MINIREVIEW

On the Structure and Function of Platelet Integrin $\alpha_{IIb}\beta_3$, the Fibrinogen Receptor (43863A)

JUAN J. CALVETE[1]

Instituto de Química-Física "Rocasolano" C.S.I.C., Madrid, Spain and Institut für Reproduktionsmedizin, Tierärztliche Hochschule Hannover, Germany

Abstract. **Platelet membrane glycoprotein (GP) IIb/IIIa ($\alpha_{IIb}\beta_3$), a Ca^{2+}-dependent heterodimer, serves as an inducible receptor for fibrinogen and other adhesive plasma proteins, and is the most thoroughly studied integrin receptor. Intensive research during the past several years has elucidated the major features of its biosynthetic pathway, covalent structure, domain organization, and topography, and we are beginning to get an insight into the cellular mechanisms controlling integrin function. The emerging picture indicates that platelet-specific elements initiate at the cytoplasmic domains of GPIIb/IIIa a signal that leads to conformational changes within the integrin's extracellular domains and expression of the fibrinogen receptor. The simultaneous occupancy on adjacent platelets of receptors with dimeric fibrinogen molecules leads to platelet aggregation. Further structural alterations promote clustering of occupied GPIIb/IIIa complexes and their attachment to the remodeling cytoskeletal network. This interaction provides the physical link for clot retraction to occur and appears to regulate the compartmentalization, and local activation, of a multienzymatic complex which translates the ligand-binding information into time-dependent irreversibility of the fibrinogen-GPIIb/IIIa interaction. Platelet GPIIb/IIIa plays, thus, a central role in thrombus formation both in health and disease: abnormalities in the platelet adhesive mechanisms responsible for the formation of the hemostatic plug, lead to major pathophysiologic disorders, ranging from severe bleeding to thrombosis. It is, therefore, not surprising that GPIIb/IIIa has been the subject of intensive research during the last decades, since a detailed knowledge of the molecular biology and the mechanism underlying the platelet activation and aggregation processes may aid in the rational design of both an effective gene replacement therapy, and of potent and specific anti-thrombotic drugs. The aim of this minireview is to summarize many functional and structural data from different laboratories in the perspective of an emerging model that may help us to understand structure-function relationships of GPIIb/IIIa and of other members of the integrin family.** [P.S.E.B.M. 1995, Vol 208]

[1] To whom requests for reprints should be addressed at Institut für Reproduktionsmedizin, Tierärztliche Hochschule Hannover, Bünteweg 15, 30559 Hannover-Kirchrode, Germany.

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Identification of GPIIb/IIIa as the Platelet Fibrinogen Receptor: Historical Background

The observation made 20 years ago that two platelet surface glycoproteins, GPIIb (135 kDa) and GPIIIa (95 kDa) were absent, or their content greatly diminished, in platelets from patients with Glanzmann

thrombasthenia, a rare autosomal recessive congenital bleeding disorder characterized by the absence of macroscopic platelet aggregation in response to all physiologic stimuli, provided the first evidence for assigning a platelet function to specific platelet membrane components (reviewed in Ref. 1). Furthermore, a correlation was established between the failure of thrombasthenic platelets to aggregate and their inability to bind plasma fibrinogen (340 kDa) (2), an essential cofactor for platelet aggregation which binds to a single class (Kd, dissociation constant = 10^{-7} *M*) of stimulation-inducible, specific, and saturable (40–80 × 10^3 binding sites/platelet) receptor (reviewed in Ref. 3). Altogether, these evidences suggested a role for GPIIb and/or GPIIIa as part of the platelet fibrinogen receptor system.

Subsequently, it was shown that GPIIb and GPIIIa form a 1:1 Ca^{2+}-dependent heterodimer both in solution and in the plasma membrane of intact resting platelets (reviewed in Ref. 4 and 5). Further studies from different laboratories supported the concept that the GPIIb/IIIa complex is the platelet fibrinogen receptor (consult Ref. 6 and references therein). Among them, (ii) a direct interaction between GPIIb/IIIa and fibrinogen was demonstrated using either an enzyme-linked immunosorbent assay (ELISA), crossed-immunoelectrophoresis, or photoaffinity labeling of fibrinogen to activated platelets; (ii) several monoclonal antibodies against GPIIb and/or GPIIIa inhibited the binding of fibrinogen to activated platelets; (iii) purified glycoprotein IIb/IIIa reconstituted in liposomes bound fibrinogen with an affinity constant similar to that determined when whole platelets or isolated platelet membranes were used; and (iv) recombinant GPIIb/IIIa was expressed in mammalian cells as a functional fibrinogen-binding complex.

Though mounting evidence indicates that the fibrinogen receptor activity is an intrinsic property of the quaternary structure of GPIIb/IIIa, when removed from the intact platelet or expressed in a nonplatelet environment, GPIIb/IIIa does neither retain the ability to bind stoichiometric quantities of soluble fibrinogen. Furthermore, although the fibrinogen-binding capability of GPIIb/IIIa can be triggered by interaction with certain anti-GPIIb/IIIa antibodies (7) or soluble small RGD-containing peptides (8), it cannot be induced in an agonist-mediated manner as observed in activated platelets (reviewed in Ref. 9). This strongly indicates that physiologically the binding function of GPIIb/IIIa may be modulated by specific platelet moieties (see below).

The stability of the noncovalent association between GPIIb and GPIIIa depends on the presence of submicromolar extracellular calcium concentration (10), and the heterodimeric form is necessary for the expression of fibrinogen receptors (11). GPIIb/IIIa is a major platelet integral plasma membrane protein that accounts for approximately 3% of the total platelet proteins (i.e., 17% of the platelet membrane protein mass); on average, a normal human platelet contains around 100,000 copies of GPIIb/IIIa complexes (12), 80% of which are randomly distributed on the external platelet plasma membrane (13). The other GPIIb/IIIa pool is cryptic and located in invaginations of the plasma membrane (SCCS, surface-connected canalicular system) and in the inner membrane of the α-granules, but becomes surface available, and fibrinogen binding competent, upon platelet activation and shape change from a disc to a spiny sphere with many filopodia, and fusion of the α-granule membrane with the platelet surface (reviewed in Ref. 14).

Glycoprotein IIb/IIIa is a Member of the Integrin Family

Immunological work in the mid-1980s demonstrated the puzzling presence of proteins immunologically related to platelet GPIIb/IIIa in a variety of human cells (15). Elucidation of the amino acid sequences of GPIIb (16) and GPIIIa (17) from cDNA clones led to the realization that GPIIb/IIIa is actually a member of a large, widely cell-distributed family of structurally, immunologically, and functionally related cell membrane adhesion receptors (18). Collectively, they are termed, "integrins," to highlight the role of these receptors in integrating extracellular ligand binding information with intracellular cytoskeleton-mediated events (19).

