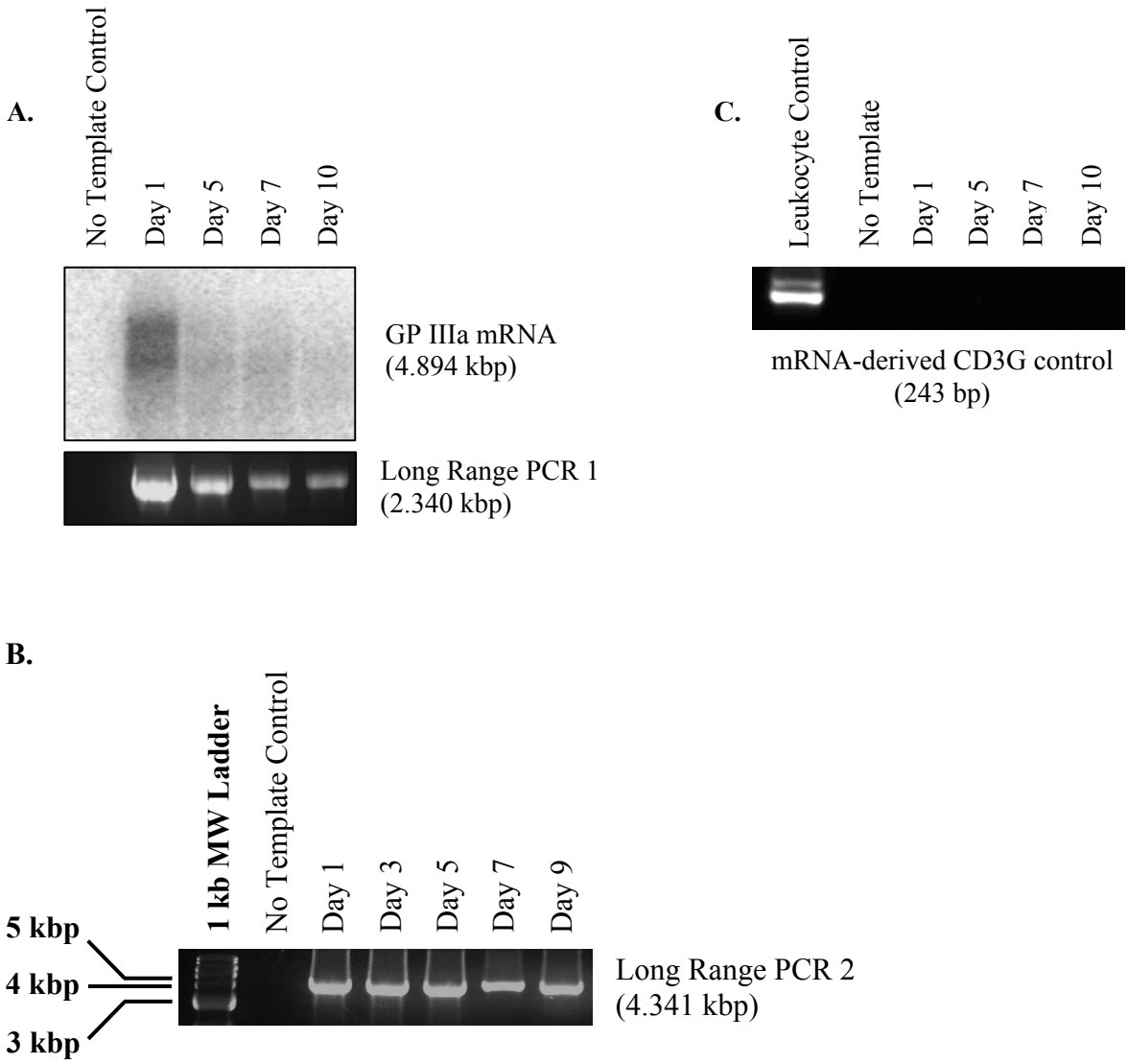


Figure 15. Northern blot hybridisation and PCR amplification of GP IIIa from stored platelet units.

Platelet units were sampled on days 1 through 10 of storage. *(A)* Northern blot of total platelet RNA demonstrating the presence of full length GP IIIa mRNA (4.894 kbp). Results confirmed by PCR amplification of GP IIIa transcript exons 1 to 15 and *(B)* exons 1 to 16 following reverse transcription of platelet mRNA. *(C)* Primers were designed such that they flanked a 243 bp fragment of the gamma chain of T cell antigen receptor-associated T3 complex (CD3G) mRNA, the absence of which is indicative of sufficiently pure platelet RNA in the original phenol/chloroform extraction (cDNA control). Figures are representative of at least 3 different BC units.

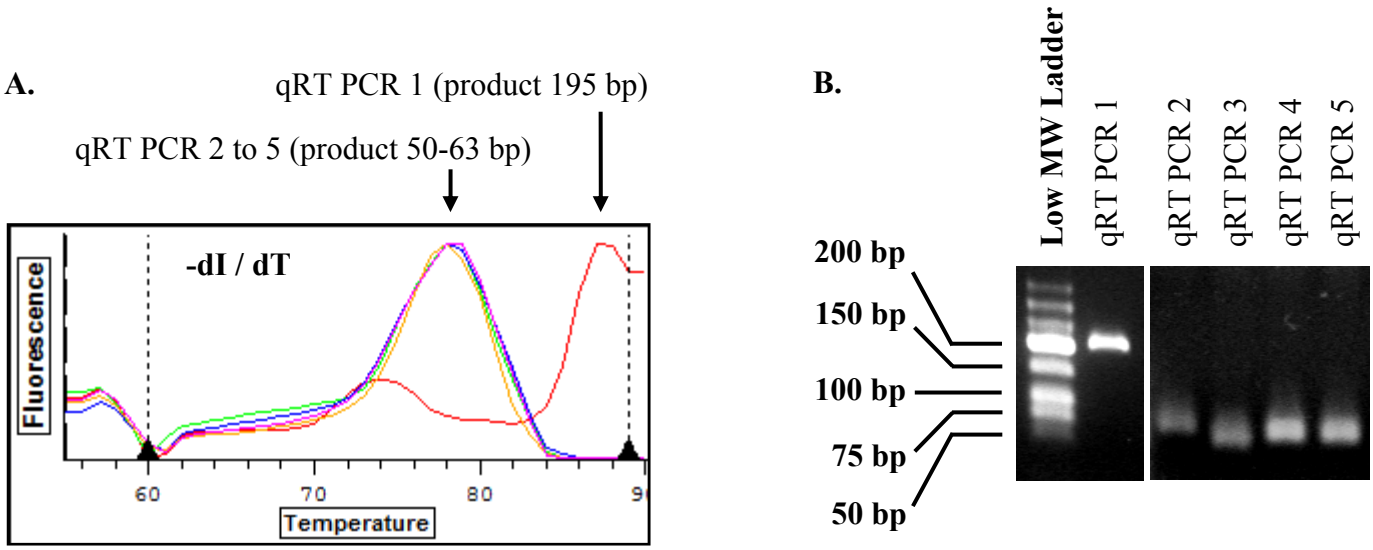


Figure 16. Representative melting curve analysis and PCR amplification products of GP IIIa for qRT PCR primers.

(A) The figure demonstrates the presence of a single cDNA product resulting from the specific amplification of GP IIIa for each of the listed primer pairs. Platelet unit was sampled on day 7 of storage. Total RNA was isolated by phenol/chloroform extraction and reverse transcribed to yield cDNA. Quantitative real-time PCR amplification using each of the listed primer pairs (qRT PCR 1 through 5) was performed with Syber Green Jumpstart Taq ready mix (Sigma) as per the manufacturer's instructions and run in a DNA Engine Opticon 3 continuous fluorescence detector (MJ Research) under stated reaction conditions. *(B)* Samples were run on a 2.0% agarose gel containing 10 µg ethidium bromide and visualized on the ChemiGenius2 bio-imaging system transilluminator (Perkin Elmer, Waltham, MA). Bands confirm the presence of a single amplification product of expected molecular weight for each of the 5 primer pairs.

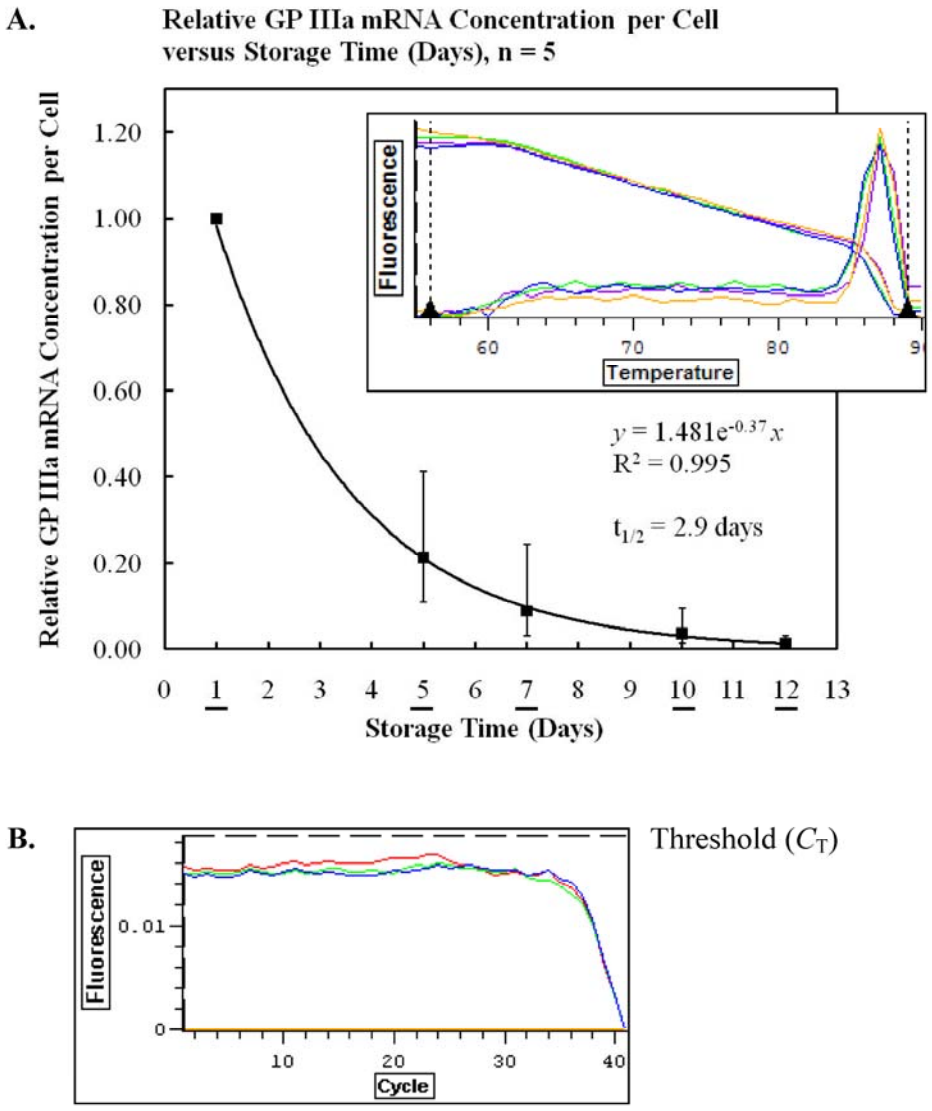


Figure 17. Quantitative real-time PCR amplification of reverse transcribed GP IIIa mRNA sampled over a 12-day storage period by a single qRT PCR primer pair.

(A) Platelet units were sampled on days 1, 5, 7, 10 and 12 of storage and normalised for cell counts. Total RNA was isolated by phenol/chloroform extraction and reverse transcribed to yield cDNA. Quantitative real-time PCR amplification using qRT PCR primer 1 was performed with Syber Green Jumpstart Taq ready mix (Sigma) as per the manufacturer's instructions and run in a DNA Engine Opticon 3 continuous fluorescence detector (MJ Research) under stated reaction conditions. The insert demonstrates the presence of a single cDNA product resulting from the specific amplification of GP IIIa. *(B)* Baseline leukocyte control; total RNA isolation, reverse transcription and qRT PCR amplification of the exact amount of leukocyte contamination identified in a standard platelet RNA preparation as previously described. Quantification graph reveals that reporter dye fluorescence generated from amplification of GP IIIa cDNA in leukocyte controls is below selected baseline (C_T).

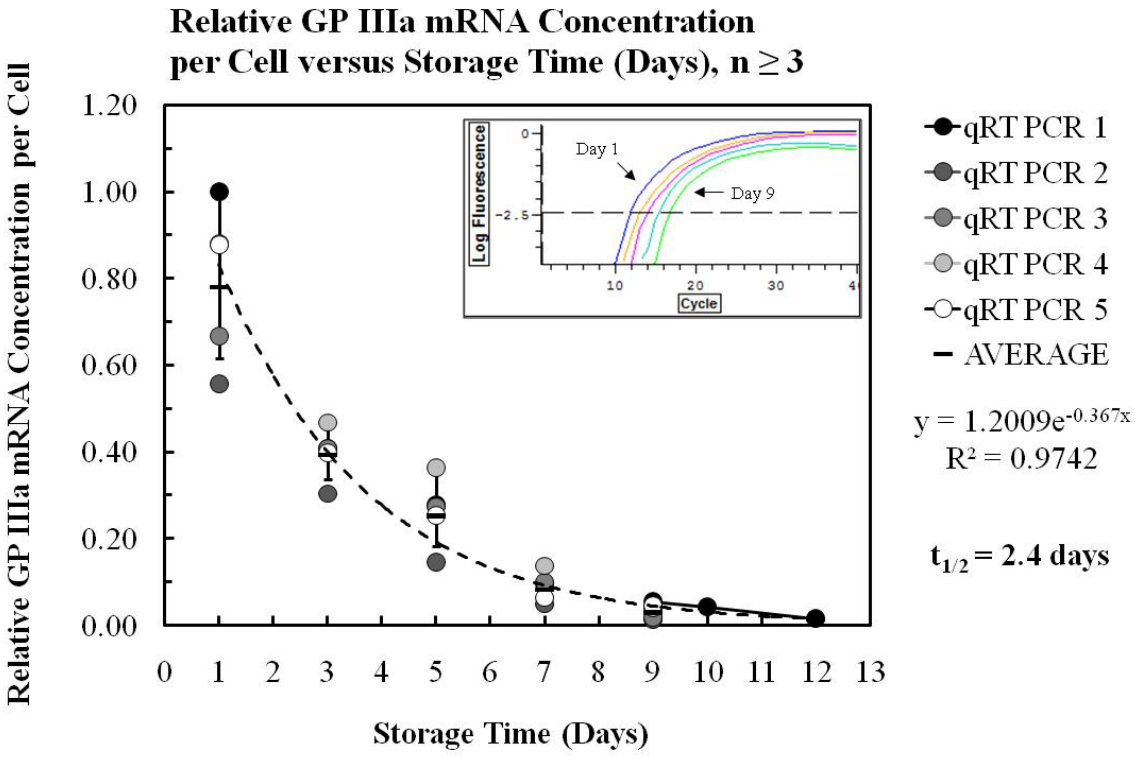


Figure 18. Quantitative real-time PCR amplification of reverse transcribed GP IIIa mRNA sampled over a 12-day storage period by multiple qRT PCR primer pairs.

Platelet units were sampled on days 1, 3, 5, 7, 9, 10 and 12 of storage and normalised for cell counts. Total RNA was isolated by phenol/chloroform extraction and reverse transcribed to yield cDNA. Quantitative real-time PCR amplification using each of the listed primer pairs (qRT PCR 1 through 5) was performed with Syber Green Jumpstart Taq ready mix (Sigma) as per the manufacturer's instructions and run in a DNA Engine Opticon 3 continuous fluorescence detector (MJ Research) under stated reaction conditions. The insert demonstrates a representative graph of the log reporter dye fluorescence intensity per cell cycle of amplified GP IIIa cDNA collected from a single platelet unit on days 1 though 9 of storage. The dotted horizontal line in the insert represents a selected baseline (C_T).

4.3. GP IIIa Immunoprecipitation of ^{35}S-methionine-Labelled-Proteins

The presence of mRNA does not necessitate protein synthesis. Translation of platelet proteins during storage was therefore confirmed by ^{35}S-methionine labelling. Platelet units were sampled on days 1, 5, 7 and 10 of storage. Easy Tag Express Protein Labelling Mix containing ^{35}S-methionine was added to washed platelets, and cells were left to incubate at room temperature for 90 min prior to cell lysis. Samples were run on a 7.5% SDS-PAGE gel and novel protein synthesis detected by autoradiography. A Coomassie-blue stain of a day 1 platelet lysate prior to GP IIIa immunoprecipitation revealed the presence of GP IIb and GP IIIa; as determined from an in-gel digestion of the excised bands by trypsin, and the subsequent detection of the resultant peptides by LC-MS/MS (Figure 19*A*). Peptides were searched against the SwissProt database for human proteins to identify the parent protein (protein identification cut off set at 95% confidence, Figure 20). Autoradiography of ^{35}S-methionine-labelled proteins prior to and following GP IIIa immunoprecipitation reveal GP IIb and GP IIIa synthesis, as well as significant protein translation occurring in stored platelets over a 90 min period (Figure 19*B*).

