PART I

GENERAL OVERVIEW: PLATELETS, SAMPLE PREPARATION, AND MASS SPECTROMETRY-BASED PROTEOMICS

1

PLATELETS AND THEIR ROLE IN THROMBOTIC AND CARDIOVASCULAR DISEASE: THE IMPACT OF PROTEOMIC ANALYSIS

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Abstract

This chapter provides an overview of how proteomics research impacts on our understanding of platelets. In addition to their role in hemostasis, inappropriate platelet activation is strongly related to the leading cause of death in Western societies: thrombotic cardiovascular disease. The known processes of platelet activation and the signaling mechanisms that regulate these are detailed here, but this knowledge is incomplete. Mass-spectrometry-based proteomics has already contributed to a growth in the understanding of platelets and presents itself as a tool that can unravel the details of the control of platelet function in health and disease through continuing refinements in technology and experimental design toward the development of diagnostic tools and antithrombotic drugs.

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1.1 INTRODUCTION

Platelets are anucleate blood cells derived from megakaryocytes that perform a pivotal role in the regulation of hemostasis, a physiologic response to injury that prevents excessive bleeding at sites of injury. Inappropriate platelet activation, however, can lead to the pathological condition *arterial thrombosis*, the formation of a blood clot within a blood vessel resulting in occlusion of bloodflow. This is a critical event that occurs at the site of lipid-rich atherosclerotic plaques to trigger both heart attack [myocardial infarction (MI)] and stroke.

Cardiovascular disease (CVD) is the main cause of death in Westernized societies, and the World Health Organization (WHO) estimated that by 2010 CVD will also be the leading cause of death in developing countries [1]. Similar rates and trends of heart disease are seen across northern Europe and North America so that in both the United States and the United Kingdom CVD is responsible for 35% of deaths each year, with about half of these deaths attributed to coronary heart disease (CHD) and about a quarter to stroke [2,3]. With changes in lifestyle and improvements in pharmaceutical and surgical intervention, mortality rates have been falling since the early 1970s, with reduction rates slowest in younger individuals and fastest in those over 55 years old. This reduction in death rate is confounded by reported increases (6.0–7.4% in men, 4.1–4.5% in women) in the incidence and prevalence of cardiovascular disease and stroke across most age ranges [3]. This increase is most consistently found in people over 75 years old and may reflect the fact that more people in developed countries are living longer [4].

Investigations using basic methods of cell biology and targeting specific signaling molecules or pathways have led to a deeper understanding of platelet biology and the mechanisms that regulate platelet activity. This has resulted in the development of safer and more efficacious antithrombotic strategies of medication [5–7] and to the identification of a number of platelet proteins as potential therapeutic targets [8,9]. Mass-spectrometry (MS)-based methods of proteomics have brought additional tools to the study of cells and tissues: direct measurement and characterization of proteins and peptides, providing information such as identity and *de novo* amino acid sequence. Hence, in the absence of appreciable levels of regulation at the genome level (although, curiously, protein synthesis by platelets has been reported [10,11]), where the analysis of platelet biology is not complicated by significantly changing levels of total protein, proteomics methods are particularly suitable. The principal regulation of platelets is achieved by changes in protein interactions, translocation within the cell, and posttranslational modifications. Hence, the unparalleled levels of sensitivity inherent in proteomic methods of analysis that enable sequence isoform and post-translational modification of low abundance proteins and of protein complexes enables the examination of both normal and disease-induced changes in platelet proteins. This level of information can provide insights not easily gained by alternative methods.

1.1.1 Regulation of Platelet Function

Knowledge of the normal regulation of platelet function may establish new mechanisms by which platelet activity in diseased blood vessels can be controlled and the risk of atherosclerosis be reduced. By examining platelet signaling processes and understanding the molecular interactions that are articulated as platelet function, it becomes possible to identify molecules or groups of molecules as potential targets for therapeutic treatment or for use as biomarkers—diagnostic molecular markers of disease. The intention of the following section is to outline the molecular processes involved in platelet activation.

The process of platelet activation and thrombus formation, controlled by ligand–receptor interactions and intracellular signaling events, is outlined in Figure 1.1, while the principal platelet receptors, ligands, and the key signaling events associated with them are described below and illustrated in Figure 1.2.

