

## Anniversary Issue Contribution

# Platelet membrane glycoproteins: A look back into the past and a view to the future

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To contribute to this Anniversary Issue of *Thrombosis and Haemostasis* is a great pleasure for me, not in the least because first as *Thrombosis et Diathesis Haemorrhagica* and subsequently under its current name, this journal has such an important place in the history of our discipline. I well remember meeting Dr. Rosemary Biggs, editor of the journal, early in my days as an assistant at the Sir William Dunn School of Pathology in Oxford (1968–1970). Dr. Biggs worked in Oxford, a town with a history of pioneering studies on thrombosis and coagulation. At this time, Professor R. G. Macfarlane, author of the 'Cascade' hypothesis of coagulation, was nearing the end of his career at the Dunn School where the bulk of the research in haemostasis and thrombosis was being led by Professor John French, the then Reader in Pathology. An electron microscopist studying animal models of experimental thrombosis and atherosclerosis, John French and his team published in 1970 some of the first images showing the fine details of platelet attachment and spreading on subendothelium (1).

### Early personal recollections

In 1968, Olaf Behnke (2) published some fundamental observations to which I referred earlier in a previous manuscript on the history of platelet glycoproteins (3) and which I will touch on again here. Behnke was also an electron microscopist and he showed, I think for the first time, that blood platelets possessed an amorphous surface coat extending some 150–200 Å that was called the 'glycocalyx'. Behnke showed that preservation of the coat was much improved in the presence of the cation dye, alcian blue, and that it was further stained by agents such as colloidal iron and ruthenium red. Acidic mucopolysaccharides were the most likely contributors to this layer, although a loss of staining after treatment with proteases also indicated a protein component. John French and his staff were impressed by this work and adopted a similar approach. An image from their work is shown in Figure 1A. Here, colloidal iron has clearly labelled the plasma membrane and certain elements of the surface-connected

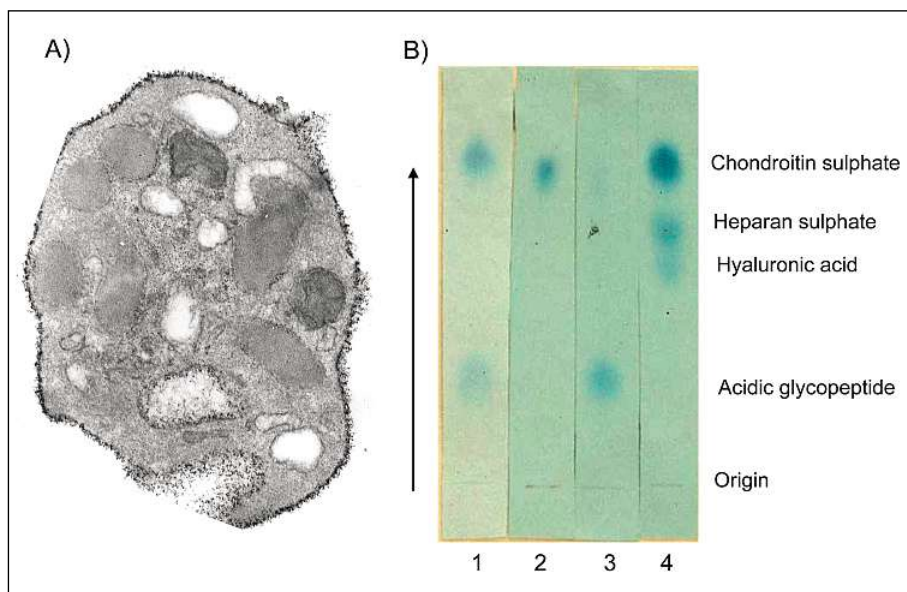
canalicular system (SCCS) showing that these were truly connected with the surface. The alpha-granules were not labelled. So, how were the components of the platelet glycocalyx to be identified? Aaron Marcus and his co-workers in New York analyzed the sialic acid-containing gangliosides and showed that they constituted 0.5% of the platelet lipids and accounted for 6% of platelet sialic acid, a major component of the platelet surface (4). It was concluded that the orientation and specific distribution of platelet membrane glycolipids could be important determinants of the unique surface properties of platelets.