Integrins are heterodimers composed of one α and one β transmembrane subunit. Up to now, eight β-chains (β_{1-8}) and 15 α-chains (α_{1-9}, α_{IIb}, α_v, α_L, α_M, α_X, α_E) have been identified in human cells, which can pair to form at least 20 distinct heterodimers (6, 19–21). Although there is no structural relationship between both subunits, any pair of α or β subunits share 35%–60% amino acid sequence identity. Depending of the cell type, the α and β subunits associate in various combinations, and more than one integrin is often expressed on individual cells (19). The variety of heterodimer combinations, together with the expression of alternate forms of cytoplasmic domains (22–24 and references cited), post-translational modifications (25, 26), and the existence of multiple affinity states for many integrins (9), contribute to the ligand-binding specificity of the heterodimer and provide cells with the potential to regulate integrin expression and function and to express, along the cell life, a wide variety of adhesion phenotypes with varied capabilities to mediate cell-cell and cell-matrix interactions (reviewed in Ref. 19, 27–33).

The importance of integrins in many biological processes have brought many investigators with diverse backgrounds (cell biology, molecular biology, protein chemistry, etc.) to the field, and led to an ex-

plosion of work done on integrins during the last years. Platelet GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) is probably the most thoroughly characterized integrin. It shares the β subunit with the widely distributed vitronectin receptor (integrin $\alpha_V\beta_3$), and together constitute the cytoadhesin, or β_3, family (34).

Biosynthesis and Expression of Integrin $\alpha_{IIb}\beta_3$

The biosynthesis of integrin $\alpha_{IIb}\beta_3$ has been examined in detail in human megakaryocytes, the human erythroleukemic (HEL) cell line, and COS-1 cells (reviewed in Ref. 6 and 35). The genes for GPIIb and GPIIIa are physically linked on the q21–22 region of the long arm of human chromosome 17. However, the differential pattern of tissue expression for GPIIb (which only pairs with GPIIIa and its expression is normally restricted to platelets and their bone marrow precursor cells, the megakaryocyte) and GPIIIa (which forms with α_V the widely distributed integrin $\alpha_V\beta_3$) suggests that independent factors control gene transcription. Like those of other integrins, both the α_{IIb} and β_3 promoters lack canonical consensus TATA and CAAT cis-acting elements but have binding sites for the ubiquitous transcription factor Sp1 (36, 37 and references cited).

The gene coding for GPIIb spans 17.2 kb, contains 30 exons and exist as a single copy in the human genome. The information for tissue-specific expression has been localized in a region extending from nucleotides -460 to -439 of the GPIIb promoter. The gene for GPIIIa spans 46 kb and is divided into 14 exons. The promoter of the avian β_3 subunit gene contains a classical vitamin D response element (36). Although no typical consensus sequence for transcription start have been identified, the 5′ region of the β_3 gene, which contains a stretch of 145 nucleotides immediately upstream of the ATG initiation codon that are GC-rich and similar to those found in the promoter sequences of other TATA-less genes, has been proposed to play a functional role in the initiation of transcription (37).

Human GPIIb and GPIIIa are synthesized as single-chain precursors to which N-linked mannose-rich oligosaccharides are cotranslationally added in the endoplasmic reticulum. A 5-fold excess of pre-GPIIb is synthesized relative to GPIIIa. Efficient surface expression of platelet GPIIb/IIIa requires both subunits and takes 4–6 hr for completion. All GPIIIa molecules are assembled into heterodimers, while the excess of pre-GPIIb is intracellularly degraded. Nascent GPIIb and GPIIIa contain "retention signals" located in the extracellular domains of the former and within the transmembrane or cytoplasmic moieties of the latter. Formation of a GPIIb/IIIa heterodimer abrogates intracellular retention (38). A similar processing has been reported for human integrin $\alpha_1\beta_1$ (39).

Following assembly, the heterodimers are rapidly transported to the Golgi apparatus where the oligosaccharide chains of pre-GPIIb, and some carbohydrate chains of pre-GPIIIa, are processed into the complex-type, O-linked oligosaccharides are added to pre-GPIIb, and its polypeptide chain undergoes proteolytic cleavage to yield mature GPIIb (136 kDa) made up of disulphide-linked heavy (GPIIbH, 114 kDa) and light (GPIIbL, 22 kDa) chains (6). Cleavage of pre-GPIIb/IIIa seems to be important for maturation into a functioning receptor, since expression of uncleaved GPIIb message produces an abnormal GPIIb/IIIa and a platelet thrombasthenic phenotype (40 and references therein).

The extracellular part of GPIIbL might be required for the efficient assembly and secretion of GPIIb/IIIa since a construct of the extracellular GPIIb/IIIa domains is secreted (38), whereas whole GPIIbH coexpressed with the extracellular part of GPIIIa is not secreted (41). In addition, the point mutation GPIIIa $Arg^{216} \rightarrow$ Gln (42) completely abolishes surface expression of GPIIb/IIIa. Since these two regions of the GPIIb/IIIa complex form part of the heterodimer subunit interface (43) (Fig. 2), it follows that alterations within subunit contacts may critically destabilize the correct conformation for assembly and/or surface transport.

The importance of the structural integrity of the Ca^{2+}-binding sites of GPIIb and the disulphide bridge pairing of both subunits of GPIIb/IIIa (Fig. 1) for surface expression of the heterodimer is underscored by the findings that the following structural alterations result in aberrant complexes, which are intracellularly assembled but not surface-expressed, and produce Glanzmann thrombasthenic phenotypes (see Ref. 35 for original references): (i) point mutations adjacents to the calcium-binding regions of GPIIb ($Gly^{242} \rightarrow$ Asp; $Arg^{327} \rightarrow$ His; and $Gly^{418} \rightarrow$ Asp) (44–46). Substitution of Asp^{418} for Ala rescued intracellular transport and surface expression, suggesting that a small, uncharged residue at the N terminus of the Ca^{2+} coordination site may be crucial for proper folding and/or maturation of GPIIb/IIIa (45); (ii) a 13-base deletion in exon 4 of GPIIb producing a 6 amino acid which includes Cys^{107}; (iii) the mutation GPIIIa $Cys^{374} \rightarrow$ Tyr; and (iv) an 11-base deletion within exon 12 of GPIIIa leading to a stop codon shortly before Cys^{655}. The altered form of GPIIIa, lacking the long-range Cys^{406}-Cys^{655} disulphide bond, may be highly unstable and rapidly degraded intracellularly after biosynthesis, since the transmembrane and cytoplasmic domains of GPIIIa seem not to be essential for association with pro-GPIIb, a correct cellular transit, and exposure of the truncated complex at the cell surface (41).