The generation of a thrombus involves the initial formation of a platelet plug followed by stabilization of this plug through fibrin deposition (coagulation). The

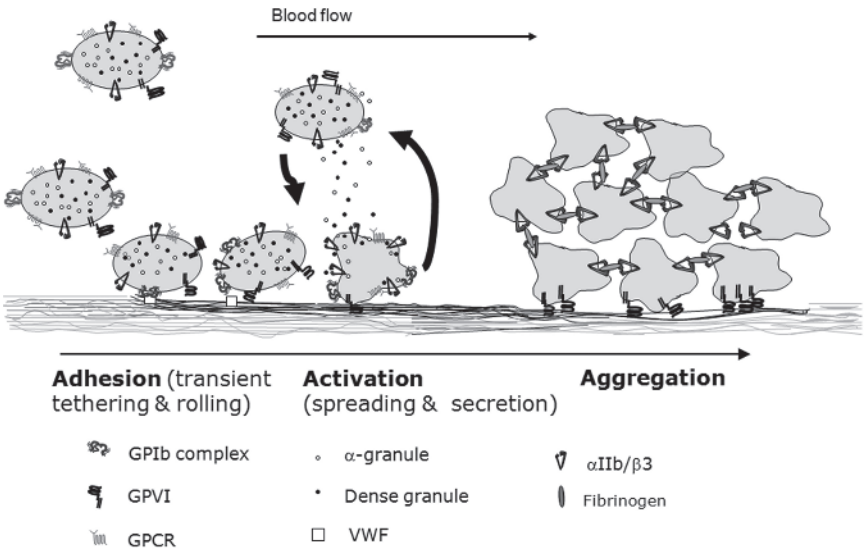

Figure 1.1 Stages of platelet activation (adapted from Barrett et al. [8]). *Adhesion:* The glycoprotein von Willebrand factor (vWF) binds to exposed collagen and under conditions of high blood shear a transient and unstable interaction with the platelet GPIb-V-IX receptor complex slows the movement of the platelets, allowing other interactions to occur. More stable and direct interactions between collagen and the platelet integrin α2β1 [16] enable binding to the collagen receptor GPVI. *Activation:* GPVI molecules, complexed with the Fc receptor (FcR) γ-chain, cluster and stimulate signaling that results in rapid shape change so that platelets spread to cover the damaged endothelium. The secretion of positive-feedback signals from α-granules and dense granules attract more platelets to the growing thrombus and activate them. *Aggregation:* Platelet activation increases the affinity of integrin αIIbβ3 for its plasma fibrinogen ligand, leading to aggregation [17–22].

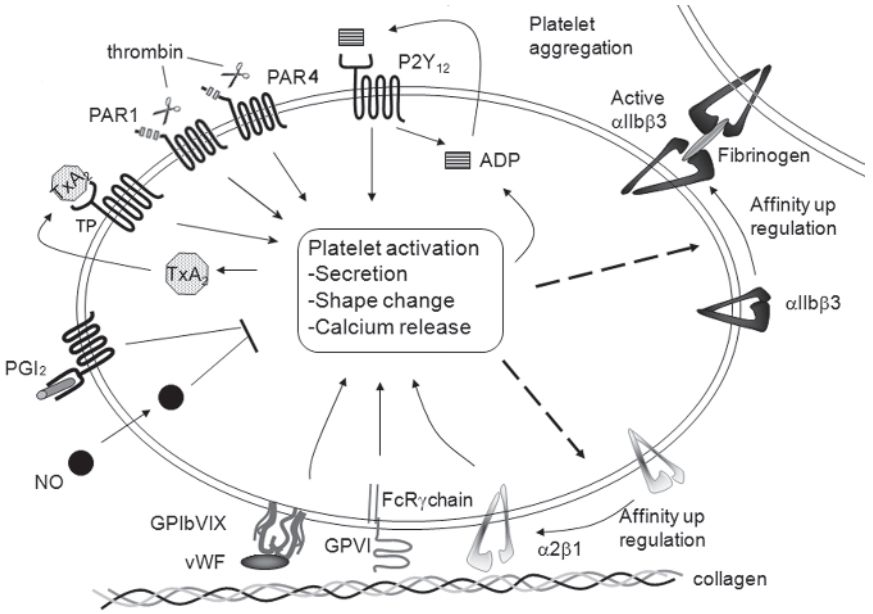

Figure 1.2 Key platelet receptors and signaling molecules. The principal platelet receptors and their ligands are illustrated. Nitric oxide [12] and PGI_2 [13] inhibit platelet aggregation under normal conditions of circulation. The process of platelet binding to collagen commences with transient interaction of plasma von Willebrand factor (vWF) with collagen and platelet GPIb-V-IX [15] followed by more stable interactions with integrin α2β1 [16] and finally with the collagen receptor GPVI [17]. Subsequent activatory signaling processes lead to increased intracellular calcium concentration, secretion of α-granule and dense granule contents, and platelet shape change. Secreted products, including thromboxane A_2 (TXA_2) and ADP, bind to their specific receptors, the thromboxane (TP) receptor and ADP receptors $P2Y_1$ and $P2Y_{12}$, adding to platelet activation by positive feedback [47–53]. Thrombin activation of protease-activated receptors (PAR1 and PAR4) stimulates further signaling activities [54,56,163]. The affinity of integrin αIIbβ3 for fibrinogen is increased via *inside-out* signaling [17]. This facilitates the formation of platelet aggregates through cross-binding with fibrinogen.

conversion of platelets from their circulating quiescent form to a thrombus may be characterized in three distinct phases: adhesion, activation, and aggregation [8].