The first biochemical evidence for the presence of glycoproteins on platelets came from Graham Jamieson's group in Washington. An elegant series of studies analyzed in fine detail the carbohydrate composition of glycopeptides released from both platelets and isolated platelet membranes by proteases such as trypsin, papain and pronase (5–7). Included in this work was the first recognition that platelets contained in their glycocalyx a sialic acid-rich macroglycopeptide, a major contributor to the platelet surface charge. My first approach in Oxford was to subject lyophilised platelets to extensive proteolytic digestion and to analyze the products by electrophoresis on cellulose acetate membrane. I tested the agents used by Behnke in electron microscopy as potential stains. Alcian blue proved to be an excellent reagent for visualizing charged components of platelets. Figure 1B shows that two classes of components were detected in platelets. The first co-migrated with chondroitin 4- or 6-sulphate standards, while the second was no longer seen when platelets were incubated with neuraminidase, an enzyme that releases negatively charged sialic acid residue present in a terminal position on many glycoproteins and the gangliosides. Note that heparan sulphate and hyaluronic acid were not present, and that the acidic glycopeptide was resistant to enzymes that cleaved mucopolysaccharides. Elution of the bands and analysis on polyacrylamide gels subsequently showed that the large chondroitin sulphate molecules failed to enter the gels, whereas the second component migrated readily and almost certainly corresponded to the macroglycopeptide (8). While the chondroitin sulphates

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**Figure 1: Studies from the late 1960s showing the presence of charged groups on the platelet surface.** A) Electron micrograph of a platelet that had been labelled with colloidal iron (given to ATN by the late John French). Note the heavy labelling of the surface and elements of the SCCS. B) Results of electrophoresis of samples of delipidated and lyophilised platelet proteins that had been subjected to protease digestion. Charged molecules were located with 1% alcian blue at acid pH. Shown are results for 1) the platelet digest or platelet proteins pre-treated 2) with neuraminidase, 3) with a mixture of hyaluronidase and chondroitinase enzymes and 4) a mixture of chondroitin-4 and -6 sulphate, heparan sulphate and hyaluronic acid standards. The acidic glycopeptide was subsequently shown to correspond to the GPIb-derived sialic acid-rich macroglycopeptide.

are known to be present in alpha-granules, their possible contribution to the surface properties of platelets has never been totally excluded.

### SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A major advance came with the development of SDS-PAGE. This technique allowed the separation of intrinsic glycoproteins following the solubilisation of platelets, or isolated membranes, by the ionic detergent SDS. Electrophoresis in the presence of the detergent allowed the separation of proteins according to their size largely due to their capacity to bind the detergent. When SDS-PAGE was applied to the study of platelets, three principal glycoprotein bands termed GPI, II and III were identified by carbohydrate staining using the periodate-Schiff (PAS) reaction. Here, Ralph Nachman in New York and David Phillips in Memphis were the pioneers (9–11). The technique became even more powerful when associated with radiolabelling of the surface proteins of platelets by lactoperoxidase-catalyzed iodination (described by David Phillips in [3]). In this procedure, lactoperoxidase, a 90 kDa enzyme unable to penetrate the plasma membrane of cells, was used to catalyze the incorporation of  $^{125}\text{I}$  into tyrosine residues at the platelet surface. In fact GPII (later termed GPIIb) and GPIII (later termed GPIIIa), were the major  $^{125}\text{I}$ -labeled constituents.

### Glanzmann thrombasthenia (GT) and the Bernard-Soulier syndrome (BSS)

The question arose as to how to identify the functional elements of the platelet surface. Why were platelets but not red cells able to aggregate and form a thrombus in response to a defined stimulus? The logic was that platelets possess surface components that specifically mediate platelet aggregation and platelet adhesion.

In France, Jacques Caen and his group were specializing in studies of patients with inherited disorders of platelet function (12). GT is the major genetically transmitted disorder of platelets. It is characterized by a total inability of platelets to aggregate in response to all physiologic agonists. Not only is aggregation affected, in most patients clot retraction is also absent although occasionally it is partial or subnormal. Fibrinogen and calcium had already been identified as essential cofactors of ADP-induced human platelet aggregation (13). It seemed to me that if ever there was a disease that would give essential clues on the role of glycoproteins in platelet aggregation then GT was it, and Jacques Caen was of the same opinion. So, this led me to Paris and in 1973 I examined GT platelets 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 GPII (GPIIb) and GPIII (GPIIIa) were clearly absent (Fig. 2) (14, 15). Three patients were examined and they all gave the same abnormal pattern. So a link between membrane glycoproteins and platelet aggregation was established.

