Anniversary Issue Contribution

A short history of platelet glycoprotein lb complex

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"Mine is a long and sad tale" said the Mouse, turning to Alice and sighing. "It IS a long tail, certainly," said Alice, looking down with wonder at the Mouse's tail; "but why do you call it sad?" And she kept on puzzling about it...(1).

1940s

The history of platelet GPIb is generally recognised as starting with the description of Bernard-Soulier syndrome (2), an inherited bleeding disorder, now known to be caused by the absence or deficiency in GPIb complex components (3, 4).

1960s

At that time it was not clear that a platelet receptor was deficient, and the first clues in that direction came from studies towards the end of 1960s showing first that Bernard-Soulier platelets had less negative charge than normal platelets and this was due to decreased amounts of sialic acid (5). Studies on the major ligand for GPIb, von Willebrand factor (vWF), and its interactions with platelets were also starting around that time. At first this was not recognised as such but came as a side effect of the observation that bovine "fibrinogen" agglutinated human platelets. Of course, it was not the fibrinogen that was responsible for this but the contaminating vWF, described then as "factor VIII related protein" (6). In 1969 came the first biochemical isolation of (part of) a platelet receptor, the extracellular domain of GPIb, proteolytic fragments later called macroglycopeptide derived from "glycocalicin" (7).

1970s

The next stage was to make the connection between platelets and vWF. This came in the early 1970s when Bithell et al. (8) and Weiss et al. (9) showed that Bernard-Soulier platelets did not agglutinate with bovine fibrinogen preparations. The discovery of the effect of ristocetin allowed interactions between human platelets and human vWF to be studied (10). This period was also noted for the first applications of acrylamide gel electrophoresis methods to platelet membranes (11, 12). Three major glycopro-

tein bands were detected giving rise to the GPI, GPII and GPIII of early platelet receptor nomenclature. In the mid-1970s these techniques were applied to platelets from the bleeding disorders Glanzmann's thrombasthenia (3, 13) and Bernard-Soulier syndrome (3, 14). These clearly showed that a major part of the GPI band was absent in Bernard-Soulier syndrome. The mid-1970s added two-dimensional non-reduced/reduced electrophoresis, which showed that many if not all membrane glycoproteins contained disulphide bonds and some consisted of separate chains linked by disulphide bridges (15). Thus, GPIb was shown to contain α and β disulphide-linked subunits. Surface labelling techniques were extensively applied during this period to enable easy recognition of membrane glycoproteins. Lactoperoxidase-catalysed iodination (14) and periodate/tritiated borohydride (16) gave different labelling patterns related to glycosylation levels and tyrosine content of the individual glycoproteins.

Another technique applied to platelets at that stage was crossed immunoelectrophoresis using non-ionic detergents, which has the advantage that the proteins are not denatured. This was able to distinguish between several of the proteins classed as GPI and also establish some of their characteristics, including the presence of functional complexes (17, 18). This technique, as well as ¹²⁵I-labelled 2D peptide mapping (19), clearly showed that glycocalicin is the hydrophilic, extracellular domain of GPIba and that GPIba contains additional peptides that are presumably transmembrane and cytoplasmic domains. It was also during this period that GPIb was shown to bind to wheat germ agglutinin (20) and also to thrombin (21). The latter observation raised considerable interest but later also controversy. GPV was now shown to be cleaved by thrombin (22). Since Bernard-Soulier platelets have reduced thrombin binding, and thrombin induced responses, this was ascribed to their lack of GPIb and GPV (21, 23).

1980s

By this stage most investigators were convinced of the role of GPIb as vWF receptor and its absence in Bernard-Soulier syndrome. Attention now turned to additional components of the GPIb "complex" and to defining function more clearly. Com-

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parison of the surface proteins on Bernard-Soulier platelets with those on normal platelets now showed that two additional glycoproteins were missing (4). These were glycoprotein V and glycoprotein IX. The first monoclonal antibodies against platelet glycoproteins were also developed. One against GPIb α could block vWF-induced platelet aggregation, thus definitively establishing GPIb α as the vWF receptor on platelets (24). The same antibody co-precipitated a 22 kDa band corresponding to GPIX from detergent lysates of platelets providing additional evidence that GPIX was an integral part of the GPIb complex. The availability of antibodies permitted functional studies in new systems in particular the first approximations to in-vivo systems (25, 26).