4.4. Discussion

It is becoming clear that platelets, while anucleate cytoplasts, are quite complex and capable of translational regulation as well as protein synthesis [51]. Although transcription is a major step in gene regulation, freshly drawn platelets have been shown to synthesise a number of biologically relevant proteins that are regulated via gene expression programs at the translational level including Bcl-3, IL-1β, Rho-GDIα and Rho-GDIβ [51].

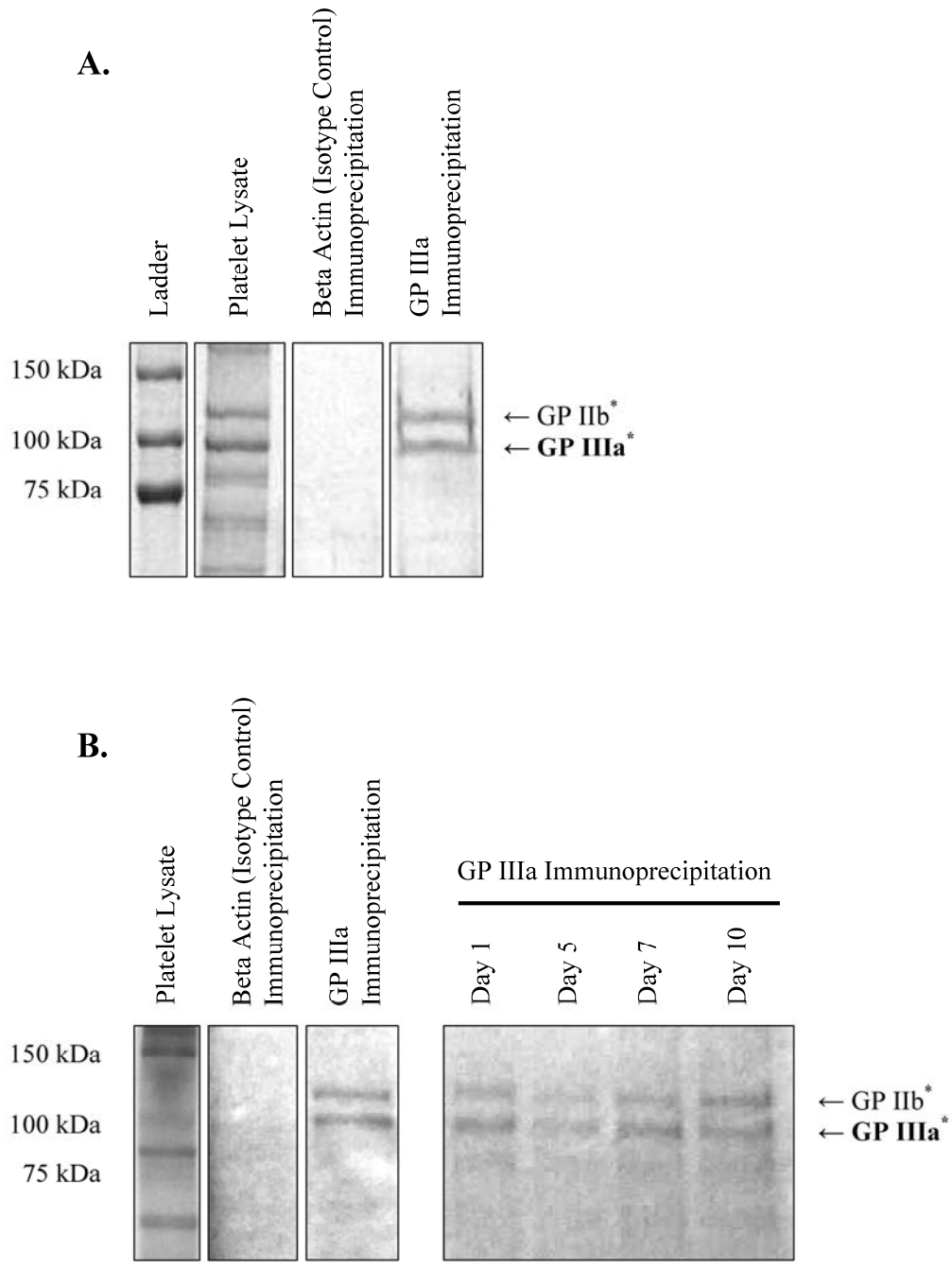


Figure 19. Glycoprotein IIIa immunoprecipitation.

(A) Coomassie blue stain of day 1 platelet lysate prior to and following GP IIIa immunoprecipitation. Gel bands were excised from polyacrylamide gel and peptides loaded onto a LC-MS/MS to resolve contents. Peptides were searched against the SwissProt database for human proteins (*, $p < 0.05$). *(B)* Autoradiography of ^{35}S-methionine-labelled platelet proteins prior to and following GP IIIa immunoprecipitation. Platelet units were sampled on days 1, 5, 7 and 10 of storage and permitted to incubate with Easy Tag Express Protein Labelling Mix (32.7 MBq ^{35}S-methionine) for 90 min prior to cell lysis.

GP IIb
P08514|ITA2B_HU **Mass:** 114460 **Unique Peptides Matched:** 22

```
ORIGIN
        1 maralcplqa lwllewvlll lgpcaappaw alnldpvqlt fyagpngsqf gfsldfhkds
       61 hgrvaivvga prtlgpsqee tggvflcpwr aeggqcpsll fdlrdetrnv gsqtlqtfka
      121 rqglgasvvs wsdvivacap wqhwnvlekt eeaektpvgs cflaqpesgr raeyspcrgn
      181 tlsriyvend fswdkrycea gfssvvtqag elvlgapggy yflgllaqap vadifssyrp
      241 gillwhvssq slsfdssnpe yfdgywgysv avgefdgdln tteyvvgapt wswtlgavei
      301 ldsyyqrlhr lraeqmasyf ghsvavtdvn gdgrhdllvg aplymesrad rklaevgrvy
      361 lflqprgpha lgapsllltg tqlygrfgsa iaplgdldrd gyndiavaap yggpsgrgqv
      421 lvflgqsegl rsrpsqvlds pfptgsafgf slrgavdidd ngypdlivga yganqvavyr
      481 aqpvvkasvq llvqdslnpa vkscvlpqtk tpvscfniqm cvgatghnip qklslnaelq
      541 ldrqkprqgr rvlllgsqqa gttlnldlgg khspichttm aflrdeadfr dklspivlsl
      601 nvslppteag mapavvlhgd thvqeqtriv ldcgeddvcv pqlqltasvt gspllvgadn
      661 vlelqmdaan egegayeael avhlpqgahy mralsnvegf erlicnqkke netrvvlcel
      721 gnpmkknaqi giamlvsvgn leeagesvsf qlqirsknsq npnskivlld vpvraeaqve
      781 lrgnsfpasl vvaaeegere qnsldswgpk vehtyelhnn gpgtvnglhl sihlpgqsqp
      841 sdllyildiq pqgglqcfpq ppvnplkvdw glpipspspi hpahhkrdrr qiflpepeqp
      901 srlqdpvlvs cdsapctvvq cdlqemargq ramvtvlafl wlpslyqrpl dqfvlqshaw
      961 fnvsslpyav pplslprgea qvwtqllral eeraipiwwv lvgvlgglll ltilvlamwk
     1021 vgffkrnrpp leeddeege
//
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GP IIIa
P05106|ITB3_HUM **Mass:** 90350 **Unique Peptides Matched:** 21

```
ORIGIN
        1 mrarprprpl watvlalgal agvgvggpni cttrgvsscq qclavspmca wcsdealplg
       61 sprcdlkenl lkdncapesi efpvsearvl edrplsdkgs gdssqvtqvs pqrialrlrp
      121 ddsknfsiqv rqvedypvdi yylmdlsysm kddlwsiqnl gtklatqmrk ltsnlrigfg
      181 afvdkpvspy myisppeale npcydmkttc lpmfgykhvl tltdqvtrfn eevkkqsvsr
      241 nrdapeggfd aimqatvcde kigwrndash llvfttdakt hialdgrlag ivqpndgqch
      301 vgsdnhysas ttmdypslgl mteklsqkni nlifavtenv vnlyqnysel ipgttvgvls
      361 mdssnvlqli vdaygkirsk velevrdlpe elslsfnatc lnnevipglk scmglkigdt
      421 vsfsieakvr gcpqekeksf tikpvgfkds livqvtfdcd cacqaqaepn shrcnngngt
      481 fecgvcrcgp gwlgsqcecs eedyrpsqqd ecspregqpv csqrgeclcg qcvchssdfg
      541 kitgkycecd dfscvrykge mcsghgqcsc gdclcdsdwt gyycncttrt dtcmssngll
      601 csgrgkcecg scvciqpgsy gdtcekcptc pdactfkkec veckkfdrga lhdentcnry
      661 crdeiesvke lkdtgkdavn ctykneddcv vrfqyyedss gksilyvvee pecpkgpdil
      721 vvllsvmgai lliglaalli wkllitihdr kefakfeeer arakwdtann plykeatstf
      781 tnityrgt
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Figure 20. Protein sequence coverage by MS analysis for GP IIb and GP IIIa.

Protein sequence coverage of gel-excised bands from day 1 platelet lysate following GP IIIa immunoprecipitation and SDS-PAGE. Protein band excision protocol for liquid chromatography (LC) tandem mass spectrometry (MS/MS) was adapted from Wilm, M. [196]. The extracted peptide samples were run on an API QSTAR Pulsar mass spectrometer and searched against the SwissProt database for human proteins. A cut-off of 95% confidence was established for each analysis.

Moreover, constitutive translation has been suggested for actin, thrombosthenin, GP Ib, GP IIb, GP IIIa, fibrinogen, thrombospondin, and VWF [51, 52, 54]. While these proteins all originate from mRNAs that are abundantly expressed in platelets and have features that predict constitutive translation, it is unknown whether constitutive translation is necessary to maintain threshold concentrations of these critical factors in platelets during storage [51, 56, 68]. In the previous chapter it was demonstrated that platelets undergo significant and reproducible changes to their proteome during storage, which may be related to the general reduction in therapeutic efficacy of platelet units that have been stored for this same period of time. Glycoprotein IIb and GP IIIa were amongst those proteins found increasing in concentration by iTRAQ and ICAT analyses, and have been implicated in the exacerbation of the PSL; however, it was unknown whether platelets were capable of translating these proteins during storage given the limited lifespan of eukaryotic mRNA and a general lack of transcription [97, 189].

Most notably due to the absence of a nucleus most, if not all, platelet transcripts must be expressed at the megakaryocytic stage and packaged into platelets during proplatelet formation. This transfer step is a result of a complex assembly system that is organised in parent megakaryocytes and delivered to platelets in an efficient, ordered process [35]. Along with a specific set of transcripts, platelets acquire ribosomal RNA, small regulatory RNAs and a multitude of proteins necessary for protein translation [48, 51-61]. Full length GP IIIa mRNA was confirmed as present in stored platelets throughout the 10-day storage period by northern blot hybridisation and PCR amplification of exons 1 to 16 of the reverse transcribed product. While it is known that GP IIIa mRNA is abundantly expressed in fresh platelets, the rate at which platelets gradually lose their RNA pool is thought to be a determining factor in understanding the extent to which platelets are able to regulate protein synthesis during storage

[51, 56, 68]. Quantitative real-time PCR amplification of the GP IIIa cDNA revealed a half-life for the mRNA of 2.4 days, which is considerably longer than is documented in other cell types [225, 226]. The regulation of mRNA decay is a major control point in protein expression and can be regulated by specific interactions between the structural elements of the mRNA and general or mRNA-specific RNA-binding proteins [227, 228]. The observation that the half-life of GP IIIa mRNA in platelets is nearly 24 to 48 hrs longer than that observed in human dermal microvascular endothelial cells and mouse embryonic stem cells may suggest the presence of RNA stabilising factors—which preserve GP IIIa mRNA during storage—and/or the inability of the exosome to recognise and degrade GP IIIa mRNA [225-227, 229].

Glycoprotein IIb/IIIa functions as a transmembrane receptor whose activation is both bidirectional and reciprocal [97]. Glycoprotein IIb/IIIa is present in roughly 500 000 to 800 000 copies per platelet—60 000 of which are expressed on the surface of the platelet in its inactive form—and is required for platelet interactions with proteins of the plasma and the ECM, most notably fibrinogen, fibrin, VWF, fibronectin, vitronectin, and collagen [97, 98, 101]. Ligand binding to GP IIb/IIIa modulates receptor clustering and promotes progressively irreversible conformational changes in the protein that are transmitted to the cytoplasmic tails causing platelet activation. Western blot analysis of GP IIIa has shown a significant increase in relative protein concentration in platelets stored over a period of 10 days that is expressed, in part, on the surface of the cell. These observations are consistent with comparative analysis of the platelet proteome at days 1 and 7 of storage by iTRAQ and ICAT analysis. Those studies have shown that talin, which connects the GP IIb/IIIa complex to the cytoskeleton, as well as other actin binding proteins such as 14-3-3, actinin and zyxin appeared to increase in concentration over the 7-day storage period. Incubation of washed platelet samples with ^{35}S-methionine has

demonstrated significant protein translation in buffy coat pooled platelet units. This was particularly true of GP IIb and IIIa which were shown to incorporate ^{35}S-methionine throughout storage. Interestingly, the rate of GP IIb and IIIa translation does not appear to change significantly during storage and suggests that the increase in platelet GP IIIa concentration and GP IIb/IIIa surface expression observed by western blotting and flow cytometry may be due to a loss of protein degradation following day 5 of storage resulting in protein accumulation. Conversely, GP Ib and GP V, subcomponents of the GP Ib-V-IX complex, which were shown to decrease in overall concentration during platelet storage by comparative analysis of the platelet proteome at days 1 and 7, were also found to decrease in surface expression over a 10-day storage period in this study [230]. Actin, a major component of the cytoskeleton, showed no change in its total amount in both studies, but revealed a shift toward more acidic isoforms during platelet storage [231, 232]. ^{35}S-methionine labelling however, suggests that actin may be constitutively translated during platelet storage. Full length beta-actin mRNA was not well conserved in stored platelets and showed a half-life of roughly 1.9 days (unpublished observations). Interestingly, the amount of total GP IIIa protein appears not to correlate directly with its mRNA transcript abundance and is maintained throughout a 10-day storage period. This is not altogether surprising as platelets have a finite lifespan in normal individuals of 9.5 ± 0.6 days in circulation [37], and suggests that GP IIIa synthesis plays a functional role in platelets over time. The loss of regulation of both the total concentration and cell surface expression of GP IIIa may contribute to the exacerbation of platelet-storage defects and constitutes a basis for the development of inhibitors to test the role of GP IIb/IIIa in PSL.

CHAPTER 5 Measurement of ^{35}S-Methionine Incorporation by Stored Human Platelets

Validation of the proteomics results by western blotting, flow cytometry, quantitative real-time PCR and ^{35}S-methionine incorporation confirmed that platelets are capable of synthesising biologically relevant proteins *ex vivo* throughout a 10-day storage period. Nevertheless, it is unknown whether constitutive translation is necessary to maintain threshold concentrations of these proteins in stored platelets, and to what extent (if any) protein translation is regulated upon platelet activation and throughout storage. A ^{35}S-methionine incorporation assay was therefore developed to identify differences in protein translation rates among freshly drawn and stored human platelets in the presence and absence of agonist. Analysis of when and how changes in the relative concentration of platelet proteins develop during storage will certainly contribute to our understanding of the PSL.

5.1. Conditions of the Assay

Whole blood collection and platelet isolation was carried out by Canadian Blood Services under standard conditions and from separate donors. Buffy coat (BC) units were sampled on days 1 through 10, as previously described, and all samples were normalised for cell count at physiological platelet concentrations (1.5 x 10^8 platelets/mL). Incubation of platelets with EasyTag™ Express protein labelling mix (Perkin Elmer, Waltham, MA) containing ^{35}S-methionine led to the incorporation of radioactivity into the TCA-precipitable fraction of the platelets (Figure 21). The extent of ^{35}S-methionine incorporation was proportional to the duration

of incubation while EasyTag™ Express protein labelling mix (^{35}S-methionine) concentrations remained non-limiting. Increasing the concentration of EasyTag™ Express protein labelling mix (^{35}S-methionine) in the incubation mixture resulted in increased incorporation of the amino acid. Heating the platelet suspension in a boiling water bath (95°C) reduced the net counts per minute of ^{35}S-methionine incorporated to levels comparable those observed after subjection to 0.6 J UV light (254 nm) exposure and served as a negative control for this experiment. Ultra-violet irradiation of the platelet sample arrested protein translation during the assay and was used both as a negative control and to end further translation following sample incubation. The rate of ^{35}S-methionine incorporation in newly synthesised protein by stored platelets at day 6 was calculated from the quantitative linear range of the assay and discovered to be approximately 0.091 pmol/1x10^9 platelets/min.