Both GPIIb and GPIIIa are polymorphic proteins (Fig. 1) (i.e. they carry a number of clinically relevant

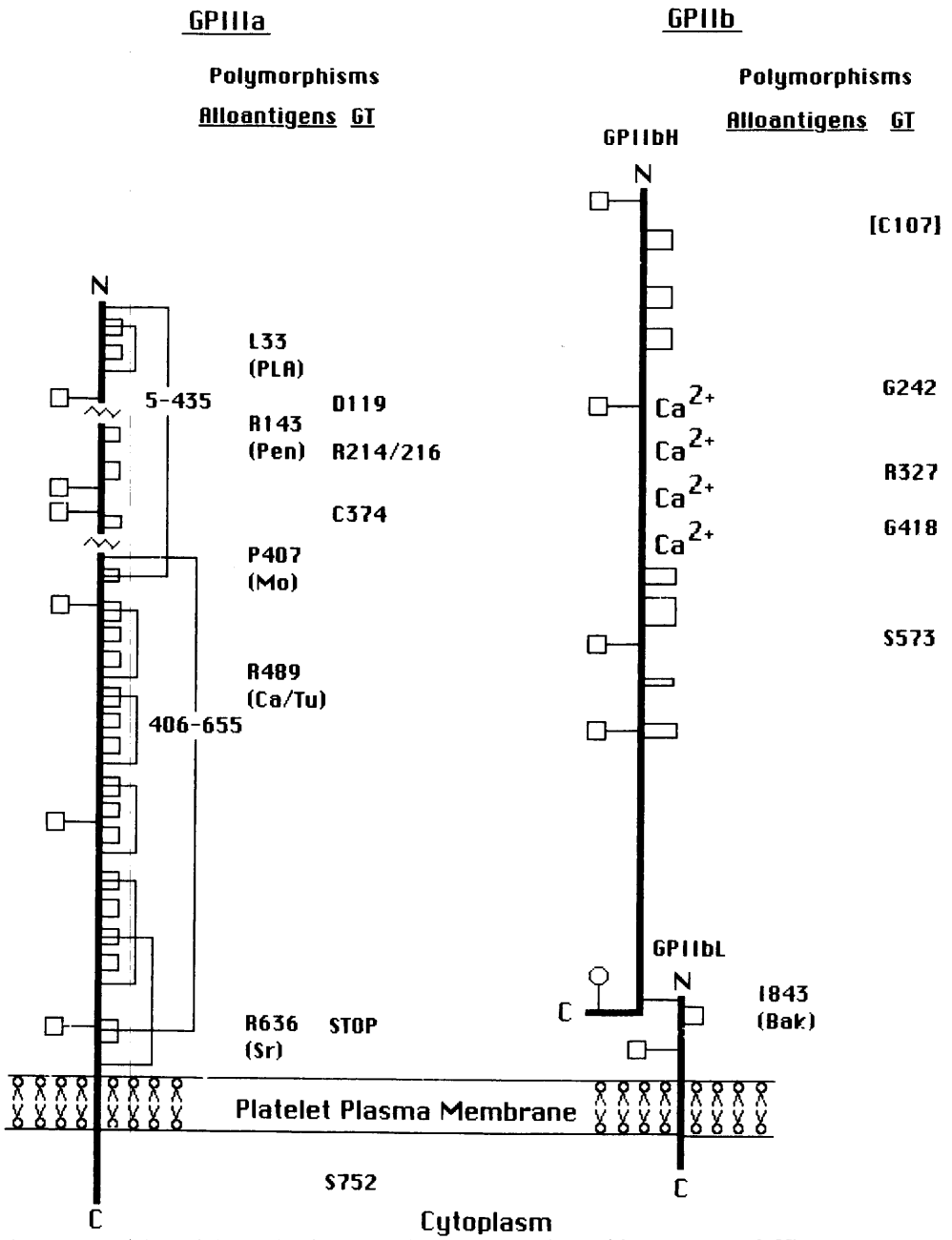

Figure 1. Schematic representation of the major features of the linear amino acid sequences of GPIIIa and the subunits of GPIIb, GPIIbH and GPIIbL. N, N-terminus; C, C-terminus. The thin lines show the disulphide bonding pattern. The position of the two long-range disulphide bridges of GPIIIa (5–435 and 406–655) is indicated. Ca^{2+}, calcium-binding site; —❑, N-glycosylation site; —○, O-glycosylation site. The relative position of residues primarily involved in the formation of either alloantigen systems (PLA, Pen, Mo, Ca/Tu, Sr, and Bak) or Glanzmann thrombasthenia (GT) phenotypes are shown. ⩙, indicates that the polypeptide chain of GPIIIa is interrupted due to the large size of that region. The drawing is not at scale.

human platelet alloantigen systems [for a recent review see Ref. 47]). Besides their importance in the development of anti-allotype antibodies, functionally speaking, there does not appear to be any deleterious effect nor evolutionary advantage associated with a given alloantigenic phenotype. Although amino acid dimorphisms control the formation of all GPIIb and GPIIIa alloantigens thus far examined (45), post-translational modifications (i.e., glycosylation) and protein tertiary structure determinants may, in some instances (Bak [=Lek] and PLA systems, respectively), play a role in the formation of the epitopes (48–50).

Molecular Characterization of Isolated GPIIb/IIIa

The morphology of isolated GPIIb/IIIa complexes determined by electron microscopy of rotary-

shadowed specimens is heterogeneous and consists of filled globular, empty oval, bilobular or head-two-tails shapes, among others (15, 51). It is not clear whether these forms are preparation artifacts or represent different conformations of the heterodimer which could arise by the inherent flexibility of the integrin (52). The most abundant structure consists of a globular head (8 × 10 × 10 nm) with two rod-like tails extending 14–16 nm from one side, which often show a small globular domain at their ends. This morphology has also been reported for integrin $\alpha_5\beta_1$, a prototypic fibronectin receptor (53). It has been proposed that the tails may contain the transmembrane and carboxy-terminal portions of GPIIbL and GPIIIa, with the N-terminal domains of both subunits being extracellular and forming the bulk of the oblong head (15, 53). In addition, Wippler and colleagues (54) have shown that the cysteine-rich domain of GPIIIa and the extracellular part of GPIIbL are structural parts of the GPIIb/IIIa tails, while a recombinant truncated GPIIIa species (residues 1–469), which forms a functional complex with a construct of GPIIb consisting of the whole heavy chain (GPIIbH 1–856) disulphide-bonded to the first 13 residues of GPIIbL, retains the characteristic globular head with shorter (~10 nm) tails. Therefore, the cysteine-rich domain of GPIIIa may not interact with GPIIb in the quaternary structure of the heterodimer.