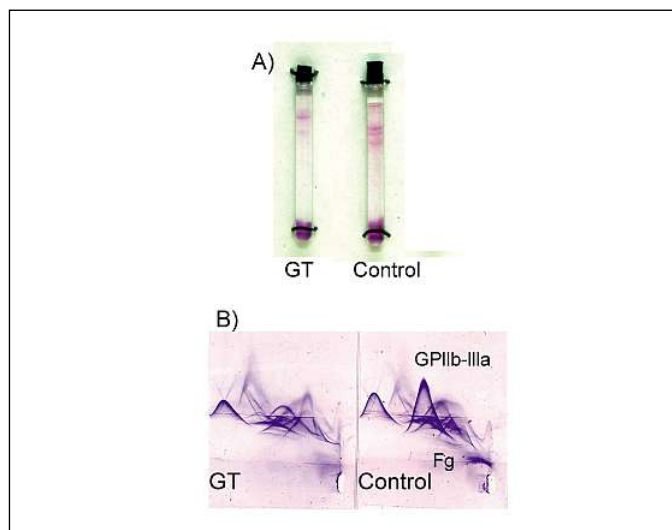
This result did not answer the question of the role of the sialic acid-rich GPI (GPIb). In the BSS, giant platelets and thrombocytopenia were associated with decreased surface sialic acid and the inability of the platelets to interact with bovine factor VIII (containing VWF) or with ristocetin in the presence of plasma (16–18). It appeared logical to also study glycoproteins of these platelets, and a second visit to Paris led to the discovery that, in BSS, platelets lacked both the alcian-blue staining macroglycopeptide and GPI (GPIb), while GPII (GPIIb) and GPIII (GPIIIa) were present. Rapidly it was established that BSS platelets exhibited a defective adhesion to subendothelium, and this discovery led to the identification of the GPIb-VWF interaction (19, 20). Meanwhile, studies using lactoperoxidase-catalysed  $^{125}\text{I}$ -labeling had clearly and elegantly both confirmed and extended the specificity of the GT platelet surface abnormality on single-dimension gels (21).

## Two-dimensional electrophoresis

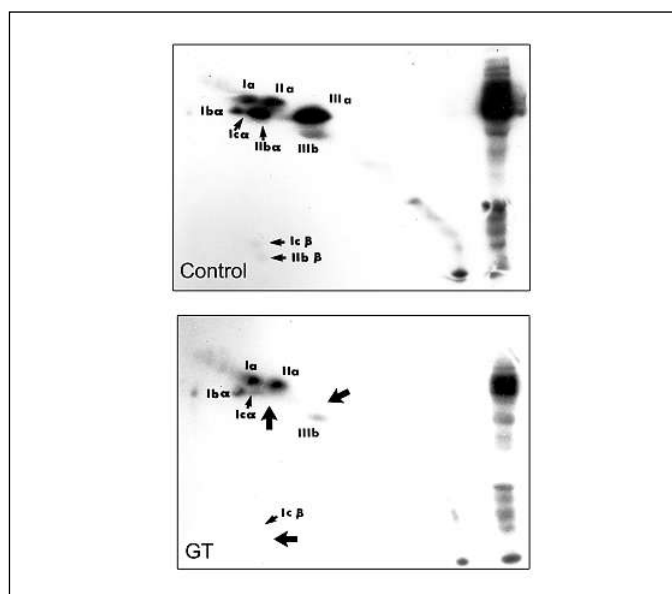
In 1979, Inger Hagen and her colleagues in Oslo applied crossed immunoelectrophoresis (CIE) to the study of platelets, and she applied the procedure to GT and BSS platelets in a stay with us in Paris (22, 23). This procedure involved the separation by electrophoresis in agarose gels of proteins solubilised from platelets with a non-ionic detergent, followed by a second dimension electrophoresis into an agarose gel containing immunoglobulins isolated from the sera of rabbits immunized with normal human platelets. Quite a complex pattern of immunoprecipitates was seen on Coomassie blue (C-B) staining and comparison of the patterns obtained for platelets from GT and BSS patients showed that different immunoprecipitates were lacking, thereby confirming the specific nature of the glycoprotein deficiencies in these disorders. One of the early CIE gels from this period showing the results for normal and GT platelets is illustrated in Figure 2. At this time, Tom Kunicki came from Milwaukee to join us in Paris; the CIE studies were continued, and the platelet antigens giving rise to the bulk of the immunoprecipitates were identified. Most importantly, it was recognized that GPIIb and GPIIIa colocalized to the same immunoprecipitate and that they existed in the Triton X-100 soluble extracts of platelets as  $\text{Ca}^{2+}$ -dependent complexes (24, 25). The trail of the  $\alpha\text{IIb}\beta 3$  integrin had begun. Important to note here were studies on the IgG L., an isoantibody isolated from a polytransfused Glanzmann's patient that both bound to GPIIb-IIIa when incorporated in an intermediate gel during CIE, and inhibited the aggregation of control platelets (25).