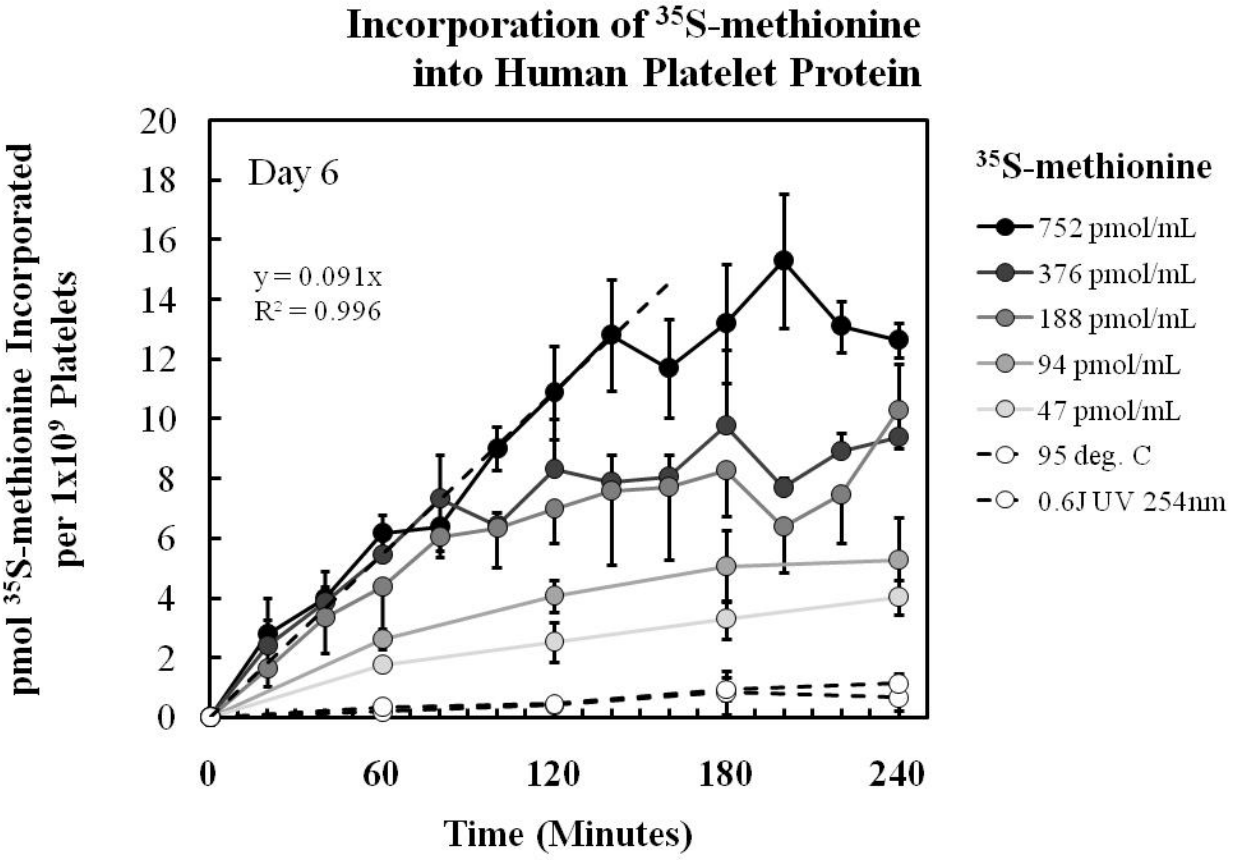


Figure 21. Incorporation of ^{35}S-methionine into TCA-precipitable human platelet protein as a function of time.

Platelet units were sampled on day 6 of storage and normalised for cell count (3.0 x 10^7 platelets per 200 µL aliquot). EasyTag™ Express protein labelling mix containing ^{35}S-methionine was added to each platelet sample at the following concentrations: 47, 94, 188, 376 and 752 pmol/mL. Samples were incubated at RT and with gentle agitation for increasing periods of time. As negative controls, platelet samples were suspended in a boiling water bath (95°C) for the duration of the assay or exposed to 0.6 J UV light (254 nm) prior to the addition of the ^{35}S-methionine protein labelling mix. Translation was arrested by exposure of the platelet sample to 0.6 J UV light. The amount of ^{35}S-methionine incorporated per 1x10^9 platelets was quantified by scintillation count and normalised against the 20 min time point. Error bars represent ± 1 standard deviation about the mean. Translation rate was calculated within the quantitative linear range of the assay.

5.2. ^{35}S-methionine Incorporation in Fresh versus Stored Platelets

Platelets sampled from fresh blood prior to buffy coat unit production showed a significantly slower rate of ^{35}S-methionine incorporation in newly synthesised protein when compared to that of stored platelets at day 6 (Figure 22). Nevertheless, this rate increased significantly in both the fresh and the day 6 buffy-coat platelets following agonist exposure (10 μM ADP). When the rate of ^{35}S-methionine incorporation per μg total protein was measured for fresh and pooled buffy coat platelets during a 10-day storage period, stored platelets showed a modest increase in this rate, reaching significance at day 8, and an overall increase in ^{35}S-methionine incorporation when compared to the fresh (day 1) platelets (Figure 23*A*). Pooled buffy coat platelet units demonstrated no microbial growth following storage by the BacT/ALERT 3D system (Biomérieux Industry, Hazelwood, MO), and total protein concentrations between samples varied by less that 0.25-fold (Table 4). While the variation in total protein concentration between sampling days was low, samples were nevertheless normalised for cell counts to ensure that any changes in the rate of ^{35}S-methionine incorporation were due to protein production by platelets and not the result of bacterial contamination of the platelet unit or loss of radiolabelled protein by degranulation.

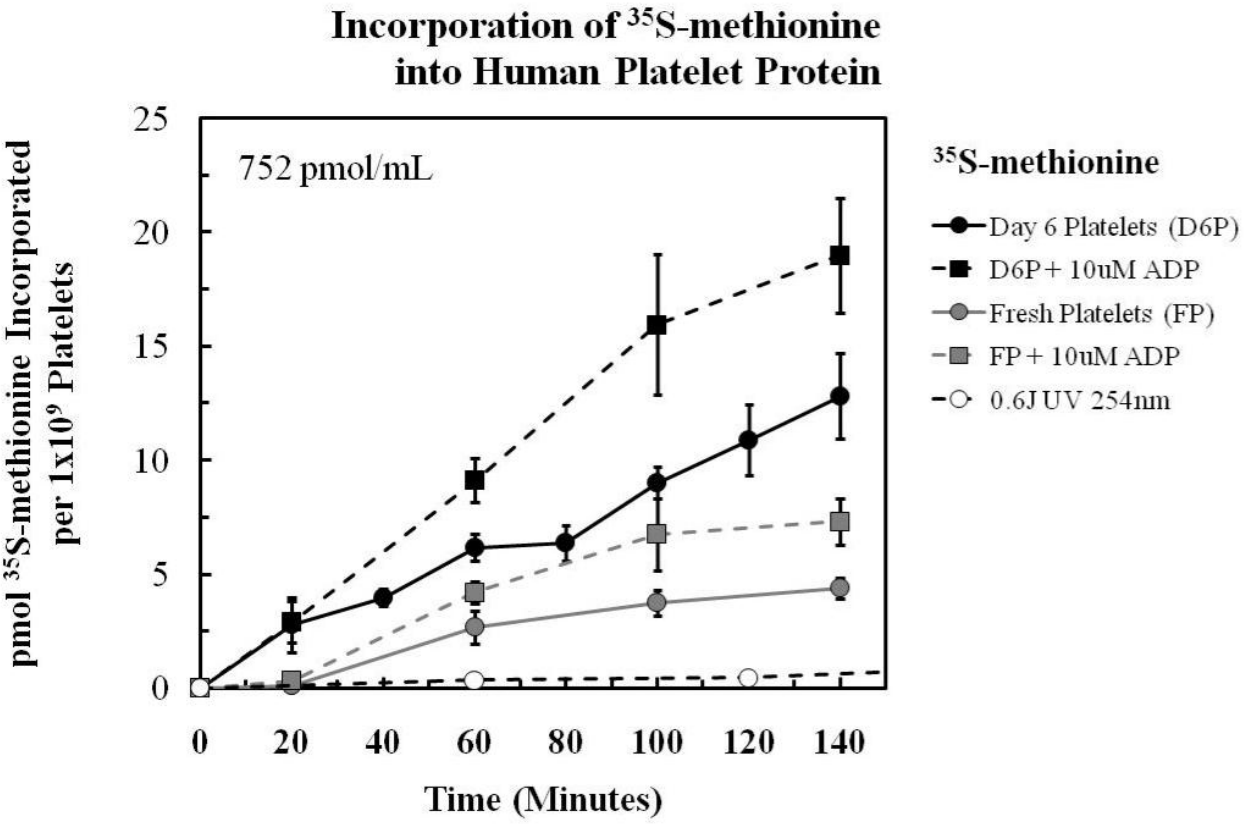
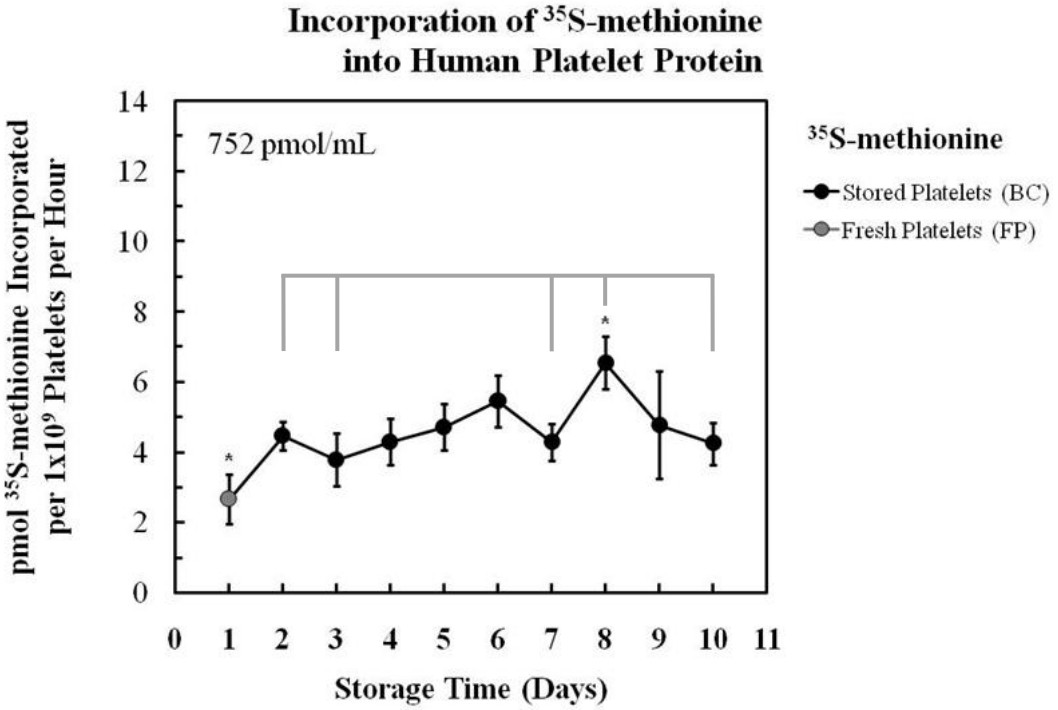


Figure 22. Incorporation of ^{35}S-methionine in fresh versus stored human platelets as a function of time.

Platelets were sampled from fresh blood donations and pooled buffy coat platelet units on day 6 of storage as previously described. Adenosine diphosphate-activated platelets were incubated with 10 μM ADP for 10 min prior to ^{35}S-methionine addition. As a negative control, platelet samples were exposed to 0.6 J UV light (254 nm) prior to the addition of the ^{35}S-methionine protein labelling mix. Translation was arrested by exposure of the platelet sample to 0.6 J UV light. The amount of ^{35}S-methionine incorporation per $1x10^9$ platelets was quantified by scintillation count and normalised against the 20 min time point. Error bars represent ± 1 standard deviation about the mean. Graph demonstrates similar expression profile when normalised for total protein concentration (data not shown).

A.



B.

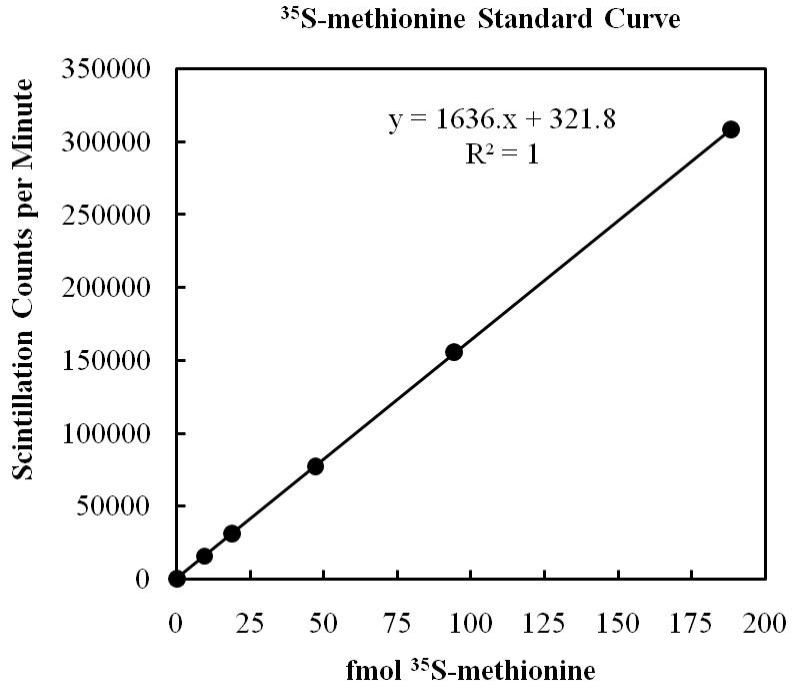


Figure 23. Incorporation rate of ^{35}S-methionine in stored human platelets as a function of time.

(*A*) Quantification of ^{35}S-methioinine incorporation rate in fresh and stored human platelets over a 9-day storage period. Fresh platelets were collected from fresh blood immediately following the donation, and stored platelets were sampled from pooled buffy coat platelet units on days 2 to

10 of storage as previously described. Translation was permitted to proceed for 20 and 80 min for each sample, and arrested by exposure of the platelet sample to 0.6 J UV light (254 nm). The amount of ^{35}S-methioinine incorporation per $1x10^9$ platelets was quantified by scintillation count and normalised against the 20 min time point to obtain a rate (per hr) value. Error bars represent ± 1 standard deviation about the mean. Data were subject to one-way ANOVA for 3 independent samples and Tukey HSD analysis. (*B*) Standard curve for scintillation count quantification of ^{35}S-methioinine incorporation rate. Relative intensity readings were taken within the quantitative linear range of detection for all samples. Graph demonstrates similar expression profile when normalised for total protein concentration (data not shown).

Table 4. Total protein concentration of platelet preparations control for ^{35}S-methionine incorporation assay.

Fresh platelets (FPs) were collected from fresh blood immediately following the donation and stored platelets (SPs) were sampled from pooled buffy coat platelet units on days 2 to 10 of storage as previously described. Preparations were normalised for cell count at physiological platelet concentrations (1.5×10^8 platelets/mL) and incubated with EasyTag™ Express protein labelling mix (Perkin Elmer, Waltham, MA) at the stated concentrations. Samples were then divided into 200 µL aliquots and incubated at RT and with gentle agitation for increasing periods of time. Translation was arrested by exposure of the platelet sample to 0.6 J UV light (254 nm). Platelets were sedimented as before, and washed once in an equal volume of ETS before being solubilised over ice in RIPA lysis buffer (50 mmol/L HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 20 µg/mL PMSF, 1µg/mL leupeptin, 1µg/mL pepstatin A, and 2 µg/mL aprotinin) and stored at -80°C. Proteins were precipitated and washed using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA). Protein concentrations were determined by running samples on a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA) alongside a molecular weight standard.