On the other hand, proteolytic dissection of isolated resting GPIIb/IIIa complex (43) showed that sequences of the GPIIIa loop region between the cysteine-rich domains (residues [217–235]-S-S-[262–298]; 324–366; and 403–421), regions of the C-terminal half of GPIIbH (486–553; 696–734; and 780–817), and the midregion of GPIIbL (residues 30–75) are all involved in the heterodimer intersubunit surface (schematically depicted in Fig. 2). Noteworthy, the intersubunits regions correspond well to exon boundaries within the gene structure of GPIIb and GPIIIa. Thus, sequences within GPIIIa exons IV–V (residues 180–287), VII–VIII (320–396), and the N-terminal part of exon IX (residues 396–537) form part of the heterodimer surface together with the product of GPIIb exons XVI–XVII (GPIIbH 485–553), XXII–XXIII (GPIIbH 699–752), XXV–XXVI (GPIIbH [786–857]-S-S-GPIIbL [1–19]), and XXVII (GPIIbL 20-57) (Fig. 2). Such correlations were not apparent from analysis of the linear primary structure of GPIIb and GPIIIa.

Though at first sight these biochemical data appear to be in contrast with the electron microscopy picture of GPIIb/IIIa, Rocco and colleagues (55) have elaborated a model for the solution conformation of GPIIb/IIIa according to which the biochemical constraints can be fitted in the overall shape of GPIIb/IIIa determined by electron microscopy, and is in good agreement with dynamic light scattering measure-

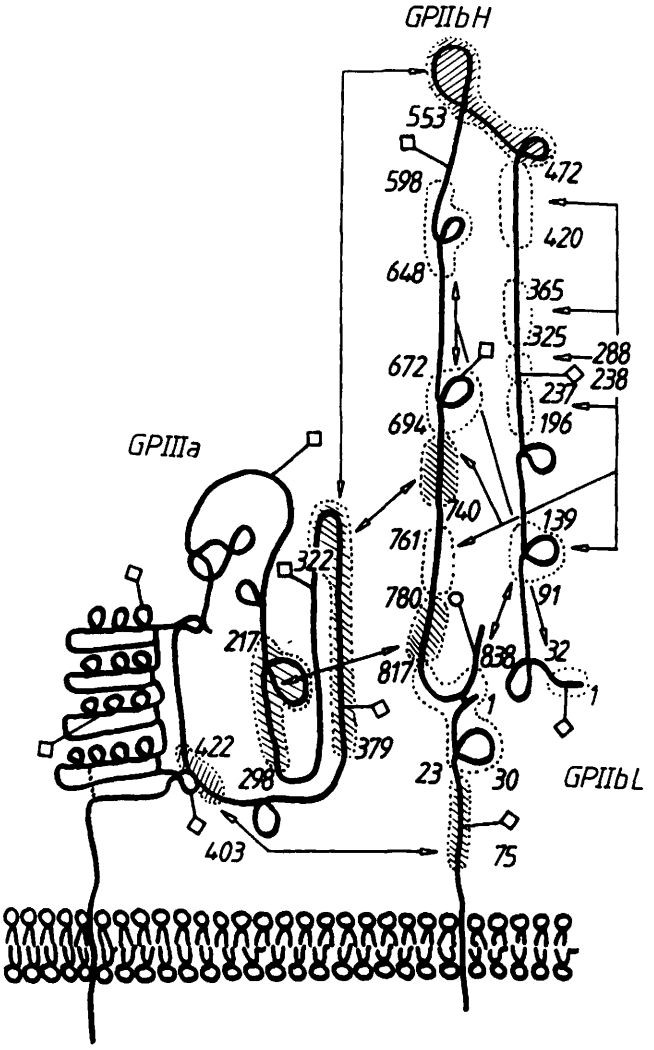

Figure 2. Representation of intra- and intermolecular domain connectivities (dotted areas and shadowed regions, respectively) within resting GPIIb/IIIa identified by proteolytic dissection of the isolated integrin. The arrows indicate interdomain associations.—❑, N-glycosylation site;—○, O-glycosylation site.

ments of GPIIb/IIIa solubilized with octyl glucoside (52) (schematically depicted in Fig. 3A).

The structure and binding properties of GPIIb/IIIa indicate the presence of domains involved in a number of major functions including: subunit association, conformational changes exposing the ligand-binding pocket, extracellular ligand recognition and binding, ligand binding-induced expression of neoepitopes, membrane association, receptor clustering, and cytoskeletal interactions. In the following, I will summarize many structural and functional data into an emerging model. It must be emphasized that most of the interpretations based on structural elements are necessarily subjective and require independent confirmation. Nevertheless, this initial biochemical model of GPIIb/IIIa may help us understand structure-function