The first two-dimensional SDS-PAGE procedure to be applied to platelets involved migrating SDS-soluble platelet membrane glycoproteins in a first dimension rod-gel, and reducing disulphides prior to a second dimension electrophoresis across a slab gel as described by Phillips and Poh Agin (26). Reducing disulphides resulted in the separation of the small light or beta-chains from the heavy or alpha-chains or the loss of intra-molecular disulfides of some membrane glycoproteins altering their mobility. Not in the least, this procedure was instrumental in showing that the GP composition of the platelet surface was more complicated than first thought, with GPIa, GPIc and GPIV (or GPIIb) being identified. When combined with lactoperoxidase-catalysed  $^{125}\text{I}$ -labeling, this procedure was excellent for showing the defects in GT platelets (26). An illustration from my work is shown in Figure 3. It also clearly illustrated the specificity of the BSS platelet surface defect despite GPIb $\alpha$  being relatively poorly labeled (27). The introduction of isoelectric focusing in the first dimension provided a much better separation of both membrane glycoproteins and cytosolic proteins and this is shown for a typical GT patient in Figure 4. On close inspection, a series of vertical parallel spots given by the fibrinogen  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains is also seen to be missing from the GT platelet pattern. This was proteomics as performed in 1981.

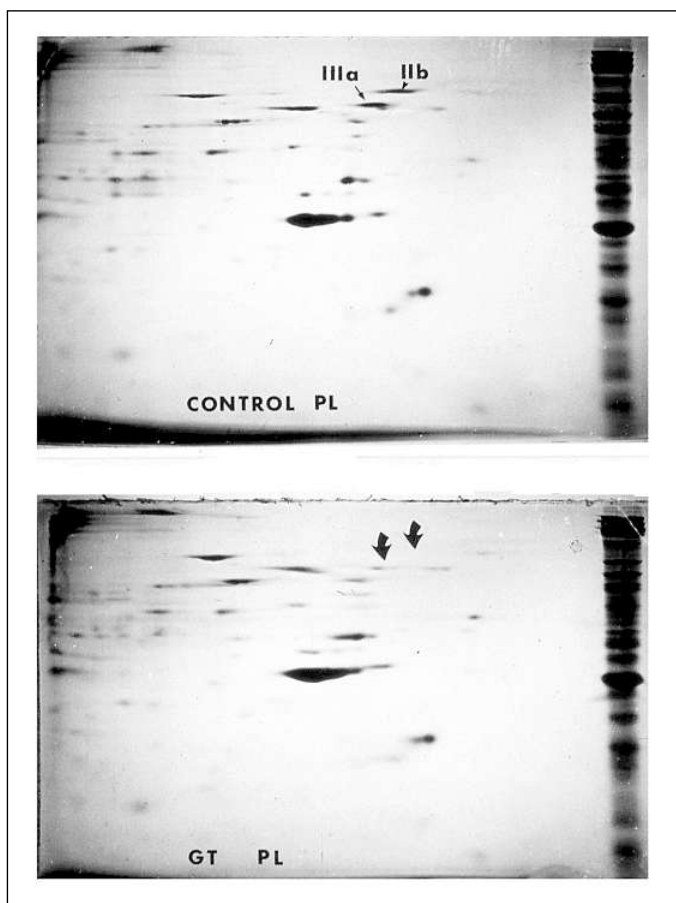
Ken Clemetson's group was instrumental in applying high-resolution two-dimensional gel electrophoresis to platelets (28). They were also among the first to use the incorporation of  $^3\text{H}$  into subterminal galactose residues of membrane glycoproteins and this proved ideal for visualizing highly glycosylated GPs such as GPIb. This procedure involved removal of the terminal sialic