1.1.1.1 Adhesion Under normal conditions of blood circulation, molecules such as nitric oxide (NO) [12] and prostaglandin I_2 (PGI_2) [13] released from healthy endothelial cells inhibit platelet activation. When the endothelium is damaged, however, this antiactivatory signaling is disrupted and the proactivatory subendothelial matrix is exposed, initiating a series of events that initially cause platelet binding to the damaged surface. The multimeric plasma glycoprotein von Willebrand factor (vWF) is released from endothelial cells into the plasma at high concentrations [14]. This vWF binds to exposed collagen and, under

conditions of high shear, undergoes conformational changes that enable interaction with the glycoprotein (GP)Ib component of the platelet GPIb-V-IX receptor complex [15]. This initial binding is transient and unstable, but is sufficient to slow the platelets, enabling direct adhesion to collagen via the integrin α2β1. This stabilizes platelet–collagen interactions [16], allowing interaction of the collagen receptor glycoprotein VI (GPVI).

1.1.1.2 Activation Activation of platelets occurs in two rapid phases, amplified by positive feedback, and results in irreversible aggregation. Phase 1 is initiated by binding of collagen to GPVI, inducing rapid activation of a kinase cascade. This cascade results in multiple signaling events that lead to platelet shape change and secretion of many positive-feedback factors. Phase 2 consists of further activation downstream of these (and other) factors, recruiting more platelets, and propelling platelets into aggregation [17–22].

As a pivotal platelet-specific activatory receptor, GPVI and its signaling pathway has been the subject of much research and is described in detail in many articles and reviews [22–25]. Platelets deficient in GPVI are unable to aggregate on collagen stimulation, yet have no major bleeding defect [25,26], thus highlighting the possible benefit of therapeutically targeting GPVI or its signaling pathway components. The binding of collagen to GPVI results in receptor clustering and subsequent tyrosine phosphorylation of the noncovalently associated Fc receptor (FcR) γ-chain. Two conserved tyrosine residues found within the immunoreceptor tyrosine-based activatory motif (ITAM) of the FcR γ-chain are phosphorylated by the Src family kinases Fyn and Lyn [27]. This enables docking of the tyrosine kinase Syk via its two Src homology 2 (SH2) domains, and thus the kinase cascade is initiated [18–21]. Syk becomes autophosphorylated on several tyrosine residues and induces tyrosine phosphorylation of residues in the adaptor protein linker for activation of T cells (LAT) [26–28]. LAT acts as a scaffold for signaling molecules such as phospholipase Cγ2 (PLCγ2) and phosphatidylinositol 3-kinase (PI3K). PLCγ2 catalyzes the conversion of phosphatidylinositol(4,5)bisphosphate to inositol(1,4,5)trisphosphate and diacylglycerol, triggering a rise in intracellular calcium. The increased intracellular concentration of calcium initiates secretion of both α-granules and dense granules [31–33], the contents of which include fibrinogen, vWF, coagulation factors V and XIII, ADP, and serotonin, and act in an autocrine and paracrine fashion to further stimulate platelets [17,22,34–38]. PI3K catalyzes the conversion of phosphatidylinositol (4,5) bisphosphate to phosphatidylinositol (3,4,5) trisphosphate. This phosphorylated lipid recruits Pleckstrin homology (PH) domain containing molecules to the cell membrane, such as protein-dependent kinases (PDKs) and protein kinase B (PKB; also known as Akt) [17,39–42], where they become activated and induce further signaling, ultimately leading to upregulation in the affinity of the fibrinogen receptor integrin αIIbβ3 [38,43–45].

Factors secreted from platelets act as positive-feedback signals to attract more platelets to join the growing thrombus and to activate them. These factors include locally high concentrations of secondary agonists such as

ADP, adrenaline, 5-hydroxytryptamine (5HT) and thromboxane A_2 (TXA_2), each of which binds to specific receptors on the platelet plasma membrane. Activation of phospholipase A_2 results in the liberation of arachidonic acid from membranes, which is, in turn, converted to thromboxane A_2 (TXA_2) via the actions of cyclooxygenase (COX) and thromboxane synthase [44]. Liberated TXA_2 then binds to thromboxane–prostaglandin (TP) receptors, contributing to positive-feedback activation of platelets [47,48].