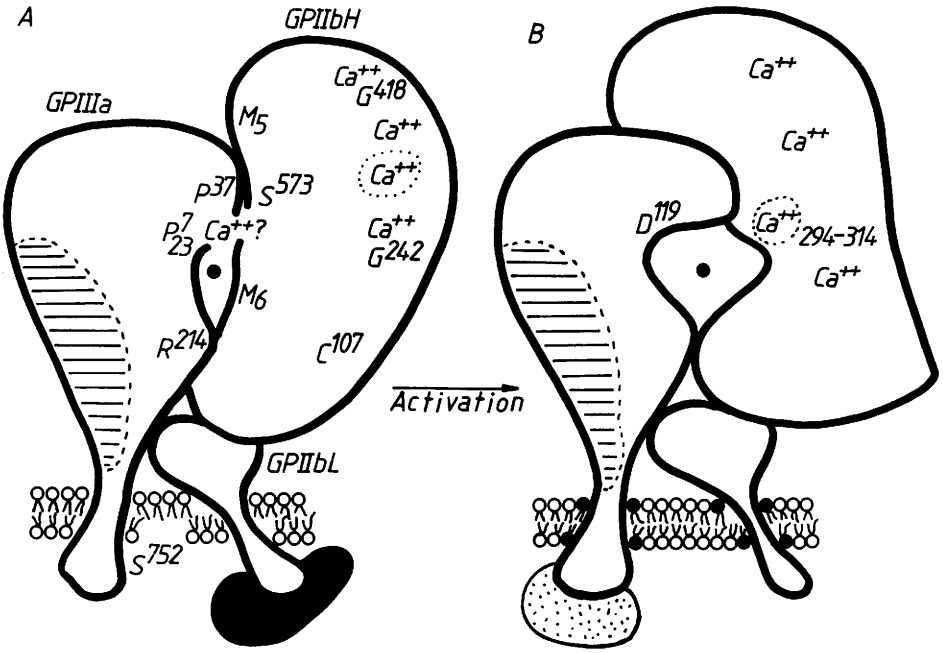

Figure 3. Schematic model of the overall structure of resting GPIIb/IIIa (A) and the activation-dependent conformational transition that opens the mouth of the ligand binding pocket (B). The filled circle shows the arbitrary location of the ligand binding site. The shadowed surface represents the disulphide-rich core of GPIIIa. , putative lipid moiety involved in modulating the receptor capability of GPIIb/IIIa. The black moiety at the cytoplasmic tail of GPIIbL is a hypothetical repressor of the receptor's high-affinity state, whereas the dotted moiety associated with the cytoplasmic tail of GPIIIa represents a putative activator. The proposed relative distribution of some relevant epitopes discussed in the text is indicated: M5 (GPIIbH 550-558) and P37 (GPIIIa 101–109), epitopes recognized by fibrinogen-binding inhibitory monoclonal antibodies; M6 (GPIIbH 657–665) and $P_{23}7$ (GPIIIa 114–122), activation-dependent monoclonal antibody epitopes; R^{214}, S^{752}, S^{573}, C^{107}, G^{242}, G^{418}, D^{119}, residues whose mutation affect GPIIb/IIIa biosynthesis or function; Ca^{++}, calcium-binding site; Ca^{++}?, a putative calcium-binding site around GPIIIa D^{119}. The region around the calcium binding site encircled (GPIIbH 294–314) contains the cross-linking site for fibrinogen γ400–411 peptide. It is hypothesized that this GPIIb region contributes to the topography of the ligand binding site within activated GPIIb/IIIa.

relationships of the fibrinogen receptor, and of other members of the integrin family, as well as to design experimental work to test current hypotheses.

Inside-Out Induction of the Fibrinogen Binding Site of GPIIb/IIIa

On unstimulated platelets, GPIIb/IIIa is maintained in an inactive (resting) conformation and serves as a low-affinity adhesion receptor for surface-coated fibrinogen. This interaction occurs in the absence of platelet activation, and is irreversible and mediated by the fibrinogen γ-chain C-terminal dodecapeptide site (56 and references cited). In addition, the ligand binding pocket of GPIIb/IIIa on resting platelets is accesible to molecules which contain RGD-, OrnGD-, KGD-, or RYD-amino acid sequence motifs located at the tip of a recognition loop which protrudes 14–17 Å from the protein core (reviewed in Ref. 35 and 57; see also Ref. 58 and 59). Similarly, Beer *et al.* (60) have shown that the majority of RGD binding sites on GPIIb/IIIa can be reached by peptides that extend out 11–32 Å from the surface of polyacrylonitrile beads, and that peptides in the lower length limit (~11 Å) showed the greatest sensitivity to the platelet activation state. The presence of the RGD sequence is, however, not always necessary for binding, since disagregin, a fibrinogen receptor antagonist from the tick *Ornithodoros moubata* lacking the RGD sequence, compete with the RGD-containing snake venom disintegrin echistatin for its binding to GPIIb/IIIa (61). Nevertheless, the current evidence suggests that the geometry of the binding pocket in nonactivated GPIIb/IIIa may resemble a narrow cavity buried 10–20 Å below the protein exterior.

Following platelet stimulation, GPIIb/IIIa becomes competent to bind soluble plasma fibrinogen and some other Arg-Gly-Asp (RGD) sequence-containing macromolecules, like fibronectin, vitronectin, and von Willebrand factor. In addition, at least fibronectin and fibrinogen contain additional regions which independently interact with the integrin (62, 63) and, in the case of fibrinogen, the C-terminal regions of the γ-chains contain the major GPIIb/IIIa binding sites (56, 63, 64). The relationship among the RGD-, the γ-chain-, and other binding site(s) on GPIIb/IIIa remains obscure, however (reviewed in Ref. 6 and 35). They may share a common binding site or may compete for related, mutually exclusive, nonidentical binding sites (i.e., subsites within a large ligand-binding surface).

Studies on the regulation of GPIIb/IIIa activation indicate that it is tightly controlled by a complex net-

work of intracellular signaling reactions that transmit the state of platelet activation to the extracellular part of the integrin (65). It has been demonstrated that GPIIb/IIIa undergoes a measurable conformational change in its quaternary structure upon platelet activation (66), which causes a relative reorientation of the subunit's exodomains (67) that might be responsible for opening the mouth of the ligand-binding cavity to allow the entry of macromolecular ligands (Fig. 3). Moreover, cells other than platelets transfected with GPIIb/IIIa are unable to modulate the receptor capability of the integrin, indicating the existence of platelet-specific elements along the GPIIb/IIIa activation pathway(s).

Platelet stimulation activate lipid-metabolizing enzymes, which may alter the lipid composition of the plasma membrane. A specific lipid environment has been shown to modulate the receptor properties of integrins from the β_1, β_2, and β_3 families (7, 35). Smyth *et al.* (68) have reported that a lipid fraction present in activated but not in resting platelets increases the proportion of purified GPIIb/IIIa complexes competent for fibrinogen-binding without affecting the affinity of the interaction. Purified phosphatidic and lysophosphatidic acids mimicked this effect. However, since production of these lipid species does not correlate with GPIIb/IIIa activation (9), their biological potential remains to be established.

On the other hand, the cytoplasmic domain of GPIIb appears to control the ligand-binding affinity of GPIIb/IIIa, suggesting the existence of an inside-out transmembrane signaling mechanism (69). Since expression of a GPIIb/IIIa construct in which the cytoplasmic portion of GPIIb had been deleted resulted in a constitutively ligand-binding active Δ^{cyt}GPIIb/IIIa, whereas truncation of the cytoplasmic tail of GPIIIa did not activate GPIIb/Δ^{cyt}IIIa, the authors suggested that an intracellular repressor might maintain GPIIb/IIIa in its resting state and that modification of this interaction during platelet activation could induce the high-affinity state of the integrin (69) (Fig. 3). In addition, mutations that disrupt the highly conserved α subunit cytoplasmic domain GFFKR result in high-affinity binding of ligands to GPIIb/IIIa (70). Thus, this motif might also be involved in maintaining the default low-affinity state of the integrin.

Furthermore, a point mutation ($Ser^{752} \rightarrow$ Pro) blocks the platelet activation-dependent upregulation of fibrinogen-binding capability of GPIIb/IIIa without affecting its ability to bind RGD-peptides (71). As a whole, these data point out that the affinity of GPIIb/IIIa for fibrinogen may be regulated by both negative and positive intracellular moieties through the cytoplasmic domains of GPIIb and GPIIIa, respectively (Fig. 3).