**Figure 2: Initial studies on the characterization of the GPIIb-IIIa abnormality in GT patients.** A) One of the original PAS-stained gels from my stay in Paris in 1973. Note that 3 PAS-stained bands were clearly seen for normal platelets, while only the first band (GPI or GPIb) was normally present on the GT platelet profile. Reproduced from [14] with the publisher's permission. B) C-B stained CIE gels showing the predominant GPIIb-IIIa precipitate on the normal platelet pattern. Note its absence from the GT platelet pattern. This specific loss was accompanied by a parallel decrease in the immunoprecipitate given by fibrinogen (Fg). (Reproduced from [23] with the publisher's permission.) These studies were performed on platelet proteins extracted into Triton X-100 and electrophoresed in the second dimension against the isolated immunoglobulins of a rabbit antibody to normal human platelets.



**Figure 3: Mapping of the surface composition of platelets using two-dimensional non-reduced/reduced SDS-PAGE.** The surface proteins of washed platelets were labelled with  $^{125}\text{I}$  by lactoperoxidase-catalysed iodination. Platelet proteins were first separated by rod gel electrophoresis without disulphide reduction. Disulfides were then reduced within the rod gels that were then over-layered onto a slab prior to the second dimension electrophoresis (26, 27). Radiolabelled proteins were detected by autoradiography. Note the specific absence of the GPIIb heavy and light chains and GPIIIa from the GT pattern.

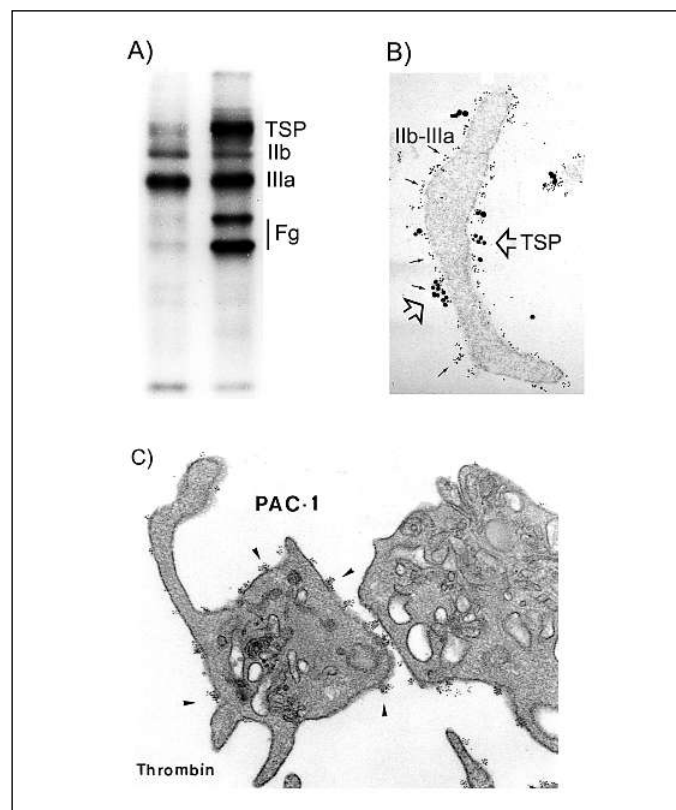


**Figure 4: Proteomic analysis of platelet proteins in the early 1980s.** Platelet proteins were solubilised using a non-ionic detergent and separated by isoelectric focusing in rod gels prior to separation after disulfide reduction by SDS-PAGE in the second dimension. C-B stained gels are shown. Note the clear absence of the spots given by GPIIb and GPIIIa. To the left of the gel, three vertically aligned spots corresponding to the fibrinogen  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains are also missing.

acid residues from the oligosaccharide chains by neuraminidase treatment, oxidation of the exposed galactose residues using galactose oxidase, and then reduction of aldehyde groups using sodium ( $^3\text{H}$ )-borohydride.

