Platelet Membrane Glycoproteins: A Historical Review*

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I am very pleased to contribute to this anniversary edition of Seminars in Thrombosis & Hemostasis. My aim here is to provide some personal reflections on the discovery and initial characterization of the platelet membrane glycoprotein (GP) mediators of platelet aggregation and adhesion to the vessel wall. In view of space limitations, I will confine myself to the critical 25-year period from 1968 to 1983. Most early studies were based on the hypothesis that unidentified membrane constituents were responsible for the surface contact interactions necessary for the participation of platelets in the hemostatic plug. I will emphasize studies on inherited disorders of platelets although I will try to avoid repeating as much as possible my previous historical reviews on this subject.1,2 The reader is also asked to note that I use the GP nomenclature of that time; GPIIb and GPIIIa are now known as the integrin αIIbβ3 subunits, respectively.

Platelets and the Surface Glycocalyx

It was through the use of electron microscopy (EM) that workers such as John French in Oxford were able to define the ultrastructure of platelets and show how their morphology changed following platelet activation and during adhesion to the injured vessel wall.3,4 But it was a Danish researcher, Olaf

Abstract

The search for the components of the platelet surface that mediate platelet adhesion and platelet aggregation began for earnest in the late 1960s when electron microscopy demonstrated the presence of a carbohydrate-rich, negatively charged outer coat that was called the “glycocalyx.” Progressively, electrophoretic procedures were developed that identified the major membrane glycoproteins (GP) that constitute this layer. Studies on inherited disorders of platelets then permitted the designation of the major effectors of platelet function. This began with the discovery in Paris that platelets of patients with Glanzmann thrombasthenia, an inherited disorder of platelet aggregation, lacked two major GP. Subsequent studies established the role for the GPIIb-IIIa complex (now known as integrin αIIbβ3) in binding fibrinogen and other adhesive proteins on activated platelets and the formation of the protein bridges that join platelets together in the platelet aggregate. This was quickly followed by the observation that platelets of patients with the Bernard–Soulier syndrome, with macrothrombocytopenia and a distinct disorder of platelet adhesion, lacked the carbohydrate-rich, negatively charged, GPIb. It was shown that GPIb, through its interaction with von Willebrand factor, mediated platelet attachment to injured sites in the vessel wall. What follows is a personal reflection on the studies that were performed in the early pioneering days.

Keywords

- platelet
- inherited disorder
- membrane glycoproteins
- Glanzmann thrombasthenia
- Bernard–Soulier syndrome

* This article is dedicated to Professors Jacques Caen and Peter Castaldi. Jacques Caen is the father of studies on inherited disorders of platelets and gave me my chance during my early years in Paris. His knowledge and drive meant that he was always pushing back the boundaries of knowledge. Peter Castaldi was one of the early pioneers of studies on Glanzmann thrombasthenia and provided much encouragement to me during my early years in Paris. I wish them both a long and happy retirement.
investigate the

Somewhat later, Richard Skaer in Cambridge used EM to

gap-containing material that was proteinaceous in nature.

aggregated, the surface membranes remained separated by a

proteases also suggested a GP component. When platelets

glycocalyx although a loss of staining after treatment with

charides were thought the most likely contributors to the

membrane.

spanned by protein bridges attached to stubs on the platelet

added to the glutaraldehyde

or other stains such as ruthenium red or colloidal iron, were

be identified.

Sialic acid is a negatively charged monosaccharide that terminates many of the O-linked and N-linked oligosaccha-

ride side chains of membrane GP as well as a class of
glycolipids termed gangliosides. Aaron Marcus et al in New