	Storage Day, SPs									
	FPs	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
No Agonist*	0.561 ± 0.028	0.516 ± 0.064	0.472 ± 0.018	0.446 ± 0.059	0.489 ± 0.034	0.470 ± 0.029	0.457 ± 0.012	0.457 ± 0.046	0.456 ± 0.015	0.457 ± 0.037
+10uM ADP*	0.536 ± 0.042	-	-	-	-	0.437 ± 0.017	-	-	-	-

***values listed in mg/mL ± standard deviation, n ≥ 3**

5.3. Discussion

These experiments have shown that stored human blood platelets incorporate ^{35}S-methionine at a rate that is proportional to time and substrate concentration (Figure 21). The rate of ^{35}S-methionine incorporation in newly synthesised protein by stored platelets at day 6 is consistent with those published for ^{14}C-leucine by fresh platelets [62, 65], and can be prevented by heating platelets during incubation [62] or irradiating them with 0.6 J UV light (254 nm). While the former results in denaturation of the translational machinery in platelets, the latter is known to produce crosslinks between RNA and protein in close contact [233, 234] and induce phosphorylation of eIF2α, inhibiting protein translation [235, 236]. Interestingly, platelets sampled from fresh blood prior to buffy coat unit production showed a significantly slower rate of ^{35}S-methionine incorporation when compared to that of stored platelets at day 6, which could not be directly attributed to platelet activation or degranulation (Figure 22, Table 4). Nevertheless, the sharp increase in the rate of ^{35}S-methionine incorporation per $1x10^9$ platelets (relative to platelets collected from freshly drawn blood) observed in pooled buffy coat platelet units immediately following preparation suggests that the process of buffy coat platelet unit production may have an effect on overall protein translation rates in these cells (Figure 23*A*). Moreover, the constitutive rate of increase in ^{35}S-methionine incorporation during storage correlates well with previous findings relating to GP IIIa expression and synthesis (Figure 19), and further confirms that messenger RNA derived from the megakaryocyte precursor persists in the platelet throughout a 10-day storage period, and remains metabolically stable. The fact that the ^{35}S-methionine incorporation rate on day 8 was roughly 1.5-fold higher than that measured on days 2, 3, 7 and 10 of storage, and that all of these values were higher than those measured for

fresh (day 1) platelets could be indicative of translational regulation of the platelet proteome during storage and may be related to the PSL.

While much progress has been made in documenting the characteristics of the PSL, and understanding how the changes in relative concentration of various platelet proteins during storage might contribute to the initiation or exacerbation of this process, much remains to be done in terms of elucidating the underlying biochemical mechanisms involved. This is to a large extent due to the fact that for most cells, changes in protein phenotype have largely been described in terms of gene transcription. For platelets, the absence of a nucleus has been limiting from a biosynthetic standpoint and stereotyped it as a cell without synthetic potential. It is now clear that this viewpoint is far too simplistic. Platelets synthesise, and in some cases regulate, the expression of biologically relevant proteins at the translational level in the absence of a nucleus [56, 58, 237]. Protein synthesis in platelets was first discovered by Booyse and Rafelson [53, 238], and Warshaw *et al.*[62] in 1967 when it was observed that puromycin could block incorporation of ^{14}C-leucine into platelet TCA precipitable material. Since platelets are anucleate, it was suggested that long-lived mRNA derived from the megakaryocyte could be responsible for directing protein synthesis. This observation appears now to have been correct. Although a number of proteins have since been shown to be translated by platelets during storage and upon agonist exposure, the method by which platelets synthesise new protein and the system through which translation takes place in the platelet is still unclear [51, 52, 62, 65, 239]. In this regard, the development of a translation assay capable of resolving changes in overall protein synthesis (^{35}S-methionine incorporation) in fresh and stored platelets under different experimental conditions may prove to be invaluable.

FUTURE DIRECTIONS

Bacterial risk receives and deserves a lot of attention when it comes to extension of platelet storage; however, determining the quality of platelets during storage needs to be treated with equal importance. Since the platelet life span is 7 to 10 days *in vivo*, extension of the platelet shelf life by 2 to 3 days would improve platelet inventory and efforts of donor recruitment tremendously, as well as reduce the overall cost of provision of this blood product to patients. Proteomic studies on the platelet storage lesion are aimed at understanding the changes occurring within the platelet proteome and enabling the design of biochemical and physiological experiments to understand the meaning of these proteomic findings. Proteomics thus provides an excellent tool to decode complex processes by identifying novel platelet-expressed proteins, dissecting mechanisms of signalling, or metabolic pathways, and analysing functional changes of the platelet proteome. Taken together, this work offers a potential correlation to *in vitro* observations during storage, and demonstrates the complementarity of different proteomic approaches to achieve a complete working model of the storage lesion. However, one must bear in mind that proteomic techniques are limited in sensitivity as well as in the dynamic range and are especially dependent on the protein separation method used. Therefore, translation of proteomic results into platelet biochemistry and physiology is necessary to unravel mechanisms of PSL in time and space.

Applications of proteomic "fingerprinting" for the purposes of identifying factors responsible for declining recovery and survival of stored platelets can be separated into the categories of basic research and process assessment. The cold storage lesion is a prime example of the first, as platelets stored at temperatures less than 15°C undergo extensive morphological

changes that are consistent with signal activation, and are not currently licensed for transfusion [240, 241] . Hoffmeister *et al.* revisited this issue in a murine model, and demonstrated that poor survival of cold-stored platelet units is associated with a virtually irreversible clustering of alpha subunits of glycoprotein Ib on the platelet surface [242]. In the case of cold-stored platelets it seems that rather than modifying the storage medium to improve and extend platelet storage, the platelets themselves should be treated such that they can be stored under refrigeration without a subsequent loss of viability [243]. Proteomics offers the power to characterise protein mixtures in such systems; to determine relationships among proteins, resolve their function, and identify protein-protein interactions of interest in the PSL process. In this regard, 2D-gel electrophoresis, DIGE, iTRAQ and ICAT can be used to identify protein isoforms that may enable platelets to be stored longer, and resolve conditions under which such platelets store better. As many differential effects on proteins themselves come from post-translational modifications such as phosphorylation or glycosylation, monitoring these will contribute to a better understanding of how platelets function under various storage conditions. In addition to potentially allowing for extended storage, plate additive solutions (PAS) and refrigeration would almost certainly reduce the risk of bacterial growth in contaminated units and may help in the development of platelet substitutes.

In addition to helping answer basic research questions, proteomics is an attractive tool by which to assess established processes such as the use of PAS and pathogen inactivation strategies. Generally, PAS require plasma concentrations of 20 to 30% to maintain platelet metabolism. Acetate is often added as a second metabolic fuel and buffer agent along with electrolytes such as magnesium and potassium which are commonly used to inhibit platelet activation and aggregation during storage [244]. Because additive solutions replace 70 to 80% of

the plasma in the original platelet unit, they are thought to promote a reduction in allergic and febrile transfusion reactions [245], decreased transfusion of blood type antigen (ABO) and human leukocyte antigen (HLA) antibodies, and increased plasma availability for fractionation [244]. Unfortunately, the values of *in vitro* platelet quality markers such as pH, glucose consumption and lactate production are limited in the prediction of *in vivo* function. Without a clear understanding of the factors involved in the initiation or exacerbation of the storage lesion, and the molecular mechanisms by which they function to promote platelet activation during storage, it is impossible to ameliorate the storage lesion by targeted approaches and we are left with empirical approaches that have thus far been unsuccessful.

A second major area of platelet research, in terms of quality assurance, includes pathogen inactivation strategies including photoactive psoralen compound S59 [246] (Cerus, Concord, CA), riboflavin treatment [247], and gamma irradiation [248], which are amongst the most developed approaches. Psoralen intercalates into the nucleic acids of treated cells which, upon exposure to ultraviolet (UV) light, becomes activated and binds the nucleic acid core preventing subsequent DNA/RNA replication. Riboflavin works in much the same way, intercalating between the bases of DNA or RNA. On exposure to light (UV or visible) riboflavin becomes activated and oxidizes guanine, thus preventing replication of the pathogen's genome. Similarly, gamma irradiation of platelet components eliminates the proliferative capacity of leukocytes; when performed before administration it has been shown to successfully reduce the risk of transfusion associated graft-versus-host-disease (TA-GVHD) in immunocompromised patients. External stimuli such as drug intervention for therapeutic studies can alter protein expression levels in cells. As platelets have been shown to translate functionally significant proteins during

storage, RNA crosslinking by UV and gamma irradiation should inhibit this process, and its effect on the storage lesion will certainly need to be investigated.

Proteomic tools such as iTRAQ and ICAT can be used to study phenomena such as phosphorylation and signal transduction pathways when looking for protein markers as potential drug targets. By providing a snapshot of the multifaceted response of human blood platelets to storage, inhibitors can then be used to identify and assess the effect of specific proteins, signalling pathways and translational machinery governing platelet activation during storage. This represents an important step toward designing targeted interventions that can extend the storage of platelets beyond 7 days.

In each of these examples, proteomics techniques can be used to provide a rapid, comprehensive diagnosis of proteolytic events and post-translational modifications related to the PSL and also to platelet fitness. Indeed, the use of proteomics in conjunction with an understanding of the activity, interactions, regulation, and localisation of key proteins under a combination of processing conditions, may provide an excellent starting point for examining the *in vitro* characteristics of these varied elements on platelet viability and function. Description and understanding of the platelet proteome will enable the rapid detection of protein expression and abundance profiles that may be used as a measure of platelet health, to which PAS and different storage conditions can be compared.

Although the definition of the platelet proteome during storage and the elucidation of biologically meaningful relationships between platelet storage conditions and the PSL will undoubtedly yield a better, longer-lasting product, the relative changes in protein concentration during platelet storage that have been observed by proteomic screens and validated by biochemical analyses should not be ignored. The notion that platelets are static cytoplasmic

fragments incapable of protein synthesis and with no capacity for translational regulation clearly needs to be reconsidered. In the early 1970s Agam *et al.* [63, 64] published a number of papers on RNA and DNA turnover in platelets and associated both with the mitochondrial system. A similar study to that of Warshaw *et al.* performed nearly 2 decades later by Ian Bruce and Roger Kerry [65] using chloramphenicol and cycloheximide to block incorporation of ^{14}C-leucine into platelet TCA precipitable material appeared to have confirmed this observation and suggested that the majority of platelet protein synthesis is mitochondrial and that this protein synthesis may have a role in human platelet aggregation. Nevertheless, the precise system used to generate new proteins in fresh platelets versus stored platelets, be it mitochondrial or nuclear, and the relative contribution of each during a 10-day storage period and under agonist exposure have yet to be resolved, and could be examined using the ^{35}S-methionine incorporation assay. It will also be interesting to note if these 2 systems account for all protein translation in platelets, and what more specific translational inhibition (through the use of RNA interference, for instance) can tell us about this process and the effect of protein synthesis on the PSL.

A clear disadvantage of the ^{35}S-methionine incorporation assay is that it is currently limited to a global analysis of overall changes in protein synthesis rates and therefore unable to detect changes in the translation rates of individual proteins during storage. This is of particular importance given that the overall rate of ^{35}S-methionine incorporation in stored platelet units does not (for the most part) appear to change significantly during storage. Nevertheless, significant changes in total concentration and surface expression have been observed for a number of proteins thought to be involved in platelet activation on agonist exposure and during storage, and suggest that platelets are capable of regulating their proteome at the translational level. Regulating gene expression at the translational level offers many advantages to the cell.

Like transcription, it is a biosynthetic step that is subject to a multitude of controls. It does not, however, require a nucleus and permits the cell a direct and rapid means to synthesise proteins that bypasses delays encountered with transcription. In order to shed light on how the translation of specific proteins is thought to be regulated by platelets, a protein-specific incorporation assay is required.

One such proteomic approach that can be used to study protein translation during platelet storage and possibly help link the 2 is stable isotope labelling with amino acids in cell culture (SILAC); this is an MS-based quantitative proteomic method that relies on the metabolic incorporation of amino acids with substituted stable isotopic nuclei. When 2 cell populations are incubated in culture media that are identical except for a 'light' or 'heavy' form of a particular amino acid in each (*e.g.*, ^{12}C- and ^{13}C- labelled leucine, respectively), each will incorporate its particular labelled analogue of the amino acid into all newly synthesised proteins. Since there is hardly any chemical difference between the labelled amino acid and the natural amino acid isotopes, this should enable one to detect relative changes in protein synthesis in platelets at various points during the storage period and under a number of different experimental conditions by MS.

As with iTRAQ, SILAC lacks the preliminary peptide enrichment step that is present in the ICAT approach and is therefore subject to the same sample complexity issue that can confound iTRAQ analysis. While the platelet ^{35}S-methionine incorporation assay is certainly not able to resolve translation of nearly as many proteins in a single pass (as is SILAC), by coupling it with protein immunoprecipitation it should be possible to detect quantitative changes in the synthesis of specific lower-abundant platelet proteins during storage and upon agonist exposure that may otherwise go unnoticed by the SILAC method; and may certainly serve to validate

those results obtained through SILAC for higher abundant proteins. Moreover, the direct analysis of translation rates for specific proteins during storage and upon agonist exposure will enable one to approach the specific mechanistic factors involved in propagating the morphological, biochemical and functional changes that have been observed during platelet storage [10, 11].

It is becoming increasingly clear that, like their megakaryocyte progenitor cells, platelets may also use translational mechanisms to regulate gene expression of proteins with key biologic functions during storage and upon agonist exposure. The phylogenetic counterparts of platelets, avian thrombocytes, express numerous mRNAs which are actively translated into protein. While mammalian platelet biosynthesis is always devoid of nuclear influences, translational control remains the primary mode of gene regulation in maturing oocytes [249], and circulating reticulocytes (which are also devoid of nuclei) continue to synthesise globin proteins for 2 to 3 days before they become mature erythrocytes [250]. In each example, translational control is a consequence of remarkable cellular specialisation and precise biochemical pathways which, in the case of platelets, may lead to storage-associated losses of blood component quality and must be understood before storage times can be extended.

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APPENDIX 1 Timeline for Platelet Unit Preparation and Storage

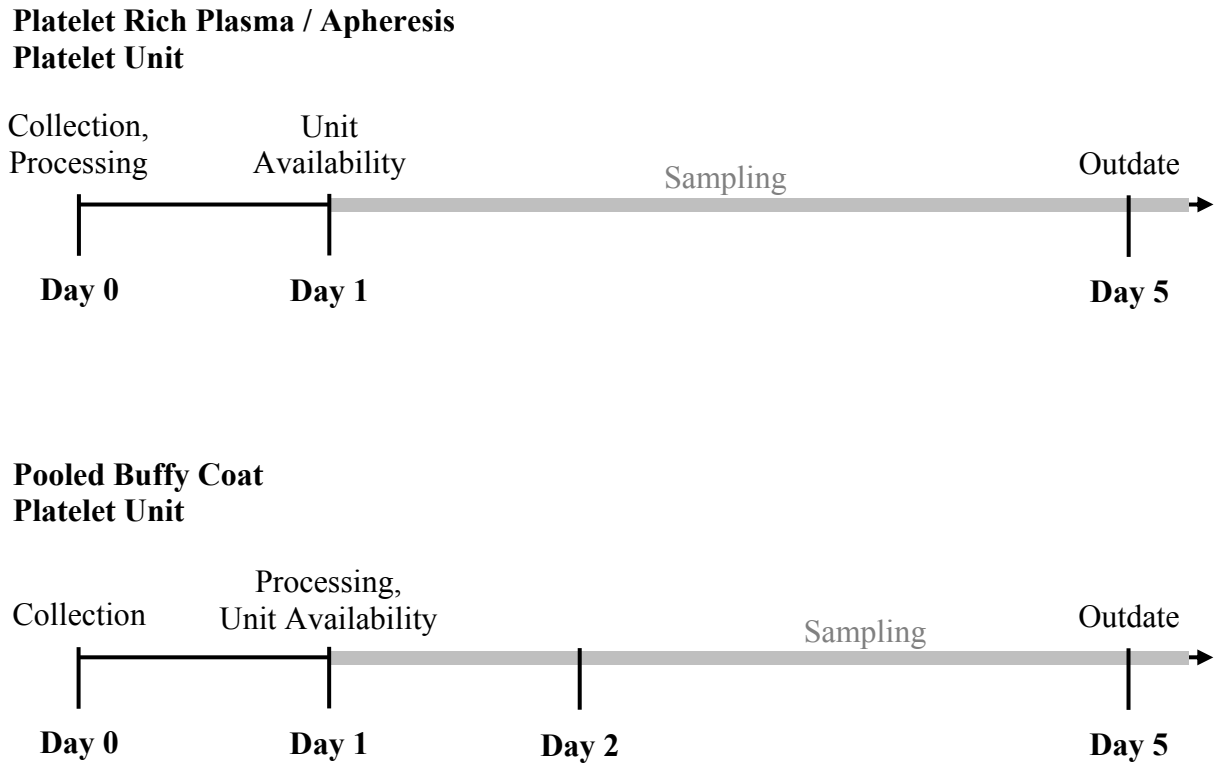

Figure 24. A schematic of the timeline for sample collection, processing and storage of PRP, apheresis and pooled BC platelet units.