Triton X-114 phase separation was applied to platelets and most of the known platelet receptors, including GPV, were isolated in the detergent phase in line with their hydrophobic properties. Surprisingly, however, GPIb-IX separated in the water phase (27). This is assumed to be due, on the one hand, to the fact that this molecular complex is highly glycosylated and, on the other hand, it is also strongly associated with cytoskeletal molecules, such as filamin, of the submembranous cytoskeleton. Efforts at establishing the stoichiometry of the GPIb complex using antibodies to GPIba, GPIX and GPV provided evidence for a 2:2:1 relationship (28, 29), which remained so until recently (30). During this period considerable effort was also expended in establishing the domain structure of GPIba using various proteinases and analysing the fragment patterns (31). Receptors were now seen as signalling molecules and GPIba was shown to be phosphorylated (32), with the phosphorylation on Ser166 increasing in response to PGE_1 or PGI_2 (33). The glycosylation of GPIba was extensively investigated by several groups and the structures of both N- and O-linked oligosaccharides were reported (34-36). Together these results gave a picture of GPIba as an extended molecule containing binding sites but with a mucinlike stem.

The next major breakthrough and one that clarified this issue was the cloning of GPIb α , which showed that it was a member of the leucine-rich repeat protein family (37). The outer 45 kDa domain consists of several leucine-rich repeats forming a curved

structure – the details would be revealed later – followed by two large disulphide-linked loops and then a long mucin-like domain containing many O-glycosylation sites. Then follows the transmembrane domain, with two cysteines near the outer surface, and the cytoplasmic domain. The cloning and sequencing of GPIb β (38) and GPIX (39) followed rapidly and showed that all three molecules had overall very similar domain compositions but the latter two are of course, much smaller.

The end of the 1980s also revealed a new protein capable of inducing human platelet aggregation to human vWF via GPIb. This was the snake venom protein botrocetin, which soon became a valuable tool for studying this interaction (40).

The presence in platelets of mRNA coding for GPIb α was also detected during this period (41), and first investigations of the structure of the genomic region encoding GPIb α including chromosomal location were published (42). In addition, acylations of GPIb complex components GPIX, by myristoylation and GPIb, by palmitoylation were described (43).

1990s

The decade opened with the molecular characterisation of the mutation(s) causing platelet-type von Willebrand's disease showing a Gly233Val (44) or a Met239Val (45) change within one of the disulphide-linked loops of GPIb α . Thus, mutations in GPIb α could not only reduce vWF binding but, depending on location, also increase it. GPIb was also shown to be removed rapidly from the platelet surface following activation (46). This could possibly be explained by a redistribution of membrane to the surface-connected canalicular system.

New snake venom proteins such as the alboaggregins, which activate platelets via GPIb, were described (47, 48). The question whether GPIb is a signalling receptor was addressed more seriously and binding of vWF was reported to result in signal transduction (49). In this period we were also able to show how the distribution of disulphide bonds in GPIb α leads to an unusual "knot" structure at the core of the double-loop domain (50). Although it has never been formally demonstrated, it has been



Figure 1: A contemporary version of a model of the GPIb-V-IX complex. GPV reaches further out than in earlier models because the leucine-rich domains are now thought to fold like those in GPIb α giving a more open arc. Two molecules of GPIb α linked covalently per GPIb β via disulphide bonds are shown, in line with recent findings. The fine detail of the binding sites for ligands other than the AI domain of von Willebrand factor, thrombin and FXI are not yet known. Further

thrombin and FXI are not yet known. Further connections to the cytoskeleton and to signaling pathways are known but are omitted for the sake of clarity. tacitly (and reasonably) assumed that the other complex components, GPIb β , GPIX and GPV contain the same type of structural element based on similar sequences. Indeed, it is likely that this is a common feature of many related leucine-rich repeat proteins.

The role of GPIb in platelet responses to thrombin were emphasised by several studies including those with new blocking monoclonal antibodies. This period also saw new interest in the cytoskeletal associations of the GPIb complex with demonstrations of actin-binding protein (now called filamin) binding (51, 52). The possibility of monitoring levels of the soluble extracellular domain, glycocalicin, in plasma raised interest in what this might provide as information about the state of the circulation and possible pathological conditions (53).

During this period the question also arose about expression of GPIb on endothelial cells and what physiological significance this might have, an area not yet satisfactorily explored (54). An interesting and still poorly understood Bernard-Soulier syndrome variant Leu57Phe in GPIba was described (55) that showed autosomal dominant inheritance unlike the recessive inheritance normally observed. A major step forward came with the cloning and sequencing of the last component of the GPIb complex, GPV (56). It was again found to be a member of the leucine-rich repeat family, this time with 16 repeats, and a similar domain structure to the other members. The thrombin cleavage site was identified just below the disulphide loop knot. The function of the thrombin cleavage remained obscure. Following snake venom proteins that agglutinated platelets by enhancing VWF binding or directly by cross-linking GPIb, the first to block VWF binding, echicetin, was described (57). Many other snake venom C-type lectins with similar sequences and activity were subsequently described (58).