York analyzed the sialic acid-containing glycolipids of plate-

lets and showed that gangliosides constituted 0.5% of the
platelet lipids and accounted for 6% of the sialic acid found on

the platelet surface. Nevertheless, it was concluded that the

orientation and organization of platelet membrane glycoli-
pids could be important determinants of the unique surface properties of platelets. But clearly, there were other com-
nents involved and Graham Jamieson’s group in Washington

brought attention to membrane GP by publishing a series of

studies detailing the carbohydrate composition of glycopep-
tides released from platelets or isolated platelet membranes

by proteases. In particular, a heavily charged sialic acid-

rich macroglycopeptide was identified as a major contributor
to the platelet surface charge; significantly, its structure was

quite different from the glycopeptides released from eryth-

rocytes. In a personal approach, I tested the agents used by

Behnke in EM as potential stains for glycopeptides released

from platelets by proteases; these were separated by electro-

phoresis on cellulose acetate paper and then, as technology

progressed, on polyacrylamide gels. Alcian blue proved to

be an excellent reagent and two classes of component were

detected (see Figure 1 of the review by Nurden). The first

comigrated with chondroitin 4- or 6-sulfate, whereas the

second was no longer seen when platelets were incubated

with neuraminidase, an enzyme that releases terminal nega-
tively charged sialic acid. The second component corre-
sponded to the macroglycopeptide previously characterized

by Jamieson and his coworkers. Interestingly, charge differ-
ces were detected between the macroglycopeptide released

from human platelets and from platelets of a series of primates.

The high sialic acid content of the macro-
glycopeptide in human platelets suggested a rigid structure

that would protrude out from the platelet surface.

Although early studies suggested that most sulfated mucopolysaccharides synthesized in megakaryocytes were

stored in granules (chondroitin-4-sulfate is now known to be

a major constituent of α granules where it complexes with platelet factor 4), evidence was also obtained for a surface

pool. A possible contribution to the surface properties of platelets was shown by the loss of mucopolysaccharide
during adenosine diphosphate (ADP)–induced platelet aggre-
gation. Surprisingly, the role of surface-bound chondroitin-
4-sulfate of platelets has never been fully elucidated. Never-
thless, chondroitin–4-sulfate and the platelet macroglyco-

peptide are the major charged components of the “glycocalyx” first revealed by Behnke and the early EM studies

were the starting point for much of the work that followed.

Identification of Major Surface Membrane Glycoproteins

The development of sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS–PAGE) allowed the study of intrinsic

GP (those that possess transmembrane and cytoplasmic domains) following the solubilizing of cells or isolated mem-

branes by the ionic detergent SDS. The migration of proteins

according to their size was largely because of their uniform
capacity to bind the detergent although exceptions occurred
to highly glycosylated proteins. When SDS–PAGE was applied
to platelets, three principal GP bands termed GPI, GPII, and

GPIII were identified by carbohydrate staining using the
colorimetric periodic acid–Schiff (PAS) reaction. Ralph Nachman in New York, David Phillips in Memphis, and

James N George in San Antonio were early pioneers in this

field. SDS–PAGE was even more powerful when combined

with radiolabeling of surface GP. Initially, lactoperox-
idase, a large (90 kDa) enzyme unable to penetrate the plasma

membrane of cells, was used to catalyze the incorporation of
into tyrosine residues exposed at the platelet surface. The principle 125I-labeled constituents revealed by autoradiography proved to be GPIIb (a major component of the previously termed GPII) and GPIIIa (a major component of GPIII) (Figs. 3 and 4). Contrasting with this enzymatic approach, direct chemical labeling was achieved using diazotized (125I)-diiodosulfanilic acid although the principle labeled surface constituents remained the same. An alternative approach, first applied to platelets by Kenneth Clemetson (Berne) and John McGregor (Lyon), along with their coworkers, was to detect carbohydrate residues in the oligosaccharide residues of the GP either using 125I-labeled lectins after SDS-PAGE or by a prelabeling procedure that involved the enzymatic removal of sialic acid by neuraminidase to expose galactose residues that were then reduced using sodium (3H)-borohydride. Incorporated 3H was located by fluorography and using this approach GPIb (the major component of GPI) predominated as a result of its high content of O-linked oligosaccharide chains (Fig. 5).