These values reflect current protocols used by CBS and this thesis when referring to platelet storage dates.

APPENDIX 2 Proteomic Data Table

Overview of all proteins found to be changing significantly in concentration ($p \leq 0.01$) over a 7-day storage period in human platelets, as determined by 2D gel, DIGE, iTRAQ and ICAT.

Platelet proteins were collected on days 1 and 7 of storage. For quantitative analysis using the differential gel electrophoresis (DIGE) technique, labelling with CyDye reagents was carried out according to the manufacturer's protocol (GE Healthcare, Chalfont St. Giles, UK). Protein quantities per spot were determined using ProFinder 2D software (Perkin Elmer, Boston, MA) and proteins were identified by MASCOT searches against current Swiss-prot databases. For ICAT, 3 different pairs of frozen samples from days 1 and 7 of storage, termed ICAT I and ICAT II and III, were sent for analysis to the University of Victoria, Genome BC Proteomics Centre (Victoria, Canada) and the Institute for Systems Biology (Seattle, USA), respectively. Possible protein identities were obtained by matching peptides to entries in the International Protein Index (IPI, http://www.ebi.ac.uk/IPI/IPIhelp.htmL). Conversely, four different pairs of frozen samples from days 1 and 7 of storage, termed iTRAQ I, II, III and IV were collected and the mean ratios for each iTRAQ-labelled peptide at these time points was calculated. In order to account for within sample and between sample differences, iTRAQ sample IV was processed under identical conditions and analysed twice; iTRAQ runs IV(a) and IV(b). iTRAQ I was searched against the Celera Discovery Systems database (CDS), iTRAQs II and III were searched against the Matrix Science database (MSDB), and iTRAQs IV(a) and IV(b) searched against both MSDB and IPI. A ratio of 1.00 indicates no change in abundance of protein detected in the platelet sample; ratios > 1.00 indicate relative accumulation of protein in the platelet; ratios < 1.00 indicate relative depletion of protein in the platelet and was reflected by the smaller numbers of peptides identified per protein. A confidence level of ≥ 99% was the inclusion criteria for the identification of tryptic peptides. The number of unique iTRAQ and ICAT-labelled tryptic peptides is shown as '(#)' with different forms of the same peptide being counted as 1 peptide; multiple identifications of the same peptide were also counted as a single identity.

Legend

↑/↓	visible increase/decrease in intensity of protein spot (2D gel)
yes	protein identified by 2D gel, no change in relative spot intensity resolved

[1]	protein identification by mass spectrometry
[2]	protein identification by comparison with 2D gel
[3]	change validated by western blot
[§]	western blots indicate no change in concentration

Accession	Protein Name	2D gel	DIGE	iTRAQ									ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00472703.1	100 kDa protein							1.20	(8)	1.04	(8)	1.01	(4)			
unk|IPI:IPI00472068.1	107 kDa protein							1.01	(4)	1.09	(5)	1.00	(4)			
	14 kDa protein,PREDICTED: similar to ribosomal protein L31															3.13
unk|IPI:IPI00216318.4	14-3-3 protein beta/alpha							1.28	(3)							
unk|IPI:IPI00000816.1	14-3-3 protein epsilon									1.01	(7)					
unk|IPI:IPI00216319.2	14-3-3 protein eta							1.30	(5)	1.00	(6)	1.10	(3)			
unk|IPI:IPI00021263.3	14-3-3 protein zeta/delta[3]	↑ shift						1.32	(10)	1.07	(11)	1.03	(8)			
unk|IPI:IPI00000877.1	150 kDa oxygen-regulated protein precursor							1.22	(3)							
unk|IPI:IPI00479262.1	176 kDa protein							1.07	(3)							
	22 kDa protein,Transforming protein RhoA															0.00
	319 kDa protein,Lipopolysaccharide-responsive and beige-like anchor protein,Hypothetical protein DKFZp686K03100															0.05
	3-ketoacyl-CoA thiolase, peroxisomal precursor														0.00	
	45 kDa protein,26S protease regulatory subunit S10B															0.00
unk|IPI:IPI00472102.1	60 kDa heat shock protein, mitochondrial precursor			0.97	(5)			1.19	(6)	1.03	(6)	1.13	(4)			
	6-phosphofructokinase, liver type														0.00	
unk|IPI:IPI00009790.1	6-phosphofructokinase, type C	yes						1.01	(6)	1.05	(4)	1.02	(5)			
unk|IPI:IPI00219525.9	6-phosphogluconate dehydrogenase, decarboxylating							1.28	(7)	1.03	(3)					
	78 kDa glucose-regulated protein		↑ 1.74[2]													
unk|IPI:IPI00003362.1	78 kDa glucose-regulated protein precursor							1.06	(8)	1.01	(12)	1.02	(9)			
unk|IPI:IPI00027257.1	A6 related protein							1.20	(6)	0.92	(3)	0.96	(4)			
BAD52439	AB191263					1.13	(38)									
	Abhydrolase domain-containing protein 14B	yes														
unk|IPI:IPI00550717.2; unk|IPI:IPI00017855.1	ACO2 protein; Aconitate hydratase, mitochondrial precursor							1.04	(3)							

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00021440.1; unk|IPI:IPI00021439.1	Actin, cytoplasmic 2; Actin, cytoplasmic 1[8]	shift	↑ 1.80[1] (3)	1.10	(10)					1.04	(17)	1.08	(11)			
unk|IPI:IPI00005159.2	Actin-like protein 2							1.20	(6)	1.10	(5)	0.99	(4)			
unk|IPI:IPI00028091.1	Actin-like protein 3							1.25	(10)	1.02	(8)	0.96	(6)			
	Acyl-protein thioesterase	yes														
	Adenine phosphoribosyltransferase	yes														
unk|IPI:IPI00007722.2	Adenosine monophosphate deaminase 2									0.89	(3)					
unk|IPI:IPI00008274.3	Adenylyl cyclase-associated protein 1	yes				0.94	(3)	1.22	(12)	0.98	(11)	1.00	(9)			
unk|IPI:IPI00007188.4	ADP,ATP carrier protein, fibroblast isoform							1.28	(5)	0.96	(5)	0.93	(3)			0.00
unk|IPI:IPI00215917.2; unk|IPI:IPI00215914.2	ADP-ribosylation factor 3; ADP-ribosylation factor 1									1.00	(5)	0.94	(4)			
	Alanyl-tRNA synthetase														0.00	
gb|AAH39235.1	ALB protein			1.04	(6)											
unk|IPI:IPI00465248.4	Alpha enolase					0.92	(3)	1.09	(11)	0.98	(14)	1.01	(10)			
unk|IPI:IPI00013508.3	Alpha-actinin 1		↑ 1.78[2]	1.03	(9)	0.93	(20)	1.29	(29)	1.04	(32)	1.02	(26)			
unk|IPI:IPI00013808.1	Alpha-actinin 4							1.19	(5)							
unk|IPI:IPI00029468.1	Alpha-centractin									0.85	(3)					
unk|IPI:IPI00420053.2	Alpha-soluble NSF attachment protein							1.03	(4)							
	νιελχυνψσ–αηπλΑ	yes														
unk|IPI:IPI00328156.8	Amine oxidase							1.18	(5)							
	AMP-activated protein kinase, noncatalytic gamma-1 subunit isoform 2,5'-AMP-activated protein kinase, gamma-1 subunit,Hypothetical protein FLJ40287														0.36	
unk|IPI:IPI00414320.1	Annexin A11							1.05	(3)							
unk|IPI:IPI00329801.9	Annexin A5							0.96	(3)							
dbK|BAA33580.1	anti-HBsAg immunoglobulin Fab kappa chain			0.95	(3)											
	Apolipoprotein A-II precursor															0.00
unk|IPI:IPI00218915.4	Arachidonate 12-lipoxygenase, 12S-type							1.23	(6)							

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00550234.2	ARP2/3 complex 16 kD subunit		↓ 0.39[1] (2)					0.93	(3)							
unk|IPI:IPI00007263.2	ARP2/3 complex 20 kDa subunit							1.22	(4)	1.07	(3)					
unk|IPI:IPI00005161.3	ARP2/3 complex 34 kDa subunit			1.00	(5)			1.61	(4)	1.05	(6)	1.00	(4)			
	Aspartyl-tRNA synthetase															0.00
unk|IPI:IPI00440493.2	ATP synthase alpha chain, mitochondrial precursor							1.30	(7)	0.99	(8)	1.02	(6)			
unk|IPI:IPI00303476.1	ATP synthase beta chain, mitochondrial precursor	yes				0.99	(6)	1.21	(12)	1.07	(12)	1.11	(9)			
	Axin-1 up-regulated gene 1 protein															0.42
AAM19736; AAM19737; BAC04220	AY093951; AY093952; AK093719					0.97	(10)									
AAH33679	BC033679					0.79	(7)									
AAH63824	BC063824					1.03	(7)									
	Beta-2-glycoprotein I precursor														5.56	
TGHU	Beta-thromboglobulin precursor					0.88	(4)									
unk|IPI:IPI00413587.2	BH3 interacting domain death agonist											1.06	(3)			
unk|IPI:IPI00550792.1; unk|IPI:IPI00553064.1	Breast cancer associated protein BRAP1; Bridging integrator 2											1.05	(3)			
	Brefeldin A-inhibited guanine nucleotide-exchange protein 1														2.56	
unk|IPI:IPI00553064.1; unk|IPI:IPI00550792.1	Bridging integrator 2; Breast cancer associated protein BRAP1					1.12	(5)	1.20	(4)	1.09	(5)					
unk|IPI:IPI00008339.2	C14orf173 protein							0.94	(6)							
unk|IPI:IPI00376502.3; unk|IPI:IPI00329757.5	Calcium and DAG-regulated guanine nucleotide exchange factor I; Guanine exchange factor MCG7 isoform 1							1.17	(6)	1.10	(5)					
	Caldesmon	yes														
unk|IPI:IPI00075248.1	Calmodulin		↓ 0.62[2]													
unk|IPI:IPI00020984.1	Calnexin precursor			1.08	(3)	1.11	(3)	1.21	(3)	1.09	(3)	0.95	(4)			
unk|IPI:IPI00011285.1	Calpain 1, large [catalytic] subunit							1.22	(11)	1.02	(6)					
unk|IPI:IPI00015262.9	Calponin-2	yes						1.29	(6)	0.99	(5)	1.16	(3)			
unk|IPI:IPI00020599.1	Calreticulin precursor	yes				1.09	(3)			1.20	(3)	1.01	(3)			
unk|IPI:IPI00554752.1	cAMP-dependent protein kinase type II-beta regulatory subunit							1.35	(3)							

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00218782.2	Capping protein (Actin filament) muscle Z-line, beta							1.29	(3)	1.04	(5)	1.09	(3)			
unk|IPI:IPI00218414.4	Carbonic anhydrase II											0.98	(4)			
	Caspase-3	yes														
unk|IPI:IPI00465436.3	Catalase							1.24	(10)							
	Cathepsin D precursor	yes														
unk|IPI:IPI00154910.2	Cbl-interacting protein Sts-1							1.46	(5)							
	Chain A, Mol_id: 1; Molecule: Neutrophil Activating Peptide-2; Chain: A, B, C, D; Synonym: Nap-2; En													0.76		
unk|IPI:IPI00010896.2	Chloride intracellular channel protein 1							1.19	(8)	1.04	(5)	0.98	(5)			
unk|IPI:IPI00028275.1	CH-TOG protein							1.15	(3)	0.83	(3)					
unk|IPI:IPI00025366.4	Citrate synthase, mitochondrial precursor														0.05 (1)	0.00 (1)
	Clathrin light chain	yes														
unk|IPI:IPI00382605.1	CLINT							1.23	(7)	1.01	(7)					
unk|IPI:IPI00291262.3; unk|IPI:IPI00400826.1	Clusterin precursor; Clusterin isoform 1							0.85	(6)	0.99	(3)					
	C-Maf-induCing protein C-mip isoform,C-Maf-induCing protein TC-mip isoform															0.00
unk|IPI:IPI00017704.1	Coactosin-like protein	yes								1.08	(4)					
unk|IPI:IPI00022937.2	Coagulation factor V							0.98	(7)			0.90	(3)			
unk|IPI:IPI00478809.3	Coagulation factor V precursor									1.02	(4)					
unk|IPI:IPI00297550.7	Coagulation factor XIII A chain precursor					1.09	(6)	1.07	(11)	1.03	(17)	0.99	(10)			
unk|IPI:IPI00295851.3	Coatomer beta subunit							0.94	(3)			1.06	(3)		0.00	
unk|IPI:IPI00220219.5	Coatomer beta' subunit							1.05	(3)							
unk|IPI:IPI00001890.5	Coatomer gamma subunit									0.96	(3)					
unk|IPI:IPI00012011.3	Cofilin, non-muscle isoform	shift		0.86	(4)					0.94	(4)					
unk|IPI:IPI00295857.5	COPA protein							1.07	(4)						50.0	
unk|IPI:IPI00010133.1	Coronin-1A							1.14	(6)	1.06	(6)				0.00 (4)	0.10 (2)
unk|IPI:IPI00008453.3	Coronin-1C							1.06	(5)			1.09	(4)			
CAH18077	CR749220					1.07	(5)									
unk|IPI:IPI00004839.1	Crk-like protein											1.08	(4)			

Accession	Protein Name	2D gel	DIGE	iTRAQ									ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
	CRM1 protein														0.54	6.67
	Crystal Structure Of A Soluble Form Of Clic1. An Intracellular Chloride Ion Channel													0.95		
gb|AAA35733.1	Cyclophilin			0.87	(4)											
unk|IPI:IPI00465315.5	Cytochrome C							1.42	(4)	1.09	(4)					
	Cytochrome C oxidase Va		↑ 4.51[2]													
	Cytokine receptor-like factor 3														0.00	
unk|IPI:IPI00172513.3	DCC-interacting protein 13 beta							0.97	(3)	0.94	(3)					
	DEAD-box protein 3, X-chromosomal,DDX3Y protein,DEAD-box protein 3, Y-chromosomal															0.00
unk|IPI:IPI00011416.1; unk|IPI:IPI00550041.1	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial precursor; ECH1 protein									1.05	(3)					
unk|IPI:IPI00550179.2	Diaphanous protein homolog 1							1.18	(7)	1.17	(5)	0.91	(4)			
unk|IPI:IPI00420108.4	Dihydrolipoyllysine-residue succinyltransferase component of 2- oxoglutarate dehydrogenase							1.08	(3)							
	Disulfide isomerase precursor	yes														
unk|IPI:IPI00298547.3	DJ-1 protein	yes						0.97	(3)							
unk|IPI:IPI00550523.2	DKFZP564J0863 protein									1.11	(3)					
unk|IPI:IPI00297084.4	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit precursor							1.30	(4)							
unk|IPI:IPI00025874.1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor							1.06	(3)	0.89	(4)	0.89	(3)			
unk|IPI:IPI00003406.1	Drebrin							0.99	(7)	1.02	(5)					
unk|IPI:IPI00220503.7	Dynactin complex 50 kDa subunit							1.03	(4)	1.03	(4)					
unk|IPI:IPI00235412.3	Dynamin 1-like protein, isoform 2							1.06	(8)							
unk|IPI:IPI00146935.1	Dynamin-like protein									1.08	(7)	1.00	(5)			
	Dynein light chain 1, cytoplasmic														0.15	
unk|IPI:IPI00017184.2	EH-domain containing protein 1							1.03	(10)	1.06	(4)					
spt|Q9NZN3	EH-domain containing protein 3			1.11	(3)			1.07	(4)	1.03	(7)	0.96	(5)			
unk|IPI:IPI00000875.5	Elongation factor 1-gamma							1.29	(5)							