Platelets possess two receptors for ADP: $P2Y_1$ and $P2Y_{12}$, both of which are G-protein-coupled receptors (GPCRs). $P2Y_1$ is essential for platelet activation, while $P2Y_{12}$ amplifies and sustains $P2Y_1$-initiated signaling [49–53]. The serine protease thrombin is generated through activation of the coagulation pathways and acts as a powerful platelet agonist by cleaving the *N* terminus of the protease-activated receptors PAR1 and PAR4. The uncleaved part of the receptor forms a "tethered ligand" that interacts with extracellular loops of the receptor to stimulate intracellular signaling [54–57].

1.1.1.3 Aggregation (Thrombus Propagation) The final step in platelet activation is platelet crosslinking via fibrinogen and its receptor integrin αIIbβ3. Many independent signaling pathways (e.g., those stimulated by collagen, thrombin, ADP, and thromboxane A_2 (TXA_2) ultimately lead to an increased affinity of αIIbβ3 for its ligand through a process known as *inside-out signaling* [17]. High-affinity αIIbβ3 mediates platelet–platelet adhesion through bivalent interaction with fibrinogen or with vWF and an aggregate begins to form. In addition to linking platelets together, the binding of the receptor to its ligand results in outside-in signaling that further amplifies platelet activation [58,59]. Therapeutic targeting of a single pathway involved in the upregulation of αIIbβ3 should, therefore, reduce platelet activation while leaving other activatory pathways intact, thus maintaining hemostasis [8].

Outside-in signaling leads to the remodeling of the actin cytoskeleton and subsequent platelet shape change, including the formation of fillopodia, lamellipodia, and platelet spreading [59,60]. Processes involved in clot retraction, thrombus stabilization, and wound repair are also receiving attention as an emerging concept of sustained signaling within the thrombus [59,61–63]. This research focuses on the roles of contact-dependent ligand–receptor signaling involving a number of platelet surface molecules. These include the junctional adhesion molecules (JAM-A and JAM-B), Eph receptor tyrosine kinases and their ephrin ligands, Sema4D and its platelet receptors CD72 and plexin-B1, Gas6 and its interactions with the Axl, Tyro3, and Mer tyrosine kinase receptors [63].

1.1.2 Cardiovascular Disease and Platelets

In the majority of instances, the development of a platelet-rich thrombus at the site of an atherosclerotic plaque is the underlying cause of acute cardiovascular events, including coronary heart disease (myocardial infarction) and ischemic

stroke [64]. Atherosclerosis is a highly complex chronic disease that is strongly linked with dyslipidemia, hypercholesterolemia, and inflammation, and which requires decades to progress from its initiation to the formation of pathogenic atherosclerotic plaques [65]. Vulnerable plaques that are structurally weak and rupture or erode [64,66,67] can lead to occlusion of blood vessels. Depending on the location of the plaque, tissues (cardiomyocytes in the case of heart attack and cerebral neurons with ischemic stroke) are deprived of oxygen and die.

Investigations into the potential roles of platelets in atherosclerosis have given rise to an increased awareness that they may be significant factors in the initiation, progression and outcome of this disease, for example [66–68]. Consistent with this, antiplatelet drugs have been found to be beneficial in reducing the incidence of nonfatal events in clinical trials [71].

1.1.2.1 Atherosclerosis The processes involved in atherosclerotic plaque formation are complex and multifactorial. While still not fully understood, atherogenesis essentially follows a sequence of initiation, fatty streak formation, mature complex plaque formation, and finally atherothrombosis—the acute pathological complication of thrombus formation on plaque lesions.

Initiation Atherosclerosis commonly occurs at bends, branches and bifurcations of the aorta and its subsidiaries such as the coronary and cerebral arteries [73,74]. There, laminar blood flow is disturbed and turbulent eddies of recirculating blood are formed [74,75] allowing increased endothelium–blood particle contact and suppressing endothelial cell expression of platelet adhesion inhibiting nitric oxide (NO) [12]. *In vivo* models have also been used to show that modified blood shear rate is related to thrombus formation [76].

The retention [77] and partial oxidation of low-density lipoprotein (LDL) molecules [78,79] within the intima of the arterial wall creates a proinflammatory environment where the expression of endothelial adhesion molecules leads to the recruitment of monocytes from the circulation [80].

Fatty Streak Formation Within the subendothelium, monocyte-derived macrophages release inflammatory cytokines and growth factors [81,82], while expressing LDL binding scavenger receptors [83,84]. LDL molecules are phagocytized and oxidized to form “foamy” cholesteryl ester-rich lipid droplets. The macrophage foam cells subsequently die and their lipid contents accumulate to form a necrotic lesion core in the developing atherosclerotic plaque [85].