**Two-Dimensional Acrylamide Gel Electrophoresis**

Resolution of surface constituents was greatly favored when two-dimensional SDS-PAGE procedures were applied to platelets. Phillips and Agin first migrated SDS-soluble platelet extracts in a first dimension tube gel, then reduced disulfides before second dimension electrophoresis across a slab gel. Reducing disulfides resulted in the separation of the small...
light or β-chains from the heavy or α-chains from some GP subunits (GPIb, GPIIb, GPIa, and GPIc), whereas the loss of intramolecular disulfides of other membrane GPs (GPIIIa) actually slowed their migration. These migration changes resulted in many GPs migrating away from the diagonal. An illustration of this procedure from my work is shown in \(\text{Fig. 3}\). A further advance came with the introduction of isoelectric focusing in the first dimension after solubilizing platelets using nonionic detergents. The first dimension isoelectric focusing gels were then incubated with SDS with or without disulfide reduction and the proteins separated in a second dimension slab gel by SDS-PAGE.\(^{17}\) The application of such high-resolution procedures to platelets whose surface components were prelabeled with \(^{125}\)I or \(^3\)H led to protein separation on the basis of both charge and size (\(\text{Fig. 4}\)). In fact, these procedures were the start of proteomic analysis of platelet proteins as performed today.

**Crossed Immunoelectrophoresis**

The use of SDS-PAGE has the disadvantage that SDS is denaturing and so noncovalently linked complexes are dissociated. This is not so with some nonionic detergents. Hagen and associates in Oslo made use of this property to analyze platelet proteins by crossed immunoelectrophoresis (CIE).\(^{19,20}\) In this procedure, detergent-soluble proteins were first separated by electrophoresis in agarose (in the presence of detergent) and then migrated in a second dimension against a rabbit antiserum prepared against washed platelets. \(\text{Fig. 2}\) shows a typical pattern with immunoprecipitates highlighted by coloration with Coomassie Blue R250. Individual precipitates were excised thereby permitting the identification of those given by major membrane antigens. Significantly, \(^{125}\)I-labeled GPIIb and GPIIIa colocalized to the same precipitate which was quite distinct from that given by GPIb. Thomas Kunicki, working with me in Paris made the fundamental discovery that GPIIb and GPIIIa were present as a \(\alpha\)C \(\alpha\)2þ-dependent dimer in the platelet membrane.\(^{21,22}\) Seen retrospectively, this was a major step in the identification of the GPIIb-IIIa complex as an integrin. CIE was a highly adaptable procedure and antigen migration changes in the first dimension helped identify platelet GPs as, for example, the marked slowing of GPIb after neuraminidase treatment of platelets.\(^{23}\) Autoradiography revealed \(^{125}\)I-labeled surface components (\(\text{Fig. 2}\)) while the incorporation of a precipitating monospecific polyclonal antibody in a thin intermediate gel in the second dimension allowed the identification of specific proteins, as did the incorporation of \(^{125}\)I-labeled monoclonal antibodies (MoAbs), lectins or ligands.\(^{19–23}\)

**Glanzmann Thrombasthenia and the Bernard–Soulier Syndrome**

Recognizing the logic that platelets possess surface components that specifically mediate platelet aggregation and
platelet adhesion. Jacques Caen in Paris pioneered studies on inherited disorders of platelets. They confirmed how Glanzmann thrombasthenia (GT), the most common of these disorders, was characterized by a defective platelet aggregation in response to all physiologic agonists. Quite simply, platelets of these patients were unable to form the cohesive bonds that linked them together during the process of aggregation. Not only was aggregation affected, in most patients clot retraction was also absent, although occasionally it was partial or subnormal.24 Fibrinogen (Fg) and Ca2+ were already known cofactors of ADP-induced human platelet aggregation.25–27 GT offered the chance to identify the surface constituent mediating platelet aggregation, and in 1973, this led me to Paris to examine the membrane GP of platelets of three GT patients using the electrophoretic techniques that were available at the time. The alcian blue–staining macroglycopeptide derived from GPI (GPIb) was normally detected, but after SDS-PAGE, the PAS-stained bands corresponding to GPI (GPIIb) and GPIII (GPIIIa) were much reduced in intensity for all the three patients (an original gel is illustrated in Fig. 2).27,28 In this way, the link between membrane GP and platelet aggregation was established. Phillips et al (also in Paris) confirmed these results and improved the analysis using 125I–labeled GT platelets with proteins separated by single or bidimensional SDS-PAGE.18,29 Quite clearly, the surface of GT platelets was deficient in two major constituents (illustrated from my work in Figs. 3 and 4). When CIE was applied to the analysis of GT platelets a dominant immunoprecipitate was missing from the profile; overall, after much work, it was concluded that GT platelets lacked the Ca2+-dependent GPIIb-IIIa complex (Fig. 2).20–22 Shulman and Karpatkin in New York, who also used CIE, confirmed this conclusion.30 Incidentally, the sensitivity of CIE permitted the detection of residual amounts of GPIIb-IIa in the platelets of some GT patients confirming heterogeneity in the disease.20 Likewise, CIE also substantiated much earlier studies from Peter Castaldi and Jacques Caen that platelets of most, but not all, GT patients lacked an intracellular secreteable store of Fg—a finding that led to the definition of type I and type II disease.31