The identity of the cytoplasmic regulators of the affinity state of GPIIb/IIIa (whether the cytoplasmic domains themselves or other intracellular moieties) and how the activation signal is transmitted to the extracellular domains of GPIIb/IIIa remain obscure. Nevertheless, several intracellular proteins have been reported to associate with GPIIb/IIIa. Thus, monoclonal antibodies against the CD9 antigen induce a specific association between CD9 and GPIIb/IIIa, and this interaction promotes fibrinogen binding (72). CD9 possesses Ser/Thr kinase activity, but whether Ser/Thr phosphorylation is actually involved in GPIIb/IIIa activation is a controversial matter (73, 74). In addition, a 21-kDa GTP-binding protein has been found in association with resting GPIIb/IIIa (75), and Morii and coworkers (76) have provided evidence that *RhoA*, a member of the *Ras*-related family of GTP-binding proteins, may play a role in regulating both GPIIb/IIIa ligand-binding avidity and cytoskeletal reorganization during the aggregation process (see below). The actual involvement of these, and possibly other, molecules in inside-out activation of GPIIb/IIIa requires further research.

Exposure and Topography of the Extracellular Ligand-Binding Surface of GPIIb/IIIa

Although the fine structure of GPIIb/IIIa has not been established, current evidence favors a model in which the ligand binding pocket is discontinuous and formed by structures from both the α and the β subunits (77, 78 and references therein), which remain cryptic in the resting state of the integrin. A variety of biochemical, immunological, and mutational approaches have provided clues for the identification of protein domains involved in the conformational change leading to the exposure of the binding surface for macromolecular adhesive ligands.

Comparison of the proteolytic degradation patterns of the resting (non-RGD-binding) and activated (RGD-binding) conformers of isolated GPIIb/IIIa (69) showed that, in the activated state, the N-terminal half of GPIIbH protect itself and GPIIIa 100–348 from proteolysis, whereas the C-terminal half of GPIIbH resulted more susceptible to degradation (Fig. 3). This indicates that, for receptor expression, the GPIIbH and GPIIIa extracellular domains undergo a major relative reorientation along the subunit interface during the conformational transition of the integrin.

Particularly, the region around GPIIIa Arg^{214} has been involved in the conformational change(s) leading to activation of GPIIb/IIIa. Thus, mutation of this residue for Trp (79, 80) abolishes fibrinogen binding in response to platelet activation agonists but does not impair the interaction of the mutated integrin with short synthetic RGD peptides in solution. Similarly, the mutation $Arg^{214} \rightarrow$ Gln (81) produces a defective GPIIb/IIIa complexes, which are expressed at the

platelet surface although they spontaneously unassemble into free subunits even in the presence of Ca^{2+} and are unable to bind ligands. This may imply that the functional defect introduced by these Arg^{214} substitutions are related to both an intrinsic structural instability of the complex and the inability of the heterodimer to change its conformation to expose the macromolecular ligand binding pocket (i.e., the mutations may change the character of the subunit interface contacts maintaining thus the quaternary structure of GPIIb/IIIa in a permanently nonactivated-like conformation). This is in agreement with the observation that monoclonal antibody AP6, directed against GPIIIa 214–218, binds to immobilized active GPIIb/IIIa and does not impair fibrinogen binding (82). Therefore, GPIIIa R^{214} may not be required for fibrinogen binding but rather may be critical for maintaining the correct tertiary structure of the fibrinogen binding site on activated GPIIb/IIIa. Moreover, the amino acid sequence of integrin β_1 207–218 (which corresponds to GPIIIa 198–209) also contains epitopes for both activating and inhibiting monoclonal antibodies, and appears to be important for the avidity regulation of β_1 integrin through conformational changes (83). These data suggest that the mechanism for avidity regulation through conformational changes of the β subunit has been conserved in members of the β_1 and β_3 integrin families.

Using different approaches, a variety of regions putatively involved in ligand binding have been identified (reviewed in Ref. 35). In the case of GPIIIa, the binding activity resides entirely within its N-terminal domain encompassing residues 1–469 (54), and the region 100–150 appears to contain critical residues for ligand binding. Concretely, several activation-dependent epitopes for (monoclonal) antibodies have been mapped to GPIIIa 100–128 ($P_{23}7$: 101–109; AC7: 109–128) (84, 85) (Fig. 3). This GPIIIa region contains the cross-linking site for a short RGD-peptide in both GPIIb/IIIa in activated platelets (86) and isolated GPIIb/IIIa (78), and includes Asp^{119}, whose mutation for Tyr produces a mutant integrin with impaired ligand recognition and perturbed interaction with divalent cations (87). The linear spacing of oxygenated residues in the surrounding of Asp^{119} (Ser^{121}, Ser^{123}, Asp^{126}, Asp^{127}, and Ser^{130}) approximates that of the residues in the calcium-binding loop of other proteins. Since the aspartate residue in both the RGD sequence and the fibrinogen γ-chain peptide (^{400}HHLGGAKQAGDV^{411}) are critical for conferring biological activity (57, 77), the hypothesis has been put forwards that the cognate sequence in the ligand may interact with divalent cation(s) occupying a divalent cation binding site in GPIIIa (77) (Ca^{++}? in Fig. 3A). Furthermore, cell lines expressing a mutant GPIIb/IIIa, in which the polypeptide $GPIIIa^{129–133}$ had been replaced by the corresponding amino acids from the integrin β_1 subunit, are hyperadherent and manifest spontaneous aggregation when fibrinogen was added to the medium (88). Recently, Bajt and Loftus (89) produced GPIIb/IIIa mutants with Ser^{121}, Ser^{123}, Asp^{126}, Asp^{127}, and Ser^{130} individually substituted by alanine. None of these replacements altered heterodimer formation or surface expression of GPIIb/IIIa. Substitution at position 121 produced a complete loss of receptor function. Similarly, GPIIb/IIIa $Ser^{123\rightarrow Ala}$ failed to adhere to fibrinogen and did not bind to immobilized RGD peptides or the ligand-mimetic antibody PAC1. This mutant, however, retained the capacity to bind a GPIIb/IIIa-specific, high-affinity peptidomimetic, but occupancy did not exhibit peptide-induced conformational changes in the receptor (see below). On the other hand, substitutions at position 126, 127, and 130 had no effect on ligand-binding function or ligand occupancy-dependent conformational changes of GPIIb/IIIa. These data indicate that Asp^{119}, Ser^{121}, and Ser^{123} may play an integral role in the coordination of Ca^{2+} and the ligand binding function of GPIIb/IIIa.

Interestingly, two synthetic peptides corresponding to the GPIIIa sequences 214–218 (90) and 217–231 (91) inhibit fibrinogen binding to GPIIb/IIIa. However, whereas the former peptide bound to GPIIb/IIIa itself (90), the latter interacted with fibrinogen (91). In addition, the epitope 217–231 is cryptic in resting GPIIb/IIIa and becomes exposed following platelet activation with thrombin (92). Altogether, it seems reasonable to postulate that the N-terminal part of GPIIIa (1–430) may be divided in two domains, the N-terminal half being critical for ligand binding and the C-terminal region playing a role in both subunit association and regulation of the expression of the active site. Similarly, structure/function analysis of the integrin β_1 subunit by epitope mapping suggests the existence of a regulatory domain located between residue 493 and the transmembrane domain (93).