Mature, Complex Plaques Vascular smooth muscle cells migrate and proliferate around and above the lipid core where they secrete extracellular matrix proteins so that the mature plaque is overlaid by a collagen-rich fibrous cap and a monolayer of endothelial cells. The growth of plaques into the arterial lumen causes partial occlusion, but this is not pathogenic in itself [64].

Atherothrombosis Rupture or erosion of the plaque exposes collagen and possibly also oxidized lipids [86] to circulating platelets leading to thrombus formation at

the site of injury. More recently there has been an increasing awareness that the pathogenesis of atherosclerosis is not dependent on plaque size, but on the likelihood of disruption to plaques and the subsequent nature of overlaying thrombi i.e. a large lipid core, thin fibrous cap with few smooth muscle cells, and an abundance of proteases such as matrix metalloproteases (MMPs), cathepsins, and collagenases [64,87].

1.1.2.2 Platelet Involvement in Atherosclerosis While platelet involvement in atherosclerosis has traditionally been confined to the final thrombotic stages of the disease, the involvement of platelets in the initiation of atherosclerosis has been suggested by Huo and colleagues [88] in which platelets adhere to undamaged arterial endothelium of the apolipoprotein E-deficient ($apoE^{-/-}$) murine model of atherosclerosis. In this model platelet adhesion leads to the expression of inflammatory molecules and the initiation of atherogenesis. Aggregates of platelets with monocytes and leukocytes have been shown to promote the formation of atherosclerotic lesions in the $apoE^{-/-}$ model [88]. There, activated circulating platelets that express the surface receptor P-selectin were shown to deliver platelet-derived proinflammatory factors to monocytes, leukocytes, and the vessel wall. Platelet-derived chemokines, stored within α-granules and rapidly released on platelet activation, may also play an important role in atherogenesis through the recruitment of monocytes to sites of vascular damage and their differentiation into macrophages [89].

Numerous factors are involved in the likelihood and clinical outcome of atherothrombosis and the direct involvement of platelets in this process. Localized inflammation, bloodflow dynamics and platelet "sensitivity" to activation all appear to be involved. For example, while the proinflammatory cytokine CD40 is expressed by endothelial cells, macrophages, smooth muscle cells, T cells, and platelets, its ligand CD40L (CD154) is released at high levels by platelets after adhesion via αIIbβ3 [90–92]. Ligation of CD40 results in the expression of adhesion molecules, matrix metalloproteases (MMPs), that digest matrix proteins such as collagen fibrils and lead to the development of unstable atherosclerotic lesions [67] and procoagulant tissue factor exposure [93,94].

Dyslipidemia, a major risk factor for atherosclerosis, has been associated with increased platelet reactivity and platelets have been shown to express receptors for both native (n)LDL and oxLDL [95]. Binding of nLDL to its receptor ApoER2′ on the platelet surface leads to increased response to platelet agonists through synthesis of thromboxane A_2 via stimulation of the p38-mitogen-activated protein kinase (MAPK) pathway [96]. Ligation of scavenger receptors CD36 and SR-A on the platelet surface by oxLDL leads to more sustained activation of p38-MAPK [97] and increased platelet binding to fibrinogen via the integrin αIIbβ3. Platelet CD36 has also been shown by Podrez and colleagues to act as a receptor for oxidised choline glycerophospholipids generated by oxidative stress [98]. This mechanism may be involved in not only increased thrombosis, but also in the generation of foam cells during early atherosclerosis [68,99,100].

1.1.3 Platelet Proteomics

Proteomics is a relatively new and rapidly developing approach to acquiring biological information. While initially intended to describe the study of the entire protein content of a biological system or organism, the term is often used to describe the use of technologies such as mass spectrometry (see Chapter 2), together with its associated methods of sample preparation, protein separation (see, e.g., Chapter 4) and subsequent analyses, to perform protein studies that utilize the high levels of sensitivity and high-density throughput within their capability.

Proteomic analysis is a conceptually simple, but powerful tool that can be used to answer specific questions while requiring little prior knowledge about the proteome under examination. It allows the investigation of changes in protein abundance over time in response to stimulus, medication, illness and genetic conditions, or changes in posttranslational modifications such as phosphorylation and glycosylation. The widescale use of robotics and automation in proteomics lends itself to a high level of experimental reproducibility and also provides the potential for high-throughput analysis when required. Hence, in addition to gaining qualitative and quantitative information from an analyte, the interpretation of data from proteomics studies can be applied to the discovery of new drug targets and to diagnostics through the identification of disease biomarkers.

Proteomic analysis has become increasingly sophisticated and has progressed from being reliant on methods such as two-dimensional gel electrophoresis–mass spectrometry [2DGE (also sometimes abbreviated 2-DE)-MS] to being highly inclusive with the incorporation of techniques such as protein array and multidimensional chromatographic procedures in conjunction with one or more mass spectrometric methods (e.g., as described by O'Neill et al. [101] and Lewandrowski et al. [102]). Hence the ability to produce lists of proteins identified from biological samples now occurs alongside the generation of more functionally relevant information. In parallel with technical advances in mass spectrometry, this has been achieved by applying a targeted approach where subsets of proteins are isolated by a common feature such as a shared affinity for a substrate or their subcellular location, as illustrated in Figure 1.3.