## How platelets aggregate

Fibrinogen was shown to bind to GPIIb-IIIa on ADP-stimulated platelets in a saturable and highly specific manner (29, 30). Monoclonal antibodies (MoAbs) to GPIIb-IIIa inhibited platelet aggregation by preventing fibrinogen binding to GPIIb-IIIa on activated platelets (see [31]). Yet, there were early indications that the aggregation mechanism was not so straightforward as a fibrinogen-dependent simple crosslinking of GPIIb-IIIa complexes on activated platelets. Kent Gartner et al. postulated that thrombin-induced platelet aggregation was mediated by a membrane-bound lectin (32). This proved to be thrombospondin-1 (TSP-1), a secreted alpha-granule protein, a finding nicely confirmed by Phillips et al. who performed lactoperoxidase-catalyzed  $^{125}\text{I}$ -labelling of platelets during thrombin-induced platelet activation (33). An example of this procedure from my work is



**Figure 5: Surface components involved in thrombin-induced platelet aggregation.** A) Washed platelets in a  $\text{Ca}^{2+}$ -containing buffer were incubated in the absence (left gel) or with thrombin (right gel) in the presence of  $^{125}\text{I}$  and lactoperoxidase. As well as GPIIb and GPIIIa, TSP and fibrinogen (fibrin) were major surface constituents suggesting that they contribute to platelet aggregation. B) Thrombin-stimulated platelets were examined by electron microscopy after double immunolabelling of platelets for GPIIb-IIIa with the IgG L. (small gold beads) and for TSP with a rabbit antibody (large gold beads) (36). Note that beads visualising GPIIb-IIIa (arrows) are distributed over the section shown here, while those labelling TSP are in distinct clusters (open arrow heads). C) Slowly stirred platelets were incubated with thrombin prior to the addition of PAC-1, a murine IgM antibody to an activation-dependent epitope on GPIIb-IIIa. After fixation, PAC-1 was detected with an anti-IgM attached to gold beads. Note the visualisation of PAC-1 in clusters (arrow heads) and the presence of labeled bridges between adjacent platelets.

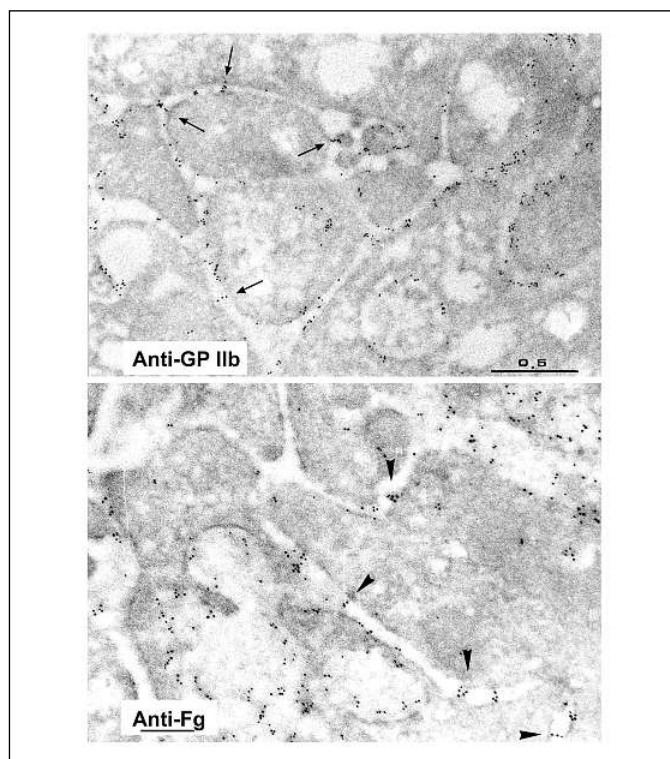
shown in Figure 5A, where clearly, secreted fibrinogen (fibrin) and TSP-1 predominate on the activated platelets. Fibronectin is another potential secreted aggregation-cofactor from platelets (34) as is VWF, although TSP-1 differs from other adhesive protein cofactors of aggregation in that there is not a significant plasma pool (35). Interestingly, when TSP-1 and to a lesser extent secreted fibrinogen are localized on thrombin-activated platelets by immuno-electron microscopy, they appear to be present as clusters (36). This is illustrated for TSP-1 in Figure 5B, an electron micrograph from one of my early collaborations with Paquita Hourdillé in Bordeaux. When platelets were stimulated with ADP in the presence of soluble fibrinogen, the clusters were smaller although still present (36). So, do fibrinogen and TSP-1 (and perhaps other adhesive proteins) co-localise to form specific focal adhesive contacts that consolidate the aggregate?