On the other hand, the GPIIb domain(s) forming part of the fibrinogen binding site appear to involve regions distantly located within its primary structure. Thus, GPIIbH 657–665 contains an activation-dependent epitope (94). In addition, GPIIbH 1–485 contains cross-linking sites for both RGD- and fibrinogen $\gamma\text{-chain}^{400–412}$ peptides (78, 95), forms a Ca^{2+}-dependent complex with GPIIIa 1–730 (96), and the recombinant fragment 171–464 binds to immobilized fibrinogen with an estimated recombinant GPIIb:fibrinogen ratio of 2.2:1, being this interaction optimal at 0.5 m*M* Ca^{2+} (i.e., when the four Ca^{2+}-binding sites of recombinant $GPIIb^{171–464}$ are saturated) (97). In addition, synthetic peptides corresponding to GPIIbH 296–312 inhibits the binding of platelets to adhesive

ligands (98, 99). Altogether, these data indicate that the contribution of GPIIbH to the ligand-binding surface of the integrin involves regions, which are brought into juxtaposition by the spatial folding of its polypeptide chain. This is in agreement with the finding that the N-terminal part of GPIIbH (1–472) is folded against the GPIIbH 600–700 domain (43) (Fig. 2).

Ligand Binding Induces Conformational Changes in GPIIb/IIIa: Outside-In Signaling

The binding of fibrinogen, and other ligands, to activated GPIIb/IIIa causes conformational changes on both fibrinogen and the integrin, which express neoepitopes called RIBS (receptor-induced binding sites) or LIBS (ligand-induced binding sites), respectively. Three sequences of fibrinogen that become exposed upon binding to GPIIb/IIIa have been localized: γ112–119 and Aα 95–98 (100), and γ375–385 (101). These sequences may create additional sites for interaction with the receptor and/or contribute to the affinity of the interaction. On the other hand, exposure of LIBS correlates with cytoskeleton-independent clustering of occupied GPIIb/IIIa complexes in the plane of the membrane (Fig. 4), followed by platelet aggregation and attachment of GPIIb/IIIa patches to the cytoskeleton network (see below) (reviewed in Ref. 102). The structure/function relationship of postreceptor occupancy events is only recently beginning to be unraveled.

The observation that GPIIIa Ser^{123} is implicated in Ca^{2+} coordination and ligand binding and that its substitution for Ala abrogates peptidomimetic binding-induced conformational changes in GPIIb/IIIa (89) suggests that the signaling of the receptor occupancy status of GPIIb/IIIa is initiated at the ligand-binding pocket itself. Monoclonal antibody AP3 binds to a discontinuous epitope within GPIIIa 348–421, does not inhibit fibrinogen binding but blocks the GPIIb/IIIa receptor occupancy signal, which leads to platelet aggregation and clot retraction (103). Thus, the AP3 epitope is not a LIBS but rather may be involved in the transmission of conformational changes following ligand binding.

The region 422–692 and the C-terminal part of the extracellular domain of GPIIIa express LIBS recognized by monoclonal antibodies D3GP3 and C5GP3 (103), and LIBS-2 (a conformational epitope assembled within GPIIIa 609–690) (104), respectively (Fig. 4A). Within this region, cleavage of the Lys^{444}-Pro^{445} bond by plasmin is dependent on fibrinogen binding (105), indicating that a conformational change may facilitate the accessibility of the enzyme to this region of the GPIIIa molecule.

As a whole, these data evidence that the alterations of the conformation of GPIIIa that follow ligand binding involve long-range propagation spanning the whole molecule, from the (N-terminal) active site to the (C-terminal) cytoplasmic tail (see below).

Another LIBS is the PMI-1 epitope localized within the C-terminal part of GPIIb, $GPIIbH^{842–857}$ (reviewed in Ref. 5) (Fig. 4). Interestingly, in resting GPIIb/IIIa this epitope is located on a hidden loop flanked by two surface-exposed epitopes, $M\alpha^2$ ($GPIIbH^{837–843}$) and M_3 ($GPIIbH^{849–857}$) (94) (Fig. 4A). Limited chymotryptic digestion of GPIIb/IIIa on resting platelets releases a 2-kDa peptide from GPIIbH

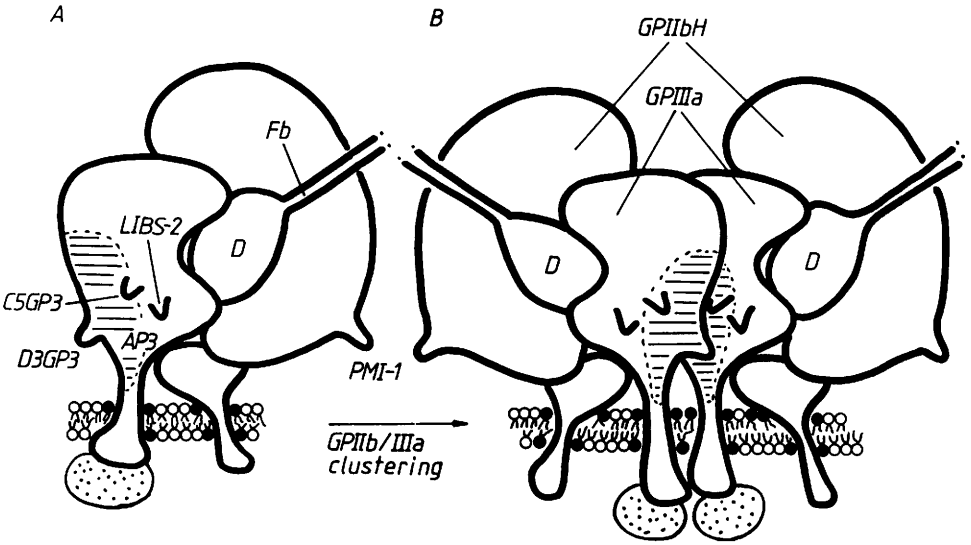

Figure 4. Model of (A) fibrinogen bound to activated GPIIb/IIIa (see Fig. 3) and the neoepitopes exposed as a consequence of ligand binding (LIBS): C5GP3, D3GP3, LIBS-2, and PMI-1. In this model the distal (D) domain is mainly responsible for fibrinogen binding to activated GPIIb/IIIa. For clarity, only a part of the fibrinogen molecule is shown. (B) Representation of the cytoskeleton-independent receptor clustering within the plane of the platelet's plasma membrane.

which contains the PMI-1 epitope. This results in the exposure of an activation-independent fibrinogen binding site on the integrin (106). In addition, the polypeptide segment 834–C-terminus of GPIIbH contains also epitope(s), which result cryptic following receptor occupation (107). Therefore, the region around the PMI-1 epitope may undergo a complex conformational transition utilized to define the ligand-binding status of GPIIb/IIIa.