These findings focus investigations on areas of particular interest while increasing the likelihood of identifying low-abundance proteins and providing evidence of the function of the identified proteins [102–108]. Although a prior knowledge of the biological system being studied is required, a targeted approach to proteomics deals with simplified systems that can supply additional dimensions of information relevant to answering specific biological and pharmacological questions. Indeed, the number of question-driven publications now exceeds those of global studies.

The proteome of an organism is complex, and there remain technical hurdles that render identification of true and relevant differences between, for example, resting and agonist-stimulated platelet samples, challenging. These include the

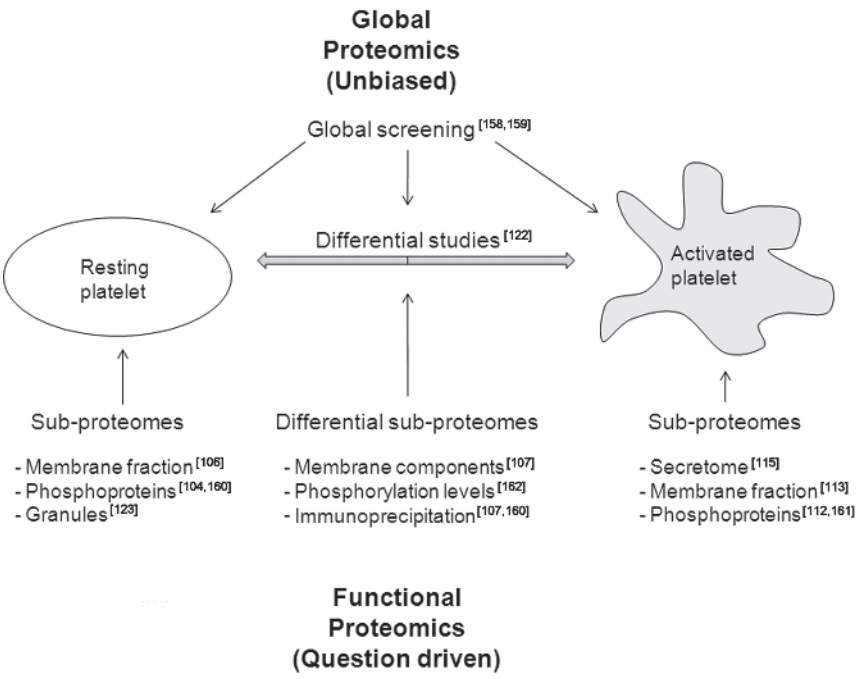

Figure 1.3 Different approaches to proteomics and mass spectrometry. Schematic representation of different approaches to proteomic analyses of platelets, indicating relevant publications.

difficulties inherent in the large dynamic range in the levels of platelet proteins, where a difference of many orders of magnitude between low- and high-abundance proteins might occur in the same sample. Also, the possible exclusion of hydrophobic, very basic and low- or high-molecular-weight proteins depending on the techniques used. Thus, proteomic studies require careful planning and understanding of the limitations that are inherently present (see Chapter 3).

The ability to identify and characterize proteins is dependent on the availability of genomewide databases. Currently data from mass spectrometry are compared against databases containing protein information that has been theoretically derived from sources such as the human genome project. It has therefore been suggested that a database based on *de novo* sequencing of platelets should be built as a common tool for people working in this field [109]. The mouse genome database is also of great importance as the mouse is the model of choice for investigating protein function in platelets (see Chapter 11).

1.1.4 Impact of Proteomics on the Understanding of Platelet Biology

As platelets are anucleate, their activities are controlled predominantly by translocation and posttranslational modification of proteins within the cell. Proteins of