The question quickly turned to the role of the sialic acid–rich GPI (GPIb). Jean Bernard and Jean Pierre Soulier in Paris had identified a giant platelet disorder, rapidly called Bernard–Soulier syndrome (BSS), where giant platelets and thrombocytopenia were accompanied by decreased surface sialic acid levels and an inability of platelets to interact with bovine Factor VIII (containing von Willebrand factor [VWF]) or with the antibiotic, ristocetin, in the presence of plasma.32–34 It was therefore logical to also study the surface GP of BSS platelets, and so I returned to Paris in 1974 to perform this work. It was quickly apparent that platelets from these patients lacked GPI (GPIb) and failed to release the alcian blue–staining macroglycopeptide; in contrast, GPII (GPIIb) and GPIII (GPIIIa) were normally present. As platelet function testing improved, the role of VWF in platelet adhesion was established. Harvey Weiss in New York showed that BSS platelets had a defective flow–dependent attachment to exposed subendothelium, a discovery that highlighted the importance of the GPIb–VWF adhesion axis.35 Although others also showed a GPI defect in BSS platelets, difficulties in separating the large platelets from leukocytes led to controversy about the specificity of the defect with leukocyte DNA and protease contaminations leading to secondary modifications of the BSS platelet protein profiles.30,36 Doubts about the specificity of the GPIb defect were quickly removed by studies combining lactoperoxidase–catalyzed 125I–labeling or 3H–labeling with CIE or one- and two-dimensional SDS-PAGE procedures. (Fig. 5)20,37,38 GPIIb was rapidly established as a major functional determinant of the platelet surface. It was also quickly apparent that the major functional chain, GPIbα, was transported to the platelet surface linked to smaller subunits; findings that resulted in the identification of the GPIb-IX-V complex. Significantly, an incomplete synthesis of the O–linked oligosaccharide chains of GPIbα associated with a defective galactosyltransferase in platelets of donors with the so-called Tn–syndrome, resulted in neither giant platelets nor a defect in ristocetin–induced platelet agglutination but led to thrombocytopenia and decreased platelet survival.33,39

How Platelets Fulfill Their Role in Thrombus Formation

The earlier noted findings describe how studies on inherited platelet disorders led to the identification of key membrane receptors involved in platelet adhesion and aggregation mechanisms. But what were the molecular mechanisms behind their mode of action?

Platelet Aggregation

Studies by several workers including Gérard Marguerie, Edward Plow, and Mark Ginsberg in the Scripps Clinic in La Jolla (US) as well as Joel Bennett in Philadelphia showed how radiolabeled Fg and other adhesive proteins (initially VWF and fibronectin) bound in a saturating and highly specific manner to GPIb-IIa on stimulated platelets.40–43 Significantly, Barry Coller (New York) and his colleagues were able to inhibit platelet aggregation by preventing Fg binding to GPIb-IIa on activated platelets with MoAbs reacting with complex-dependent determinants on GPIb-IIa.44,45 In the blood stream, protein sources were plasma and, in the event of platelet secretion, their α-granules. Nonetheless, early indications suggested that the aggregation mechanism was not so straightforward as an adhesive protein-dependent simple cross-linking of GPIb-IIa complexes on adjacent platelets. For example, Kent Gartner et al in Memphis (US) postulated that thrombin–induced platelet aggregation was mediated by a membrane–bound lectin.46 This was soon identified as thrombospondin–1 (TSP–1), a secreted α-granule protein virtually absent from plasma, a finding nicely confirmed by Phillips et al who performed lactoperoxidase–catalyzed 125I–labeling of platelets during thrombin–induced platelet activation.47 It is interesting to note in these studies just how Fg and TSP predominate on thrombin–activated platelets. Overall, these findings led to the concept that Ca2+-dependent Fg binding was at the basis of ADP–induced platelet aggregation, whereas
with strong agonists, such as thrombin or collagen, Fg is joined by other adhesive proteins to promote receptor clustering and the formation of more compact attachment sites. Such interactions provide the molecular basis of the bridges that were initially described morphologically by Skaer et al.6