In the mid-1990s came the first description of mutants in GPIX leading to Bernard-Soulier syndrome (59, 60) and the description of a new cytoplasmic protein associated with the GPIb complex, designated 14–3–3 ζ (61). This period also saw the first description of the tandem repeat polymorphisms of a 13 amino acid peptide within the highly glycosylated stem region of GPIb α that were subsequently much investigated as a possible source of variation in cardiovascular disease susceptibility (62). The thrombin-binding site on GPIba was localised to the anionic peptide region between the outer domains and the highly glycosylated stem (63). A new snake venom protein affecting GPIb function was described, this time a metalloprotease that cleaves GPIb α in the middle of the sulphated tyrosine/anionic peptide domain, and was to become a valuable tool in studying GPIb function (64). Mutations in GPIbß leading to Bernard-Soulier syndrome were now also described (65), leaving only GPV where a role in Bernard-Soulier syndrome had not been found. During this decade considerable progress was also made in understanding the regulation of GPIb complex gene expression that would be later applied to studies of expression in megakarvocytes (66). Towards the end of the 1990s the demonstration of tyrosine phosphorylation in response to GPIb specific platelet activation (67) and a proposal for a role of the FcyRIIA receptor in this process were major advances (68). The first studies appeared that implicated GPIb as a receptor in bacterial binding to platelets. Studies showed that the interactions between vWF and GPIb during platelet rolling induced actin polarisation and cytoskeleton reorganisation (69). Expression of GPIb complex genes in transfected cells was able to assemble the functional receptor. It was now shown that Ser609 on GPIb α is phosphory-lated, which had not been observed earlier, using ³²P labelling, due to the low rates of turnover at this site (70).

So far vWF and thrombin had been the only known GPIb ligands. This was now extended when P-selectin was shown to bind via the sulphated tyrosines and an O-glycosylation site of the anionic peptide domain (71). Possible signalling mechanisms were also extended by experiments suggesting that FcR γ played an important role in GPIb activation of the src kinases Fyn and Lyn (72).

Much effort went into identifying structure-function relations within GPIb either by mutating single amino acids or swapping specific domains such as leucine-rich repeats with those from other species.

2000s

Several papers appeared emphasising the important role of GPIb in platelet function and coagulation responses to thrombin (73–75). Since the discovery of the protease activated receptors (PAR) in the 1990s, doubt had been cast on the role of GPIb as a thrombin receptor. For a few years this was a very controversial area. The range of ligands using GPIb as receptor was extended further with the discovery that (Mac-1, CD11b/CD18) is a counter-receptor and a little later that factors XII and XI also bind to GPIb (76). The preparation of a mouse with GPIb α "knocked out" provided a model for BSS and showed that it could be rescued by gene therapy (77).

The anchorage of GPIb complex in the cytoskeleton via its interaction with filamin was shown to be essential for platelet rolling on vWF (78). The question of the role of the membrane-associated cytoskeleton in regulation of GPIb binding to vWF was also addressed (79). This is probably related to the changes in GPIb surface expression following platelet activation.

Another important observation in the late 1990s and early 2000s was based on the preparation of mice with GPV "knocked out" by two groups in an effort to establish the function of this component of the complex (80–83). Although the results remain controversial, evidence was obtained suggesting that the absence of GPV rendered these mice platelets more susceptible to thrombin and they were shown to form larger thrombi *in vivo* in response to thrombin. On the other hand, some experiments suggested that GPV might have a role in collagen binding (83).

During this period the first results appeared suggesting that GPIb might interact with GPVI, the recently characterised collagen signalling receptor, in its responses to its specific ligands (84). Snake venom C-type lectins interacting with both receptors to produce a maximal activation of platelets had also been characterised (85).

Binding of thrombin to GPIb caused conformational changes in the active site leading to enhanced cleavage of PAR1, suggesting this might be the physiological role (86).

In 2002 there was a burst of structural activity with several groups reporting crystallographic data on the outer domain of GPIb α alone (87, 88) or in complex with the A1 domain of vWF (88, 89). These clarified the nature of the final complex formed

Ligand	Function
AI domain of von Willebrand factor	Critical in platelet binding to exposed sub-endothelium or activated endothe- lium
Thrombin	Binding to GPIb has a major role in pla- telet responses to low concentrations of thrombin (presentation to PAR-1?)
Mac-I	Platelet binding to macrophages. Removal of "chilled" platelets from the circulation
P-selectin (CD62P)	Critical in platelet binding to activated endothelium or activated leukocytes
FXI, FXII	Critical roles in amplification of pro- coagulant cascade
High-molecular-weight kininogen	Supports GPIb interaction with Mac-I
Thrombospondin-I	Can replace von Willebrand factor in pla- telet binding to small vessel subendothe- lium at high shear
Collagen	Indirectly via A1 and A3 domains of von Willebrand factor
Soluble GPVI	May affect platelet responses to collagen

Table 1: Platelet GPlb ligands and their function.