When secretion occurs, we had already shown in Paris that alpha-granule-stored adhesive proteins are secreted together and that some can be surface localized already attached to membrane receptors (37). PAC-1 is a murine IgM that specifically recognizes an activation-dependent configuration on GPIIb-IIIa (38). Addition of PAC-1 to platelets stimulated by thrombin in slowly stirred platelet suspensions showed clearly that platelet-to-platelet bridges were labelled with PAC-1 implying that these structures contained GPIIb-IIIa molecules both occupied and unoccupied by adhesive proteins (Fig. 5C). Thus platelet aggregation, at least by thrombin, can involve both high-affinity fibrinogen binding and clustering of occupied and unoccupied integrin with the possible participation of other adhesive proteins.

After my transfer to Bordeaux in 1985, Eric Heilmann and Paquita Hourdill  used electron microscopy to confirm that protein bridges are found within thrombin-induced platelet aggregates formed under the shear conditions of a platelet aggregometer (39). Immunogold labelling again showed focal attachments and that GPIIb-IIIa, fibrinogen and VWF co-localised to these structures. This is illustrated for both GPIIb-IIIa and fibrinogen in Figure 6. All in all, it appears that the strength of the adhesive forces depends on the multivalent interactions that are necessary for bridge formation.

## The present and the future

Mainly through the outstanding efforts of Barry Coller (40), anti-GPIIb-IIIa antagonists arrived in the clinic and have since played a major role in saving lives of patients with acute coronary syndromes. Although they may participate in ‘dispersing’ platelet thrombi, they mainly act by blocking unoccupied surface GPIIb-IIIa and preventing platelet aggregation and platelet incorporation into a growing thrombus. But these antagonists do not work all of the time. Are there other mechanisms by which platelets attach together? Although it was quickly established that GPIb , by binding to surface-exposed VWF, was a necessary ligand for platelet adhesion at all shear rates, only recently has it also been shown to take part in thrombus formation at high shear by participating in platelet-to-platelet cohesion (41). The role of plasma cofactors other than fibrinogen is not clear, although by using different transgenic mouse models knocked-out for one or more adhesive proteins, Denisa Wagner and her colleagues are proceeding along a logical path towards solving this problem (42). The fine structure of the GPIIb-IIIa complex ( $\alpha$ IIb 3 integrin) is now known from crystal structures, and it has been determined that changes in affinity states parallel straightening from a bent position (43). The change in affinity of GPIIb-IIIa that brings about adhesive protein binding results from a cascade of signalling pathways originating from primary receptors that converge on the cytoskeletal protein, talin. It is the interaction of the phosphotyrosine-binding domain of talin with the GPIIIa cytoplasmic domain that causes activation (44). When occupied GPIIb-IIIa complexes are in contact with their counter-receptors on aggregating platelets, a new wave of signalling termed ‘outside-in signalling’ results in the activation of additional signalling pathways within the platelet, and this is important for thrombus stabilization (45, 46). How these pathways strengthen platelet-to-platelet bonds is not clear. It is now known from several studies that platelet-to-pla-



**Figure 6: Visualisation of protein bridges within a thrombin-induced platelet aggregate.** Immunogold labelling of lowicryl-embedded sections was performed as described (39). Shown are sections stained with a rabbit antibody to GPIIb (upper panel) and a rabbit antibody to fibrinogen (lower panel). Both GPIIb-IIIa (arrows) and fibrinogen (arrow heads) are seen in bridges joining the surfaces of adjacent platelets. (Reproduced from [39] with permission of the publisher.)

telet contacts brought about by the initial fibrinogen binding include secondary interactions between other proteins on the surfaces of opposing platelets. These include proteins that have only recently been recognized to be platelet components such as ephrins and eph kinases, SLAM family proteins and semaphorin 4D (47–49). How these proteins contribute to aggregate stability is unknown; also unknown is whether they colocalise to the protein bridges shown linking platelets in Figure 5. It is clear that the anti-GPIIb-IIIa blocking drugs that are currently being used, abciximab, eptifibatide, and tirofiban, may be difficult to improve upon in terms of their ability to prevent fibrinogen binding. But the development of a drug that will reverse the GPIIb-IIIa clasp, releasing fibrinogen and other ligands including the reversal of the secondary interactions, may give a more rapid restoration and maintenance of blood flow in acute situations either when used alone or in combination with existing agents. This, to me, is the way forward.

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