**Platelet Vessel Wall Attachment**

The early characterization of the molecular basis of von Willebrand disease and the identification of qualitative or quantitative defects of VWF involved both in platelet attachment to the vessel wall and as a carrier of FVIII, helped enhance our understanding of how platelets accumulate at sites of vascular injury.68–69 Zaverio Ruggeri and Ted Zimmerman in La Jolla (USA) and Harvey Granilck in Washington (USA) were early pioneers in this field whereas Hans Baumgartner (Basle, Switzerland), Harvey Weiss and Dominique Meyer (Paris) all contributed to show how FVIII was important for platelet adhesion at high shear rate.35,52 Many early studies characterizing the interaction of VWF with platelets involved ristocetin-induced platelet agglutination although it was quickly recognized that VWF could bind not only to GPIb but also GPIIb-IIIa.51 MoAbs to GPIb blocked both ristocetin-induced platelet agglutination and high shear-dependent adhesion by preventing the binding of VWF to GPIb.53,54 So the concept was proven that platelets attach to injured sites in the vessel wall by attaching to VWF and that this is an initial step of thrombus formation that then led to adhesive protein binding to GPIIb-IIIa.55

**Historical Discoveries As Seen in Their Current Perspective**

It is quite remarkable that so many of the early studies still stand up in 2014. Their significance grew in importance when it was shown that the mechanisms responsible for platelet function in hemostasis were also pathologically involved in ischemic syndromes. Anti-GPIIb-IIIa antagonists arrived in the clinic and have played a major role in saving lives of patients with acute ischemic disease.60,61 They mainly act by blocking unoccupied surface GPIIb-IIIa (now referred to as the αIIbβ3 integrin) and preventing platelet aggregation and platelet incorporation into a growing thrombus. But anti-GPIIb-IIIa acting drugs also induce a bleeding risk and in some ways mimic GT. While drugs blocking GPIb and platelet adhesion offer promise in animal models, they surprisingly have yet to be adequately tested in the clinic.58 Platelet deficiency of the P2Y12R receptor results in a mild bleeding syndrome and pharmacological blockade of this pathway implicated in the formation of stable platelet aggregates initiated by ADP has received widespread use particularly in preventing arterial thrombosis in patients at risk.59,60 The identification of GPVI as a major collagen receptor, whose absence gives rise to little bleeding has raised hope that antplatelet drugs can be produced that will act without inducing bleeding.61 When occupied αIIbβ3 form bridges between aggregating platelets, a new wave of signaling (termed “outside-in signaling”) promotes new contact interactions between newly identified molecules such as ephrins and eph kinases, SLAM family proteins, and semaphorin 4D.62,63 How inhibition of these proteins will contribute to aggregate instability or dissolution is unknown, while the development of a drug that will specifically block or reverse the activated αIIbβ3 clasp will represent a significant advance. Finally, the inhibition of biologically active proteins secreted from platelets to counteract ischemic or inflammatory events offers a novel therapeutic approach particularly for cerebrovascular disease.64 Accordingly, much remains to be done and these are indeed exciting times.

**References**

44 Coller BS, Peerschke EI, Scudder LE, Sullivan CA. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombathenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest 1983;72(1):325–338
56 Coller BS, Shattil SJ. The GPIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. Blood 2008;112(8):3011–3025

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