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00013079.1	EMILIN 1 precursor							0.66	(3)	0.87	(3)	0.88	(3)			
unk|IPI:IPI00060201.2	Endocrine transmitter regulatory protein							0.95	(4)							
	Endoplasmatic reticulum ATPase	yes														
unk|IPI:IPI00027230.3	Endoplasmin precursor							1.20	(12)	1.03	(15)	1.00	(10)			
	Enolase	yes														
unk|IPI:IPI00219682.5	Erythrocyte band 7 integral membrane protein					1.28	(5)	1.26	(7)			1.01	(7)			
	Esterase D\formylglutathione hydrolase, Esterase D															0.00
	Eukaryotic translation initiation factor 3 subunit 11														0.00	
	Eukaryotic translation initiation factor 5A	yes														
unk|IPI:IPI00073110.1	EVH1 domain binding protein							0.94	(3)	1.11	(3)	0.98	(4)			
	Exportin 7														3.45	
unk|IPI:IPI00005969.1	F-actin capping protein alpha-1 subunit	yes						1.30	(5)	1.08	(4)	1.05	(5)			
unk|IPI:IPI00168839.1	FAM10A5							0.92	(3)							
unk|IPI:IPI00418433.1	Fatty acid synthase									1.09	(3)					
unk|IPI:IPI00001730.1	FH1/FH2 domains-containing protein							0.98	(5)	1.07	(4)					
unk|IPI:IPI00014398.2	FHL1 protein									1.05	(3)	1.07	(3)			
spt|P02671	Fibrinogen alpha chain precursor			0.92	(6)											
unk|IPI:IPI00298497.3	Fibrinogen beta chain precursor		↓ 0.59[2]	0.94	(5)			0.99	(11)	0.95	(14)	0.89	(8)	1.11 (4)		
pir|FGHUG	Fibrinogen gamma chain precursor			0.85	(4)	1.14	(6)									
	Fibronectin 1 isoForm 7 preproprotein,Hypothetical protein DKFZp686K08164,Fibronectin 1 isoForm 2 preproprotein,Fibronectin 1 isoForm 4 preproprotein															3.45
gb|AAA92644.1	Filamin			1.21	26					1.10	(77)	1.00	(57)	1.78		
	FLJ00338 protein, HSPC084, Trinucleotide repeat containing 5														0.40	
unk|IPI:IPI00302592.1	FLJ00343 protein							1.17	(68)							

Accession	Protein Name	2D gel	DIGE	iTRAQ								ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
gb|AAH08083.2	FLK10849 protein			1.17	(4)											
unk|IPI:IPI00465439.4	Fructose-bisphosphate aldolase A	yes				0.88	(3)	1.29	(6)	1.06	(8)					
	Full-length cDNA 5-PRIME end of clone CS0DF026YA16 of Fetal brain of Homo sapiens															0.00
	Full-length cDNA clone CS0DH001YP08 of T cells,Guanosine monophosphate reductase 2 isoform 1,GMP reductase 2,38 kDa protein,GMPR2 protein															2.70
unk|IPI:IPI00107432.1; unk|IPI:IPI00216583.1	G6b-G protein precursor; G6b-A protein precursor									0.97	(4)					
unk|IPI:IPI00026314.1	Gelsolin precursor	yes		1.05	(4)					1.03	(14)					
unk|IPI:IPI00028414.3	Glia maturation factor gamma		↓ 0.27[2]													
unk|IPI:IPI00027497.4	Glucose-6-phosphate isomerase									0.98	(6)	1.02	(4)			
	Glutamate dehydrogenase 1, mitochondrial precursor														0.00	
unk|IPI:IPI00293975.3	Glutathione peroxidase 1 isoform 1	yes						1.08	(6)	0.98	(5)					
unk|IPI:IPI00219757.1	Glutathione S-transferase P	shift	↓ 0.25[2]					1.15	(5)	1.02	(4)					
unk|IPI:IPI00019755.3	Glutathione transferase omega 1			1.00	(3)					1.09	(6)	0.98	(5)			
unk|IPI:IPI00219018.4	Glyceraldehyde-3-phosphate dehydrogenase, liver	yes						1.32	(8)	1.00	(10)	1.02	(6)	1.09		
unk|IPI:IPI00017895.1	Glycerol-3-phosphate dehydrogenase, mitochondrial precursor							1.18	(4)							
unk|IPI:IPI00004358.3	Glycogen phosphorylase, brain form							1.13	(10)	1.08	(4)				0.52 (2)	0.38 (2)
unk|IPI:IPI00470525.2	GlycoGen phosphorylase, liver									1.08	(3)					
unk|IPI:IPI00217906.3	GNAI2 protein							1.42	(7)	1.03	(5)					
unk|IPI:IPI00288947.3	GNAQ protein							1.07	(5)							
	Growth factor receptor-bound protein 2	yes														0.18
unk|IPI:IPI00003348.1	Guanine nucleotide-binding protein G									0.96	(4)	0.94	(3)		0.23	2.78
	HDCMD34P,Suppressor of G2 allele of SKP1 homolog,SGT1B protein															0.39

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00304925.1	Heat shock 70 kDa protein 1									0.98	(4)	1.05	(5)			
rf|NP_694881.1; spt|P11142	Heat shock 70kDa protein 8 isoform 2; Heat shock cognate 71 kDa protein			1.00	(3)											
unk|IPI:IPI00025512.2	Heat shock protein beta 1		↑ 1.55[1] (2)					0.95	(3)							
unk|IPI:IPI00382470.2	Heat shock protein HSP 90-alpha							1.03	(8)	1.03	(9)	1.00	(3)			
unk|IPI:IPI00410714.1	Haemoglobin alpha-1 globin chain									1.05	(3)					
unk|IPI:IPI00218816.5	Haemoglobin beta chain		↑ 4.03[1] (2)					0.89	(7)	1.07	(7)	1.10	(5)			
unk|IPI:IPI00410297.1	Heparanase							1.13	(3)	1.11	(5)					
	Histidine-rich glycoprotein precursor															0.21
unk|IPI:IPI00472218.1	HLA class I histocompatibility antigen, B-18 alpha chain precursor							0.96	(4)							
unk|IPI:IPI00472138.1; unk|IPI:IPI00472045.1	HLA class I histocompatibility antigen, B-58 alpha chain precursor; HLA class I histocompatibility antigen, B-53 alpha chain precursor									0.98	(3)					
unk|IPI:IPI00026650.1	HLA class I histocompatibility antigen, Cw-1 alpha chain precursor							0.95	(3)							
CAA25833	HSGAPDR NID					0.91	(6)									
	HSP20	yes														
	HSP70	yes														
	HSP90	yes														
	Hsp90 co-chaperone Cdc37															0.26
	HSPC032															0.00
unk|IPI:IPI00023549.3	HSPC159 protein							0.92	(5)							
	HSPC300														11.11	
AAA92644; A37098	HUMFLNG6PD NID; Gelation factor ABP-280, long form					1.12	(41)									
trm|Q96EY0	Hypothetical protein			1.42	(3)											
unk|IPI:IPI00514299.1	Hypothetical protein									1.04	(6)					
	Hypothetical protein DKFZp566B1524,Phosphoglucomutase 2,MSTP006															0.09

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00334775.3	Hypothetical protein DKFZp761K0511							0.94	(3)							
	Hypothetical protein FLJ13353,WD repeat and FYVE domain containing protein 1															0.21
unk|IPI:IPI00442122.1	Hypothetical protein FLJ16459							1.28	(5)			1.04	(4)			
unk|IPI:IPI00156649.1	Hypothetical protein FLJ22570							0.85	(3)							
unk|IPI:IPI00017672.2	Hypothetical protein FLJ25678					0.86	(3)	1.23	(6)	1.10	(3)	1.12	(4)			
	Hypothetical protein FLJ26554,Galactokinase														0.00	
	Hypothetical protein FLJ36520														3.57	
unk|IPI:IPI00446294.1	Hypothetical protein FLJ42340							0.91	(4)							
unk|IPI:IPI00299571.4	Hypothetical protein FLJ45525							1.27	(6)	1.03	(5)	1.02	(4)			
	Hypothetical protein KIAA0153															2.94
trm|Q9UPX3	Hypothetical protein KIAA1027 (Fragment)			1.05	(2)7											
	Hypothetical protein PRO1855														0.00	
Q6GMY2_HUMAN; Q6GMX5_HUMAN	Hypothetical protein; Hypothetical protein					2.70	(4)									
unk|IPI:IPI00549254.1	Ig gamma-1 chain C region							1.00	(3)	0.86	(3)					
pir|A60764; emb|CAC10219.1	Ig gamma-3 chain C region, form LAT - human ; immunoglobulin heavy chain			0.97	(3)	2.04	(3)									
	Importin 7															0.34
unk|IPI:IPI00001639.2	Importin beta-1 subunit							1.05	(4)							
unk|IPI:IPI00013219.1	Integrin-linked protein kinase 1	yes		1.13	(5)			0.96	(10)	0.99	(12)	0.98	(7)			
unk|IPI:IPI00009477.3	Intercellular adhesion molecule-2 precursor							0.98	(3)							
unk|IPI:IPI00299048.3	IQ motif containing GTPase activating protein 2							1.06	(10)						50.0	0.00
unk|IPI:IPI00011107.2	Isocitrate dehydrogenase [NADP], mitochondrial precursor			1.10	(3)			1.35	(6)	1.02	(9)	0.99	(5)			
unk|IPI:IPI00001754.1	Junctional adhesion molecule 1 precursor; 28 kDa protein; Junction adhesion molecule														25.00 (1)	14.29 (1)
unk|IPI:IPI00014235.1	KIAA0066 protein														0.00 (1)	0.00 (2)
unk|IPI:IPI00030960.1	KIAA0068 protein							1.12	(6)	1.16	(4)					

Accession	Protein Name	2D gel	DIGE	iTRAQ					ICAT		
				I	II	III	IV (a)	IV (b)	I	II	III
unk|IPI:IPI00022143.3	KIAA0747 protein					1.06 (4)					
Q9UPX3_HUMAN; AAF27330	KIAA1027 protein (Fragment); AF178081S2 NID				0.93 (43)						
	KIAA1115									0.00	
unk|IPI:IPI00010368.3	Kinesin-like protein KIF2					1.23 (3)				20.0	
	Lactotransferrin precursor	yes									
unk|IPI:IPI00419237.1	LAP3 protein					0.90 (3)					
unk|IPI:IPI00410152.2	Latent transforming growth factor beta binding protein 1 isoform LTBP-1L			0.76 (4)			0.93 (3)	0.94 (3)			
unk|IPI:IPI00027444.1	Leukocyte elastase inhibitor	yes					1.05 (3)	0.94 (3)			
unk|IPI:IPI00000861.1, unk|IPI:IPI00386803.4	Lim and SH3 domain protein 1 (LASP-1)	shift									
unk|IPI:IPI00002255.4; unk|IPI:IPI00184931.3; unk|IPI:IPI00477088.1	Lipopolysaccharide-responsive and beige-like anchor protein; Hypothetical protein DKFZp686K03100; 319 kDa protein					1.06 (5)					
unk|IPI:IPI00217966.4	L-lactate dehydrogenase A chain						0.95 (5)		0.47		
unk|IPI:IPI00219217.2	L-lactate dehydrogenase B chain					1.41 (7)	0.97 (8)	0.98 (5)			
unk|IPI:IPI00456607.2	Lymphocyte antigen 6 complex, locus G6D					1.18 (3)					
unk|IPI:IPI00432416.2	LYN protein						0.96 (3)				
unk|IPI:IPI00293590.4; unk|IPI:IPI00455206.1	Lysophospholipase homolog; Monoglyceride lipase					1.02 (4)	0.94 (3)	0.98 (3)			
	Malate dehydrogenase, cytoplasmic									0.00	
unk|IPI:IPI00291006.1	Malate dehydrogenase, mitochondrial precursor					1.09 (6)		1.05 (4)			
	Metalloproteinase inhibitor 1 precursor,Tissue inhibitor of metalloproteinase 1										4.55
unk|IPI:IPI00017596.2	Microtubule-associated protein RP/EB family member 1							0.97 (4)			
unk|IPI:IPI00022471.4	Minor histocompatibility antigen HA-1					0.98 (6)					
unk|IPI:IPI00015602.1	Mitochondrial precursor proteins import receptor									0.14 (1)	0.13 (1)
unk|IPI:IPI00002857.1	Mitogen-activated protein kinase 14 isoforM 2					1.42 (4)					

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00219365.2	Moesin	yes		1.02	(4)	1.08	(3)	1.18	(12)	1.02	(14)	0.96	(8)			
	Monoamine-sulfating phenol sulfotransferase	yes														
unk|IPI:IPI00012269.1	Multimerin 1 precursor			0.96	(5)			0.89	(9)	0.87	(10)	0.82	(12)			
unk|IPI:IPI00019502.1	Myosin heavy chain, nonmuscle type A	shift		1.15	(32)			1.13	(52)	1.07	(60)	0.99	(47)	1.09 (8)		
unk|IPI:IPI00413922.2	Myosin light chain alkali, smooth-muscle isoform					1.10	(3)	1.09	(6)	1.04	(7)	1.09	(4)			
unk|IPI:IPI00413604.2	Myosin lignt chain polypeptide kinase isoform 1									0.99	(6)					
unk|IPI:IPI00220573.3	Myosin regulatory light chain 2, nonsarcomeric					0.91	(3)			1.09	(4)	1.09	(4)			
unk|IPI:IPI00220278.4	Myosin regulatory light chain 2, smooth muscle isoform									1.09	(7)	1.06	(3)			
	Myotrophin V-1 protein		↑ 2.95[2]													
	N-acylglucosamine 2-epimerase														0.25	
unk|IPI:IPI00337541.3	NAD							1.04	(3)	1.13	(3)					
	NAD-dependent malic enzyme, mitochondrial precursor														0.00	
unk|IPI:IPI00328415.8	NADH-cytochrome b5 reductase							1.05	(7)							
unk|IPI:IPI00026944.1	Nidogen precursor							0.69	(4)							
unk|IPI:IPI00465154.3	Novel protein							0.96	(3)							
	Nucleoside diphosphate kinase A	yes														
unk|IPI:IPI00026260.1	Nucleoside diphosphate kinase B									1.07	(3)					
	Nucleosome assembly protein 1	yes														
unk|IPI:IPI00023860.1	Nucleosome assembly protein 1-like 1											1.08	(4)			0.27
unk|IPI:IPI00017763.3	Nucleosome assembly protein 1-like 4							1.02	(3)	0.95	(3)					
	OTTHUMP00000022298														0.00	
unk|IPI:IPI00328867.4	OTTHUMP00000030925									0.98	(3)					
unk|IPI:IPI00020416.6	OTTHUMP00000040723							1.21	(3)							
unk|IPI:IPI00470587.2; unk|IPI:IPI00552345.1	OTTHUMP00000062781; WD repeat domain 44							0.99	(5)	1.07	(3)	0.98	(3)			
	PDZ and LIM domain 5 isoform b, Enigma homolog														3.33	

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00010414.3	PDZ and LIM domain protein 1							1.01	(8)			0.99	(8)	1.02		
unk|IPI:IPI00419262.1	Peptidyl-prolyl cis-trans isomerise									1.03	(4)					
unk|IPI:IPI00419585.6	Peptidyl-prolyl cis-trans isomerase A	yes				0.94	(3)	1.34	(5)	0.98	(5)					
	peptidylprolyl isomerase A (cyclophilin A)													0.99		
unk|IPI:IPI00000874.1	Peroxiredoxin 1	yes		0.95	(3)							0.99	(3)			
	Peroxiredoxin 3 isoform b,Thioredoxin-dependent peroxide reductase, mitochondrial precursor															3.33
unk|IPI:IPI00375306.1	Peroxiredoxin 5 Precursor, isoform b							1.19	(4)							
unk|IPI:IPI00024915.2	Peroxiredoxin 5, mitochondrial precursor									0.96	(4)	1.03	(4)			
unk|IPI:IPI00220301.4	Peroxiredoxin 6							1.06	(4)	1.04	(8)	0.98	(7)			
	PhosPhatidylinositol transfer Protein, beta, Similar to Phosphatidylinositol transfer protein beta isoform														0.14	
unk|IPI:IPI00009688.1	Phosphatidylinositol-4-phosphate 5-kinase type II alpha									0.87	(3)				0.00	
	PhosPhodiesterase 5A isoform 3,Splice Isoform PDE5A1 of cGMP-specific 3',5'-cyclic phosphodiesterase,Splice Isoform PDE5A2 of cGMP-specific 3',5'-cyclic phosphodiesterase,CGMP-specific phosphodiesterase PDE5A1														0.47	3.33
unk|IPI:IPI00219526.5	Phosphoglucomutase 1	yes		0.97	(3)			1.27	(4)	1.01	(3)					
unk|IPI:IPI00169383.2	Phosphoglycerate kinase 1			0.88	(4)			1.14	(6)	1.02	(6)	1.02	(4)			
	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor															0.41
	PINCH protein	yes														
	Placental thrombin inhibitor,Hypothetical protein DKFZp686I04222															0.00
unk|IPI:IPI00022445.1	Platelet basic protein precursor (CXCL7)	yes		0.68	(3)			0.76	(3)	0.83	(3)	0.88	(4)			