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between these two molecules and how the binding is regulated, but still leave a number of questions unanswered. These structures were quickly followed by those of complexes of the outer domain of GPIb α with thrombin which revealed a lot of new information but also raised a lot of new questions (90, 91). These structures supported earlier suggestions that thrombin bound to GPIb α might be able to interact with other GPIb α molecules on the same or other platelets, or with PAR1 or GPV.

More light was shed on how vWF/GPIb interactions cause platelet rolling by the discovery that this occurs via the formation of "tethers", long, pseudopod-like, membrane structures that are drawn out from the platelet surface following contact between a platelet and a vWF-coated surface (92, 93). Evidently, this involves a patch of GPIb molecules linked to the cytoskeleton. "Tether" formation ends with the slowing of the platelet, with the breaking of the "tether", or with the tether detaching from the surface. This process may also be involved in microparticle formation.

Recent years have shown an increased importance being given to GPIb in platelet function. It was well-known that platelets that had been chilled before transfusion were rapidly eliminated, and this is why platelet concentrates have to be kept warm. However, this naturally gives problems with bacterial growth and restricts the length of time that platelet concentrates can be stored to five days. Thus, studies showing that GPIb is clustered during chilling and that this has a critical role in platelet elimination because $\alpha M\beta 2$ -integrin on macrophages recognises exposed β -N-acetylglucosamine on GPIb (94). This has led to efforts to increase glycosylation of platelets in order to cover this sugar residue as a method to allow platelet concentrates storage at lower temperatures (95).

Recent years have shown an intensified interest in activation mechanisms and signalling pathways involving GPIb, and one question that has arisen is what role lipid rafts play in GPIb function. It seems that only a fraction of GPIb molecules are present in lipid rafts and these may well be the acylated ones which are therefore more hydrophobic (96). Calmodulin was shown to be a cytoplasmic component interacting with the GPIb complex and, perhaps, regulating proteolytic cleavage of GPIb α and GPV (97).

Evidence has been found that soluble GPVI and glycocalicin can bind together suggesting functional interactions between these molecules (98). Thrombospondin-1 was shown to be yet another ligand for GPIb, particularly in smaller vessels where vWF is less expressed (99). Other recently demonstrated ligands include factor XI (100), factor XII (101), and kininogens (102, 103).

Recent years have seen many studies using in-vivo models of haemostasis and thrombosis to examine the role of the individual components of plasma, platelets and endothelial cells in physiological and pathological processes. Depending on the type of injury inflicted and which ligands are exposed, receptors will be implicated to different degrees. It is nevertheless impressive to see the role that GPIb plays in many of these models (104). Following the failure of α IIb β 3 inhibitors to reduce cardiovascular disease in a preventative context, there has been a lot of interest in alternative strategies. GPIb has been suggested as a target in many studies indicating that it may be possible to use GPIb antagonists, such as small antibodies or similar molecules, to prevent thrombosis without having an undue effect on haemostasis. Why this should be is not completely clear, and the increasing number of GPIb ligands would suggest that this is counterintuitive. However, perhaps targeting the specific ligands rather than GPIb itself might be an alternative strategy and allow more flexibility.

Each year more publications on GPIb appear, but that does not change the fact that there is still a lot about this fascinating molecular complex that remains unknown. Despite intensive research and many interesting publications over the past few years we still understand only very poorly how clustering GPIb leads to platelet activation. Improving this is a major target for the coming decade. Some recent excellent reviews have addressed this area (105–107). On the structural side, recent studies (30) demonstrate that there are two GPIb β molecules linked to each GPIb α and not one, showing how much progress we need to make in studying the complex as a whole. There are still major technical hurdles to cross in order to solve these problems, but new methodologies may allow us this possibility.

The question whether the GPIb complex is expressed in other cells, such as endothelial cells and tumour cells, and what its role there is, which was once of intense interest, seems to have slipped into the background. New information may well bring it to the foreground again. There are undoubtedly more GPIb ligands than have been described so far, and the list will continue to lengthen.

This short review, describing how knowledge of one particular receptor complex has developed over nearly 60 years, has necessarily concentrated on only some aspects. I apologise to those colleagues whose work has not been fully addressed, due to lack of space, which also limited reference to their work. Several earlier reviews should be consulted for more detailed references and to fill these gaps.

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