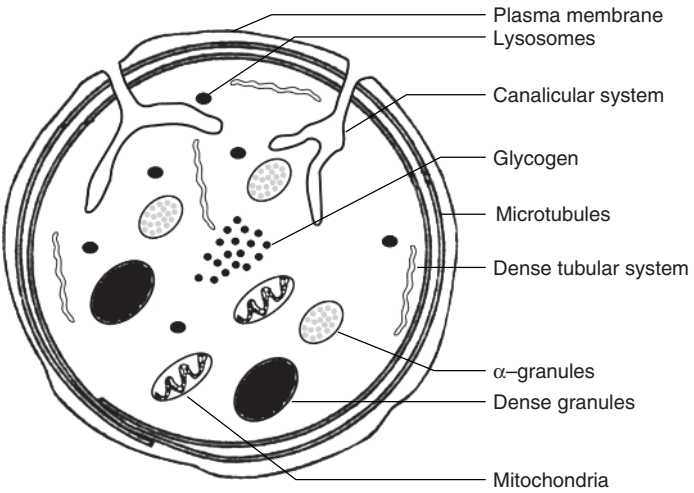

Figure 1.4 Platelet structure. Schematic cross-sectional drawing of a human platelet indicating major structural features. The *canalicular system* consists of membrane-bound channels that act as a site for granule fusion. The cytoplasm contains a coiled bundle of *microtubules* that maintain the shape of the resting platelet and centrally locate the organelles within the platelet during activation. The cytoplasm contains a number of organelles and secretory granules. *Glycogen* particles and *mitochondria* provide metabolic activity in platelets through glycolysis and oxidative phosphorylation, while small *lysosomes* contain acid phosphatase, arylsulfate, and cathepsin. α-*Granules* contain adhesive proteins, growth factors, and coagulation factors. *Dense granules* are electron-opaque granules that contain a nonmetabolic pool of adenine nucleotides, serotonin, pyrophosphate, and calcium ions. The membrane-bound *dense tubular system* is a sac-like structure containing molecules having enzymatic activity such as peroxidases and dehydrogenases.

importance to platelet function such as signaling proteins, receptors, and ion channels may be present only in low abundance and traditionally have been studied in isolation after purification from complex biological samples. This contrasts with proteomic studies where sample prefractionation techniques have been refined to enable low-abundance proteins to be investigated with a more rapid throughput of information and in the context of global signaling questions, as reviewed, for example, by García [110]. Similarly, methods involving the separation of platelets into sub-cellular components have been key to the successful identification of low-abundance proteins in several platelet proteomic studies [106,111–116].

1.1.4.1 Protein Localization Platelets, like most cells, are highly compartmentalized such that this organization collocalizes proteins with substrates and facilitates interactions in response to stimuli. In the context of signaling proteins, compartmentalization brings molecules together to form the functional units of cell biology. A schematic of the cross section of a platelet, indicating its compartments and organelles, is shown in Figure 1.4.

The plasma membrane forms the interface between a cell and its environment and as such is crucial to the exchange of information with and response to the external environment (see Chapter 5). It is not surprising, then, that membrane proteins make up about 70% of the known protein targets for drugs [117] or that platelet surface proteins and transmembrane proteins have been the focus of several studies with the aim of discovering potential targets both for drug development and as biomarkers of disease [112,113,116,118].

The platelet releasate or *secretome*, microparticles, and platelet granules, are functional aspects of platelet biology that convey molecules to the plasma membrane or to the external environment on stimulation of the cell. The importance of these systems in platelet activation, aggregation, and thrombus formation has made them a focal point of a number of proteomic investigations [115,119–123] (see Chapters 6 and 7).

1.1.4.2 Phosphoproteomics The activation of platelets is controlled by complex signaling pathways in which protein phosphorylation and dephosphorylation play important regulatory roles. It has been estimated that approximately one in three proteins are phosphorylated [124–126] by one or more of the 500+ protein kinases encoded by the human genome [127], while kinases themselves are also regulated by reversible phosphorylation [128]. The phosphoproteome is dynamic, and with most proteins being phosphorylated at multiple sites, the number of reported phosphorylation sites continues to grow. For example, the online database PhosphoSite [129] currently (as of 2010) lists over 63,000 phosphorylation sites from 11,000 proteins. Indeed, kinase inhibitor compounds constitute about 30% of all drug development programs in the pharmaceutical industry [130].

Several different approaches to the analysis of phosphoproteins have been taken. The enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC) [131] and/or titanium oxide (TiO_2) [132,133], often in conjunction with other chromatographic techniques, has become routine in the study of other cell types [134]. These methods provide varying degrees of specificity for the isolation of phosphopeptides from biological samples prior to mass spectrometry, while the immunoprecipitation of platelet proteins with an antiphosphotyrosine antibody [135] has also proved successful.

The application of multiplexed approaches to gain added information to the understanding of protein signaling is illustrated by work from the research group of Lucus Huber [136]. By combining subcellular fractionation with phosphoprotein-specific staining in 2DGE and mass spectrometry, they elucidated the signal transduction of the epidermal growth factor receptor (EGFR) and its influence on cytoskeletal proteins and also on MAPK signaling [137].

The combining of proteomic and microarray analyses of platelet proteins that become phosphorylated on platelet aggregation led to the identification of platelet endothelial aggregation receptor 1 (PEAR1), an EGFR-containing transmembrane receptor, on platelets and endothelial cells [8]. Genotyping of a cohort of patients

in conjunction with measurements of platelet activity has subsequently shown PEAR1 to be important in the regulation of platelet activity [139].