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
pdb|1RHP_A	Platelet Factor 4 (Hpf4)			0.64	(4)									9.98		
unk|IPI:IPI00022446.1	Platelet factor 4 precursor									0.89	(3)	0.84	(3)			
	Platelet factor 4 variant precursor	yes														3.03
unk|IPI:IPI00011255.3	Platelet glycoprotein Ib alpha chain precursor							0.72	(4)	0.80	(6)	0.85	(5)			
unk|IPI:IPI00464990.1	Platelet glycoprotein Ib beta									0.97	(3)					
unk|IPI:IPI00013744.1	Platelet glycoprotein IIb alpha chain precursor	yes		1.10	(6)	1.10	(9)	1.17	(4)	1.03	(5)			1.02 (5)		
gb|AAA52600.1	Platelet glycoprotein IIIa[3]			1.11	(6)	1.11	(5)									
unk|IPI:IPI00418495.4	Platelet glycoprotein IV											0.98	(3)		2.56	
	Platelet glycoprotein IX													0.97		
unk|IPI:IPI00027502.1	Platelet glycoprotein IX precursor									0.96	(3)					
unk|IPI:IPI00027410.1	Platelet glycoprotein V precursor							0.52	(7)	0.68	(6)	0.74	(3)			
	Platelet glycoprotein VI-2														0.00	
unk|IPI:IPI00306311.7	Pleckstrin	↓	↑ 1.63[2]			0.91	(5)	1.25	(5)	1.06	(6)	0.99	(5)			
unk|IPI:IPI00398002.4	Plectin 1							0.92	(8)							
unk|IPI:IPI00016610.2	Poly							0.97	(3)							
	PREDICTED: KIAA0540 protein															0.48
	PREDICTED: similar to Eukaryotic translation initiation factor 5A (eIF-5A) (eIF-4D) (Rev-binding factor),Eukaryotic initiation factor 5A isoform I variant A,Eukaryotic translation initiation factor 5A,Eukaryotic translation initiation factor 5AII														0.45	
unk|IPI:IPI00001952.5	Probable endonuclease KIAA0830 precursor							1.03	(3)			1.01	(3)			
unk|IPI:IPI00216691.4	Profilin-1			0.99	(5)	0.91	(6)	1.12	(5)	1.02	(7)	1.01	(5)	0.99		0.25
	Prohibitin	yes														
	Proline-serine-threonine phosphatase-interacting protein 2															3.57
	Prosaposin,Splice Isoform Sap-mu-6 of Proactivator polypeptide precursor,Splice Isoform Sap-mu-0 of Proactivator polypeptide precursor															3.33

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 10,Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 10,26S proteasome non-ATPase regulatory subunit 10														0.00	
	Proteasome activator complex subunit	yes														
	Proteasome subunit	yes														
	Protein BAT5														0.15	
S55507	Protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor					1.11	(3)									
	Protein disulfide-isomerase A3		↓ 0.57[1] (2)													
unk|IPI:IPI00025252.1	Protein disulfide-isomerase A3 precursor							1.16	(9)	1.04	(14)	1.03	(8)			
unk|IPI:IPI00009904.1	Protein disulfide-isomerase A4 precursor							1.20	(4)	0.98	(3)					
unk|IPI:IPI00550984.1	Protein disulfide-isomerase precursor			0.87	(4)	0.84	(3)			0.97	(11)	1.09	(7)			
	Protein transport protein Sec23A															0.22
	Protein tyrosine phosphatase PTPCAAX2															0.37
	Protein-iosaspartate-O-methyltransferase	yes														
	Prothrombin precursor														0.29	
unk|IPI:IPI00295339.3	P-selectin precursor							1.16	(5)			0.98	(3)			
	PUMA delta														0.00	
spt|P00491; trm|Q8N7G1	Purine nucleoside phosphorylase (EC 2.4.2.1) (Inosine phosphorylase) (PNP) ; Hypothetical protein FLK25678	yes		0.84	(3)											
unk|IPI:IPI00026216.4	Puromycin-sensitive aminopeptidase							1.24	(4)							
	Putative G-protein coupled receptor															0.00
unk|IPI:IPI00220644.6	Pyruvate kinase 3 isoform 2			0.94	(3)			1.32	(12)							
	Pyruvate kinase isozymes M	yes														
unk|IPI:IPI00383237.3	Pyruvate kinase M2					0.93	(5)			1.04	(13)	0.97	(13)			
	Q9P1F3	yes														

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00010154.3	Rab GDP dissociation inhibitor alpha							1.18	(10)			0.98	(5)			
unk|IPI:IPI00031461.1	Rab GDP dissociation inhibitor beta									0.92	(6)					
rf|NP_004209.1	RAB11B, member RAS oncogene family			0.87	(3)											
dbK|BAB61868.1	Raichu404X			1.12	(3)											
	Rap11A/B	yes														
unk|IPI:IPI00032267.1; unk|IPI:IPI00383401.1	Ras GTPase-activating protein 3; RAS p21 protein activator 3									0.95	(3)					
unk|IPI:IPI00017256.5	Ras suppressor protein 1					1.01	(3)			1.09	(7)	1.09	(6)			
unk|IPI:IPI00016513.3	Ras-related protein Rab-10									1.07	(6)	0.87	(4)			
unk|IPI:IPI00020436.2	Ras-related protein Rab-11B							1.37	(3)							
unk|IPI:IPI00291928.3	Ras-related protein Rab-14							1.28	(3)							0.00
unk|IPI:IPI00007755.1	Ras-related protein Rab-21									0.86	(3)					
unk|IPI:IPI00010491.1	Ras-related protein Rab-27B			0.90	(3)					1.02	(4)	1.08	(4)			
unk|IPI:IPI00016342.1	Ras-related protein Rab-7							1.37	(5)							
unk|IPI:IPI00015148.3	Ras-related protein Rap-1b[3]	↑						1.30	(6)	1.00	(7)	1.02	(6)			
	Receptor expression enhancing protein 5															0.00
unk|IPI:IPI00290328.2	Receptor-type tyrosine-protein phosphatase eta precursor							1.00	(4)							
pdb|1HKC_A	Recombinant Human Hexokinase Type I Complexed With Glucose And Phosphate			1.12	(3)											
	Rho GDP-dissociation inhibitor 2[3]	yes														4.55
	Rho kinase													0.31		
	RhoA/C	yes														
unk|IPI:IPI00307155.7	Rho-associated protein kinase 2							0.97	(4)			1.01	(3)		3.33	
unk|IPI:IPI00020567.1	Rho-GTPase-activating protein 1							1.06	(6)	0.94	(4)	1.08	(3)			
unk|IPI:IPI00550654.2; unk|IPI:IPI00550069.2	Ribonuclease/angiogenin inhibitor; Placental ribonuclease inhibitor							1.25	(3)							
	RLPR185														None on D1	0.39
unk|IPI:IPI00218442.2	Sarco/endoplaSmic reticulum Ca2+ -ATPaSe iSoform d							1.22	(6)							
unk|IPI:IPI00019376.4	Septin 11							1.25	(4)	0.89	(3)					

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00014177.1	Septin 2[3]							1.03	(5)	1.02	(4)				8.33 (2)	
unk|IPI:IPI00017731.1; unk|IPI:IPI00383573.1	Septin 5; Septin	yes								0.95	(3)					
unk|IPI:IPI00033025.6	Septin 7							1.10	(4)						2.08	
spt|Q9Y6E0	Serine/threonine protein kinase 24 (EC 2.7.1.37) (STE20-like kinase MST3) (MST-3) (Mammalian STE20-l			0.97	(3)											
unk|IPI:IPI00554737.1	Serine/threonine protein phosphatase 2A, 65 kDa regulatory subunit A, alpha isoform									0.86	(3)					
unk|IPI:IPI00550451.1	Serine/threonine protein phosphatase PP1-alpha catalytic subunit	yes								1.01	(3)					
unk|IPI:IPI00218236.4	Serine/threonine protein phosphatase PP1-beta catalytic subunit							1.32	(4)							
unk|IPI:IPI00022434.1	Serum albumin precursor	yes				1.13	(6)			0.87	(13)	0.85	(6)			
unk|IPI:IPI00005809.3	Serum deprivation response							0.99	(4)	1.15	(11)	1.11	(6)			
	Seryl-tRNA synthetase														0.00	
	SH3 domain-binding glutamic-acid-rich-like protein 3		↑ 3.19[1] (3)													
unk|IPI:IPI00220617.3	Similar to 6-phosphofructokinase, liver type							1.21	(3)							
trm|Q8IUA1; trm|Q86UX7; trm|Q8N207	Similar to hypothetical protein MGC10966 (UNC-112 related protein 2 short form URP2SF) ; UNC-112 related protein 2 long form URP2LF ; Hypothetical protein FLK36400			1.18	(7)											
unk|IPI:IPI00478005.2; unk|IPI:IPI00019600.1	Similar to MMS2; Ubiquitin-conjugating enzyme E2 variant 2							0.95	(3)							
	Similar to Retinoic acid receptor responder protein 2 precursor															2.78
unk|IPI:IPI00014572.1	SPARC precursor							0.77	(6)	0.77	(4)	0.86	(3)			
	Sperm-associated antigen 1														0.00	
	S-phase kinase-associated protein 1A isoform a,19 kDa protein,S-phase kinase-associated protein 1A isoform b,PREDICTED: similar to S-phase kinase-associated protein 1A isoform b														0.30	

Accession	Protein Name	2D gel	DIGE	iTRAQ								ICAT		
				I		II	III		IV (a)		IV (b)			I	II	III
	Splice Isoform 1 of Adenylate cyclase, type VI,Splice Isoform 2 of Adenylate cyclase, type VI															0.00
unk|IPI:IPI00477831.1	Splice Isoform 1 of Adipocyte-derived leucine aminopeptidase precursor						1.41	(4)							4.35	
unk|IPI:IPI00014516.1	Splice Isoform 1 of Caldesmon								1.22	(3)	1.13	(3)				
unk|IPI:IPI00456925.3; unk|IPI:IPI00396437.3; unk|IPI:IPI00101968.3	Splice Isoform 1 of Drebrin-like protein; Splice Isoform 2 of Drebrin-like protein; Splice Isoform 3 of Drebrin-like protein						0.90	(3)	1.06	(3)						
unk|IPI:IPI00328328.3	Splice Isoform 1 of Eukaryotic initiation factor 4A-II						1.05	(3)								
unk|IPI:IPI00003865.1	Splice Isoform 1 of Heat shock cognate 71 kDa protein						1.20	(14)	1.03	(13)						
unk|IPI:IPI00160340.2	Splice Isoform 1 of HEF-like protein						1.15	(5)								
unk|IPI:IPI00295976.4	Splice Isoform 1 of Integrin alpha-IIb precursor						1.28	(19)	0.99	(22)	0.97	(17)				
	Splice Isoform 1 of Metastasis suppressor protein 1,PRO1941															20.0
unk|IPI:IPI00336081.1	Splice Isoform 1 of Myosin light chain kinase, smooth muscle and non-muscle isozymes						1.86	(7)								
unk|IPI:IPI00549996.1	Splice Isoform 1 of PDZ domain containing RING finger protein 4														0.00 (1)	0.00 (1)
unk|IPI:IPI00014898.1	Splice Isoform 1 of Plectin 1										1.00	(6)				
unk|IPI:IPI00016620.2	Splice Isoform 1 of Proline-serine-threonine phosphatase-interacting protein 2						1.42	(3)								
	Splice Isoform 1 of Protein KIAA0513															0.36
unk|IPI:IPI00027009.2; unk|IPI:IPI00221111.1	Splice Isoform 1 of Protein kinase C and casein kinase substrate in neurons protein 2; Splice Isoform 2 of Protein kinase C and casein kinase substrate in neurons protein 2						0.91	(3)	0.87	(3)						
unk|IPI:IPI00026262.3	Splice Isoform 1 of Ras GTPase-activating protein 1; Splice Isoform 2 of Ras GTPase-activating protein 1														5.00 (2)	2.17 (1)

Accession	Protein Name	2D gel	DIGE	iTRAQ									ICAT		
				I		II		III		IV (a)		IV (b)			I	II	III
unk|IPI:IPI00298237.4; unk|IPI:IPI00554617.1; unk|IPI:IPI00554538.1	Splice Isoform 1 of Tripeptidyl-peptidase I precursor; Splice Isoform 2 of Tripeptidyl-peptidase I precursor; Splice Isoform 3 of Tripeptidyl-peptidase I precursor							0.93	(3)								
unk|IPI:IPI00456635.1	Splice Isoform 1 of Unc-13 homolog D							1.42	(9)	1.09	(10)	0.99	(4)				
	Splice Isoform 1 of Uridine 5'-monophosphate synthase,Splice Isoform 2 of Uridine 5'-monophosphate synthase															5.88	
	Splice Isoform 10 of Partitioning-defective 3 homolog															0.19	
	Splice Isoform 1B of Beta-arrestin 1,Splice Isoform 1A of Beta-arrestin 1															5.00	
	Splice Isoform 2 of Annexin A7, Annexin A7															0.00	50.0
unk|IPI:IPI00016786.1	Splice Isoform 2 of Cell division control protein 42 homolog									0.96	(3)						
unk|IPI:IPI00455383.1	Splice Isoform 2 of Clathrin heavy chain 1							1.09	(14)	1.04	(11)	0.95	(6)				
unk|IPI:IPI00217872.2	Splice Isoform 2 of Phosphoglucomutase											1.05	(4)				
unk|IPI:IPI00218372.1; unk|IPI:IPI00024175.3	Splice Isoform 2 of Proteasome subunit alpha type 7; Splice Isoform 1 of Proteasome subunit alpha type 7							1.04	(3)								
unk|IPI:IPI00298289.1	Splice Isoform 2 of Reticulon 4							1.20	(3)			1.03	(3)				
unk|IPI:IPI00216704.2	Splice Isoform 2 of Spectrin beta chain, erythrocyte							0.91	(5)							0.00	
	Splice Isoform 2 of Transportin 3															6.25	
unk|IPI:IPI00220709.3	Splice Isoform 2 of Tropomyosin beta chain							1.05	(3)								
unk|IPI:IPI00216699.1	Splice Isoform 2 of Unc-112 related protein 2							1.27	(21)	1.12	(24)	1.00	(17)				
unk|IPI:IPI00294779.1; unk|IPI:IPI00031804.1	Splice Isoform 2 of Voltage-dependent anion-selective channel protein 3; Splice Isoform 1 of Voltage-dependent anion-selective channel protein 3							1.32	(5)	1.03	(3)						

Accession	Protein Name	2D gel	DIGE	iTRAQ									ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
unk|IPI:IPI00216256.2	Splice Isoform 2 of WD-repeat protein 1							1.41	(10)	0.94	(10)	1.02 (7)				
unk|IPI:IPI00218695.1	Splice Isoform 3 of Caldesmon							0.96	(5)							
	Splice Isoform 3 of COP9 signalosome complex subunit 1,Splice Isoform 1 of COP9 signalosome complex subunit 1,G protein pathway suppressor 1 isoform 2,G protein pathway suppressor 1 isoform 1														0.47	
	Splice Isoform 3 of Fibronectin precursor,Splice Isoform 8 of Fibronectin precursor,Splice Isoform 6 of Fibronectin precursor,Splice Isoform 1 of Fibronectin precursor,Splice Isoform 11 of Fibronectin precursor,Fibronectin 1 isoForm 7 preproprotein,Hypothetical protein DKFZp686K08164,Splice Isoform 7 of Fibronectin precursor,Splice Isoform 5 of Fibronectin precursor,Splice Isoform 10 of Fibronectin precursor,Splice Isoform 4 of Fibronectin precursor,Fibronectin 1 isoForm 2 preproprotein,Fibronectin 1 isoForm 4 preproprotein														3.13	
unk|IPI:IPI00215943.1; unk|IPI:IPI00215942.1	Splice Isoform 3 of Plectin 1; Splice Isoform 2 of Plectin 1									1.00	(9)					
unk|IPI:IPI00216135.1	Splice Isoform 3 of Tropomyosin 1 alpha chain									1.17	(3)					
unk|IPI:IPI00218820.1	Splice Isoform 3 of Tropomyosin beta chain									1.12	(3)					
unk|IPI:IPI00328257.4; unk|IPI:IPI00413947.1; unk|IPI:IPI00384489.1	Splice Isoform A of Adapter-related protein complex 1 beta 1 subunit; Splice Isoform B of Adapter-related protein complex 1 beta 1 subunit; AP1B1 protein							1.15	(3)							
unk|IPI:IPI00022202.3; unk|IPI:IPI00215777.1	Splice Isoform A of Phosphate carrier protein, mitochondrial precursor; Splice Isoform B of Phosphate carrier protein, mitochondrial precursor									1.04	(3)					

Accession	Protein Name	2D gel	DIGE	iTRAQ									ICAT		
				I		II		III		IV (a)		IV (b)			I	II	III
unk|IPI:IPI00010271.3; unk|IPI:IPI00219675.1	Splice Isoform A of Ras-related C3 botulinum toxin substrate 1; Splice Isoform B of Ras-related C3 botulinum toxin substrate 1							1.27	(5)								
unk|IPI:IPI00335281.1; unk|IPI:IPI00002212.1	Splice Isoform A of Serine/threonine-protein kinase 24; Splice Isoform B of Serine/threonine-protein kinase 24							1.01	(4)			1.00	(3)				
unk|IPI:IPI00003843.1; unk|IPI:IPI00216245.1	Splice Isoform A1 of Tight junction protein ZO-2; Tax_Id=9606 Splice Isoform C1 of Tight junction protein ZO-2									0.96	(3)						
unk|IPI:IPI00029717.1; unk|IPI:IPI00021885.1	Splice Isoform Alpha of Fibrinogen alpha/alpha-E chain precursor; Splice Isoform Alpha-E of Fibrinogen alpha/alpha-E chain precursor							0.83	(8)	0.92	(11)	0.97	(7)				
unk|IPI:IPI00216222.1	Splice Isoform Alpha-6X1B of Integrin alpha-6 precursor							0.99	(5)	0.99	(3)						
unk|IPI:IPI00303882.1	Splice Isoform B of Mannose-6-phosphate receptor binding protein 1									0.96	(3)						
unk|IPI:IPI00217563.3	Splice Isoform Beta-1A of Integrin beta-1 precursor									1.01	(9)	0.97	(4)				
unk|IPI:IPI00303283.1	Splice Isoform Beta-3A of Integrin beta-3 precursor							1.22	(10)	1.06	(12)	1.00	(9)				
unk|IPI:IPI00219628.1	Splice Isoform Beta-II of Protein kinase C, beta type							1.41	(4)	1.09	(4)						
unk|IPI:IPI00470720.1	Splice Isoform Delta13 of Platelet endothelial cell adhesion molecule precursor							1.07	(5)	1.10	(3)						
unk|IPI:IPI00021891.5; unk|IPI:IPI00219713.1; unk|IPI:IPI00411626.2	Splice Isoform Gamma-B of Fibrinogen gamma chain precursor; Splice Isoform Gamma-A of Fibrinogen gamma chain precursor; Hypothetical protein DKFZp779N0926									0.89	(8)	0.89	(4)				
	Splice Isoform Long of Atrial natriuretic peptide receptor B precursor																0.00
unk|IPI:IPI00216008.2	Splice Isoform Long of Glucose-6-phosphate 1-dehydrogenase							1.21	(4)			0.98	(4)				
unk|IPI:IPI00005614.3	Splice Isoform Long of Spectrin beta chain, brain 1							0.94	(6)	0.91	(3)						

Accession	Protein Name	2D gel	DIGE	iTRAQ					ICAT		
				I	II	III	IV (a)	IV (b)	I	II	III
unk|IPI:IPI00216514.1; unk|IPI:IPI00216516.1; unk|IPI:IPI00374740.3	Splice Isoform OA3-293 of Leukocyte surface antigen CD47 precursor; Splice Isoform OA3-312 of Leukocyte surface antigen CD47 precursor; Splice Isoform OA3-323 of Leukocyte surface antigen CD47 precursor						1.06 (3)				
unk|IPI:IPI00219114.1; unk|IPI:IPI00514925.1; unk|IPI:IPI00029485.2	Splice Isoform p135 of Dynactin-1; 137 kDa protein; Splice Isoform p150 of Dynactin-1						0.97 (4)				
unk|IPI:IPI00177817.4; unk|IPI:IPI00219078.5	Splice Isoform SERCA2A of Sarcoplasmic/endoplasmic reticulum calcium ATPase 2; Splice Isoform SERCA2B of Sarcoplasmic/endoplasmic reticulum calcium ATPase 2					1.76 (4)	1.15 (4)				
unk|IPI:IPI00218440.1; unk|IPI:IPI00479855.1; unk|IPI:IPI00004092.2; unk|IPI:IPI00185231.4;	Splice Isoform SERCA3C of Sarcoplasmic/endoplasmic reticulum calcium ATPase 3; 115 kDa protein; Splice Isoform SERCA3B of Sarcoplasmic/endoplasmic reticulum calcium ATPase 3; Splice Isoform SERCA3A of Sarcoplasmic/endoplasmic reticulum calcium ATPase 3						1.05 (4)				
unk|IPI:IPI00216633.1; unk|IPI:IPI00292290.1	Splice Isoform Short of Dematin; Splice Isoform Long of Dematin					0.92 (4)	1.05 (6)				
unk|IPI:IPI00298268.1; unk|IPI:IPI00298267.3; unk|IPI:IPI00514766.1	Splice Isoform Short of Prostaglandin G/H synthase 1 precursor; Splice Isoform Long of Prostaglandin G/H synthase 1 precursor; Prostaglandin-endoperoxide synthase 1					1.20 (4)					
	Splice Isoform Short of Retina-specific copper amine oxidase precursor,Splice Isoform Long of Retina-specific copper amine oxidase precursor										3.13
spt|Q14247	Src substrate cortactin	yes		1.05 (3)		1.32 (7)	1.06 (7)	1.09 (6)			
unk|IPI:IPI00007765.5	Stress-70 protein, mitochondrial precursor					1.00 (5)	0.98 (3)				
unk|IPI:IPI00166512.1	Stromal interaction molecule 1			0.91 (3)			0.97 (6)				
	Superoxide dismutase[3]									4.76	
unk|IPI:IPI00022314.1	Superoxide dismutase [Mn], mitochondrial precursor	shift				1.12 (3)	0.93 (4)				

Accession	Protein Name	2D gel	DIGE	iTRAQ									ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
	Superoxide dismutase copper chaperone	yes														
unk|IPI:IPI00399142.3	Surfeit 4							1.34	(3)							
unk|IPI:IPI00010438.2	SynaptoSomal-aSSociated protein 23 iSoform SNAP23A	yes						1.21	(3)							
spt|Q96C24	Synaptotagmin-like protein 4 (Exophilin 2) (Granuphilin)			1.03	(3)											
unk|IPI:IPI00019971.3	Syntaxin binding protein 2							1.09	(8)	0.92	(9)	0.97	(6)			
unk|IPI:IPI00026128.1	Syntaxin-11							1.06	(4)							
unk|IPI:IPI00298994.3	Talin 1	yes						1.28	(68)	1.03	(68)	1.01	(66)	1.52 (12)		
unk|IPI:IPI00290566.1	T-complex protein 1, alpha subunit									0.98	(4)					
unk|IPI:IPI00297779.6	T-complex protein 1, beta subunit							1.20	(5)	0.95	(8)					
unk|IPI:IPI00302927.5	T-complex protein 1, delta subunit							1.28	(3)	1.02	(4)	0.91	(3)			
unk|IPI:IPI00010720.1	T-complex protein 1, epsilon subunit							1.17	(6)	0.97	(3)				2.38	
unk|IPI:IPI00553185.1	T-complex protein 1, gamma subunit							1.02	(3)	0.92	(5)					
unk|IPI:IPI00302925.3	T-complex protein 1, theta subunit	yes						1.14	(7)	1.01	(4)	1.06	(3)			
	Thioredoxin	yes														
	Thioredoxin-dependent peroxide reductase	yes														
	Thiosulfate sulfurtransferase, TST protein														0.00	
rf|NP_003237.1	Thrombospondin-1		↓ 0.47[2]	0.94	(8)											
unk|IPI:IPI00296099.3	Thrombospondin-1 precursor					1.00	(8)	0.92	(18)	0.85	(23)	0.86	(19)	2.12 (8)		
unk|IPI:IPI00329700.4	Thromboxane A synThase 1 (plaTeleT, cyTochrome P450, family 5, subfamily A) isoform TXS-							1.06	(4)	0.98	(5)	0.97	(3)			
unk|IPI:IPI00292858.3	Thymidine phosphorylase precursor							1.01	(5)	1.02	(3)					
unk|IPI:IPI00100160.2	TIP120A protein							0.98	(4)	0.89	(3)					
unk|IPI:IPI00024102.3	Transaldolase							1.07	(4)							

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
	TranscripTion facTor-like 5 proTein,Splice Isoform 2 of Transcription factor-like 5 protein															12.50
unk|IPI:IPI00000075.1	Transforming growth factor beta 1 precursor							1.07	(3)							
unk|IPI:IPI00027500.1	Transforming protein RhoA									1.09	(3)	0.96	(3)			
unk|IPI:IPI00024057.2	Transgelin 2	yes						1.31	(11)	1.09	(13)	1.06	(9)			
unk|IPI:IPI00022774.2	Transitional endoplasmic reticulum ATPase					0.94	(3)	1.00	(10)	0.97	(10)					
unk|IPI:IPI00021716.1	Transketolase							1.14	(4)	1.00	(3)					
	Transmembrane 9 superfamily protein member 2 precursor														0.32	
	Transmembrane 9 superfamily protein member 3 precursor															0.28
unk|IPI:IPI00031522.2	Trifunctional enzyme alpha subunit, mitochondrial precursor														0.25 (1)	0.25 (1)
unk|IPI:IPI00465028.4	Triosephosphate isomerase 1	yes						1.21	(6)	1.10	(6)	1.04	(6)			
	TRIP protein,Transcription repressor														0.16	
	Tropomoduline-3	yes														
unk|IPI:IPI00382894.2	Tropomyosin 3							1.08	(4)					1.14		
unk|IPI:IPI00010779.3	Tropomyosin 4	yes						1.17	(6)	1.12	(5)	1.07	(6)			
unk|IPI:IPI00218319.2	Tropomyosin alpha 3 chain (Tropomyosin 3) (Tropomyosin gamma). SPLICE ISOFORM 2									1.14	(6)	1.04	(4)			
unk|IPI:IPI00027107.3	Tu translation elongation factor, mitocondrial							1.01	(3)							
trm|Q9H4B7	TUBB1 human beta tubulin 1, class VI (DK543K19.4) (Beta tubulin 1 class VI (TUBB1)) (Tubulin, beta 1)			0.92	(3)											
unk|IPI:IPI00007750.1	Tubulin alpha-1 chain[3]		↑ 1.86[1] (3)	0.91	(5)			0.93	(15)							
unk|IPI:IPI00218343.4	Tubulin alpha-6 chain							0.92	(5)							
unk|IPI:IPI00216005.5	Tubulin alpha-8 chain							0.93	(3)	0.67	(3)	0.66	(5)			
unk|IPI:IPI00387144.4	Tubulin alpha-ubiquitous chain									0.69	(10)	0.69	(11)			
unk|IPI:IPI00007752.1	Tubulin beta-? chain							0.82	(5)	0.72	(17)	0.81	(4)			
unk|IPI:IPI00006510.1	Tubulin beta-1 chain							0.87	(19)	0.57	(12)	0.72	(10)			
unk|IPI:IPI00011654.2	Tubulin beta-2 chain							0.81	(14)	0.66	(4)	0.79	(12)			

Accession	Protein Name	2D gel	DIGE	iTRAQ										ICAT		
				I		II		III		IV (a)		IV (b)		I	II	III
spt|P05218	Tubulin beta-5 chain		↓ 0.39[1] (3)	0.90	(4)											
	Tubulin-specific chaperone A	yes														
unk|IPI:IPI00013212.1	Tyrosine-protein kinase CSK							1.29	(5)							
	Tyrosine-protein phosphatase, non-receptor type 11														4.55	
unk|IPI:IPI00289082.2	Tyrosine-protein phosphatase, non-receptor type 12							0.84	(3)							
	Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial precursor															0.00
	Ubiquitin-conjugating enzyme															0.00
unk|IPI:IPI00024466.1	UDP-glucose:glycoprotein glucosyltransferase 1 precursor							1.21	(3)							
	unk|IPI:IPI00020416.6 OTTHUMP00000040723														0.00	
unk|IPI:IPI00007682.2	Vacuolar ATP synthase catalytic subunit A, ubiquitous isoform							1.09	(4)	0.98	(3)	1.01	(3)		7.14	
unk|IPI:IPI00301058.4	Vasodilator-stimulated phosphoprotein							1.09	(7)	0.96	(4)	1.06	(4)			2.86
	Vesicular integral-membrane protein VIP36 precursor														20.00	
unk|IPI:IPI00307162.2	Vinculin (Metavinculin)	yes		0.99	(11)	0.90	(21)									
unk|IPI:IPI00307162.2; unk|IPI:IPI00291175.3	Vinculin isoform meta-VCL ; Vinculin isoform VCL							1.08	(32)	1.03	(34)	1.04	(30)			
	Vitamin D-binding protein precursor														1.96	
unk|IPI:IPI00411815.1	Voltage-dependent anion channel protein 2							1.32	(4)							
unk|IPI:IPI00216308.4	Voltage-dependent anion-selective channel protein 1							1.33	(3)							
unk|IPI:IPI00023014.1	Von Willebrand factor precursor			0.99	(3)	0.97	(3)	1.06	(15)	0.87	(10)	0.91	(6)			
unk|IPI:IPI00298625.1	V-yes-1 Yamaguchi sarcoma Viral related oncogene homolog			1.10	(3)			1.15	(3)							
unk|IPI:IPI00001545.2	Wiskott-Aldrich syndrome protein							0.91	(4)			1.08	(3)			
trm|Q86V33	YWHAZ protein (Fragment)			0.95	(4)											
unk|IPI:IPI00020513.1	Zyxin[3]		↑ 5.45[1] (4)					0.94	(7)	0.99	(8)					

APPENDIX 3 Ethical Approval Certificate

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC CREB NUMBER:
Maria Issa	UBC/Medicine, Faculty of/Pathology & Laboratory Medicine	H03-70559

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
UBC	Vancouver (excludes UBC Hospital)

Other locations where the research will be conducted:
Canadian Blood Services - Network Centre for Applied Development where, in some cases, the blood will be drawn. In most cases, blood will be drawn at the LSI Research Blood Collection Suite.

CO-INVESTIGATOR(S):

Ken Scammel
Geraldine Walsh
J Zhang
Katherine Serrano
Elena Levin
Juergen Kast
Sandra Weiss
Iren Constantinescu
Cheryl Pittendreigh
Nobu Kitamura
Peter Schubert
Elisabeth Maurer
Faith Hunter
Jonathan Thon
Brankica Culibrk
Dana V. Devine

SPONSORING AGENCIES:

Canadian Blood Services - "General Instrument Calibration and Reference Material"

PROJECT TITLE:
Human Blood for General Instrument Calibration, Reference Material and Reagent

EXPIRY DATE OF THIS APPROVAL: January 28, 2009

APPROVAL DATE: January 28, 2008

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by:

Dr. James McCormack, Associate Chair

APPENDIX 4 Notes on Publication

Dr. Peter Schubert and I contributed equally to the writing of the manuscript 'Platelet storage (lesion): A new understanding from a proteomic perspective,' including the preparation of all tables and figures therein; elements of which are found in CHAPTER 1.

CHAPTER 3 (Comprehensive Proteomic Analysis of Protein Changes During Platelet Storage Requires Complementary Proteomic Approaches) is a result of a collaboration with the Juergen Kast lab, in which we employed proteomics to examine protein changes occurring in platelet units over a period of 7 days. My role in this research project was the sampling and preparation of human blood platelets for 2 of 3 ICAT and all four iTRAQ experiments. Dr. Katherine Serrano was responsible for preparations involved in the third ICAT run, and Dr. Peter Schubert, Shujun Lin and Marie Duguay were responsible for the 2D gel electrophoresis and DIGE experiments included in this thesis. In addition to having performed the amalgamation of these data sets and their subsequent analysis myself, I was also primarily responsible for the writing of the resulting manuscript [1] alongside Dr. Peter Schubert and, with the exception of Figure 9 (immunoblot analysis of selected proteins identified as changing during platelet storage) generated by Cindy Chen and amended by me, the preparation of all tables and figures therein.

The manuscript titled 'Blood Platelet Storage Lesion: PI3-Kinase-Dependent Rap1b Activation Mediates GP IIb/IIIa Activation and Degranulation' (from which Figure 6 and Table 2 were derived) is a result of a collaboration with the Juergen Kast lab and Edwin Moore's lab, in which we employed western blot analysis, flow cytometry and microscopy to unravel the involvement of a subset of the platelet proteome found changing during storage in the exacerbation of the PSL. The nature of my major contribution to this work was in the

identification of a subset of these proteins whose identity and type of change could be determined with extreme confidence as potential markers of the PSL; this was achieved through the development and application of stringent selection criteria. I was then able to narrow down the list of 503 individual proteins found changing in relative concentration during a 7-day storage period to only 12 proteins in which this change was deemed very significant and highly reproducible, both within repetitions of individual runs and across the different proteomic approaches employed. The following assessment of protein localization by fluorescent microscopy was carried out by Dr. Peter Schubert and Cindy Chen with the assistance of Dr. Edwin Moore. Flow cytometric analysis of CD62P, CD61 and Pac-1 used to validate the changes observed by immunofluorescence were performed by Dr. Peter Schubert and Dr. Geraldine Walsh. Dr. Peter Schubert was responsible for the purification, western blotting and quantification of Rap1b during storage and following agonist exposure. Dr. Peter Schubert and I contributed equally to the experimental design and data analysis involved in this work, and shared equally the responsibilities of writing of the resulting manuscript, including the preparation of all tables and figures therein.