1.1.4.3 Quantitative Proteomics and Platelets While it is important to understand where a protein is and whether it has been modified during activation, it is equally important to know how much of that protein or modification is present. Comparative quantitation has been available for some time for researchers carrying out 2DGE by labeling two or more samples with different dyes in the same gel [differential in-gel electrophoresis (DIGE)], described, for example, by Della Corte and colleagues [140]. In addition to this, there are now several methods that can be used in non-gel-based proteomics. Most software platforms for analysis of data from mass spectrometry now incorporate label-free semiquantitative information of protein abundance utilizing methods such as the exponentially modified protein abundance index (emPAI) [141]. The relative contributions of proteins under different conditions can also be achieved by labeling with stable isotopes.

Stable isotope labeling by amino acids in cell culture (SILAC) [142] is a highly popular labeling process, but is considered unsuitable for use with platelets as the labeling is carried out during cell culture. However, studies of Kindlin-3 in red blood cells [143] showed that it is possible to SILAC-label whole mice via SILAC, and this approach could be applied to platelet research in the future. An alternative labeling method—isotope tagging for relative and absolute quantitation (iTRAQ)—does not rely on cell culture or *in vivo* methods and has been applied to the study of stored platelets [144].

1.1.4.4 Diagnostics Since the late 1990s, substantial progress has been made in understanding the regulation of platelet-function, including the characterization of new ligands, platelet specific receptors, and cell signaling pathways. Because of the asymptomatic nature of many of the stages of atherogenesis, diagnosis of early stage cardiovascular problems and the likelihood of thrombotic complications is difficult. Thus, in addition to the need for greater understanding of the biological processes involved, there is also a need for sensitive and effective predictive molecular markers of disease—biomarkers—to be used as diagnostic tools [145].

Biological fluids and in particular plasma are easy to obtain and would therefore provide an ideal medium for diagnosis. However, it can be difficult to gain distinct diagnostic information from plasma because of the complexity of its proteome [146] and the biological variation between individuals in the number and reactivity of candidate biomarker proteins [147,148]. These problems may be overcome, however by an increasing number of novel low-abundance proteins being discovered within subproteomes [149] and the possibility that a multiplex of biomarkers rather than a single diagnostic molecule could provide accurate and reliable early diagnosis [147]. Many datasets from platelet proteomic experiments are now available and bioinformatic analytic methods, combined with functional

information on candidate proteins, may be applied to the search for combinations of molecules that would yield suitable diagnostic tools.

1.1.4.5 Platelets as Antithrombotic Targets The participation of platelets in thrombosis and atherosclerosis (first proposed by Ross in 1976) [150–152] is well established, and the platelet has become a key target in therapies to combat cardiovascular disease. While antiplatelet therapies are used widely, current approaches lack efficacy, lead to drug resistance, or are associated with side effects, including problem bleeding [5,153,154].

Here, proteomic technologies have provided new insights into platelet signaling and have identified several candidate proteins as suitable targets for antithrombotic medication. For example, proteomics screening has led to the identification of two homophilic adhesion receptors in platelets, CD84 and CD150, signaling lymphocyte activation molecules (SLAM) [104,138]. Similarly, mass spectrometry and proteomics methods are being applied to the understanding of atherosclerosis as a whole and from the numerous disciplines involved in its studies [155–157] (see Chapter 14).

1.2 CONCLUDING REMARKS

Since the 1990s, proteomic studies of platelets have ranged from global approaches to changes within whole-platelet samples in response to stimuli [158,159]; to the analysis of very specific subproteomes, including the phosphoproteome of resting [104,106] and stimulated [104,160] platelets; studies on the secretome [122], the membrane fraction of unstimulated platelets [112,113], and proteins upregulated in surface fractions on stimulation [111,114,116,161]; and immunoprecipitation experiments [162]. Each of these studies has identified proteins not previously identified in platelets and has produced new and valuable information. Proteins new to platelets continue to be discovered through the use of proteomic methods. The evolution of mass spectrometry and associated technologies provides improved speed, accuracy and ease of use. Analytical software, which has often been a bottleneck in the experimental process, is becoming more integrated with the emergence of proteomics platforms and pipelines that can readily convert raw data into information with greater confidence and reduced numbers of false-positive identifications. In turn, the growing experience of expert scientists in platelet proteomics is indicated in the development of more question-oriented experiments and a trend toward integration between proteomics with functional genomics, transcriptomics, informaticists, and mathematicians to gain greater depth and relevance of information.

Chapters 2–14 in this book have been written by key scientists in the fields of platelet proteomics, transcriptomics, and functional genomics, with a special focus on the technology and the application to platelet research and platelet-related diseases.

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