Taking into account that the GPIIbH region 780–817 and GPIIIa 403–421 form part of the heterodimer interface (Fig. 2), the evidence summarized above is consistent with the idea that ligand-induced binding sites may arise as a consequence of subtle motions between the integrin subunits and that the C-terminal half of the extracellular domain of GPIIIa (downstream of residue 214) may not only be involved in regulating the inside-out expression of the ligand-binding pocket but may participate also in outside-in signaling.

Though the physiological significance of LIBS expression remains to be established, it is tempting to speculate that exposure of neoepitopes within the C-terminal GPIIIa extracellular domain could be related to expression of interaction surfaces for other platelet and/or plasma components (i.e., GPIIIa regions involved in heterodimer patching (Fig. 4B).

Postligand Occupancy and Cytoskeletal Rearrangement

At physiological temperature, GPIIb/IIIa in the plasma membrane of resting platelets is mainly free from stable associations with other membrane or cytoskeletal proteins (108). As a consequence of extracellular ligand binding, receptor clustering, and platelet aggregation, GPIIb/IIIa becomes attached to the membrane skeleton (Fig. 5), a structure composed of short actin filaments, actin-binding protein, spectrin, vinculin, and other, unidentified proteins (reviewed in Ref. 109).

The molecular determinants of the cytoplasmic tails of GPIIb/IIIa for cytoskeletal attachment appear to be located entirely on the GPIIIa cytoplasmic tail (110). This GPIIIa domain shows a high degree of homology with that of integrin β_1 subunit. Indeed, the cytoplasmic tail of β_1 can be interchanged with its homologous sequence from GPIIIa without loss of receptor function (111). The underlined amino acid within the sequences ^{743}*F*AK*F*EK*E*KMN^{752} and ^{760}*NP*IY-*S*AVTTVV*N*PK*Y*^{775} of the β_1 cytoplasmic domain are critically required for focal contact localization (112, 113). With the exception of β_1 S^{765}, these crucial residues are absolutely conserved in the corresponding GPIIIa regions (727–736 and 744–759, respectively). However, whether they are also involved in targetting GPIIb/IIIa to its (unknown) cytoskeleton attachment

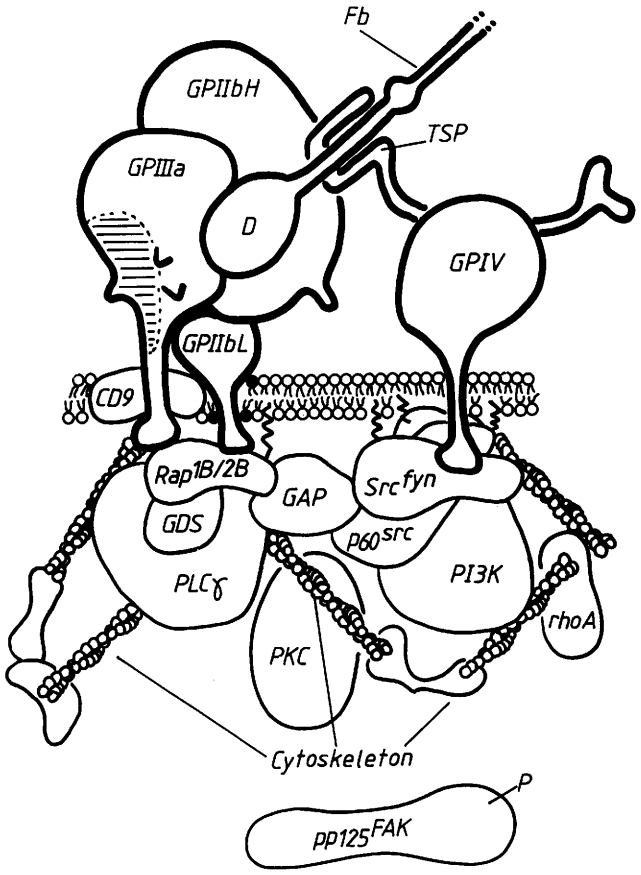

Figure 5. Cartoon of the hypothetical arrangement of a multiprotein signaling complex assembled at the cytoplasmic domains of GPIIb/IIIa as a consequence of platelet aggregation. For clarity, a single GPIIb/IIIa-fibrinogen heterotrimer rather than a patch (see Fig. 4) is shown. Only a half of the fibrinogen molecule is drawn. TSP, thrombospondin; GAP, p21^{ras} GTPase-activating protein; GDS, guanine nucleotide dissociation stimulator; PLCγ, phospholipase Cγ; PI3K, phosphoinositol-3-kinase; PKC, protein kinase C. Rap^{1B} or Rap^{2B}, Src^{fyn}, p60^{src}, and PI3K are associated with the inner leaflet of the plasma membrane through a lipid anchor. This drawing is not at scale.

site remains to be established. In addition, the molecular determinants that suppress the attachment of unoccupied GPIIb/IIIa to the cytoskeleton remain obscure.

The anchorage of GPIIb/IIIa is necessary for clot retraction to occur and appears to trigger a major cytoskeletal reorganization (Fig. 5). The monomeric GTP-binding protein *rho*A seems to regulate these GPIIb/IIIa-dependent cytoskeletal reorganization in aggregating platelets (76). The parallel incorporation of GPIIb/IIIa and a number of intracellular proteins into the thrombin-stimulated aggregating platelet cytoskeleton have been reported. These include the GTP-binding proteins p21^{rap1B} and p21^{rap2B}, protein kinase C (PKC), phospholipase Cγ (PLCγ), diacylglycerol kinase, phosphatidylinositol-3-kinase (PI3K), the src-family tyrosine kinases pp60$^{c\text{-}src}$, pp62$^{c\text{-}yes}$, and pp59^{fyn}, and the p21^{ras} GTPase-activating protein (GAP) (114–120). The incorporation of Rap 1B paral-

leled the attachment of membrane glycoproteins GPIIb/IIIa, but not the incorporation of pp60$^{c\text{-}src}$ and GAP (120). Thus, the recruitment of these, and other, signaling molecules with the reorganizing cytoskeleton occur in different steps and leads to the assembly of a multimeric signal transduction complex (Fig. 5), which may explain the causal relationship between GPIIb/IIIa occupancy and a number of intracellular events elicited by ligand binding, such as increase in Na^+/H^+ exchange, intracellular pH, and phosphatidyl inositol metabolism, calpain activation, and thrombin-induced phosphotyrosine dephosphorylation of 115- and 75-kDa proteins (121), as well as tyrosine phosphorylation of platelet proteins other than GPIIb/IIIa itself (119, 122). Among the latter, proteins with apparent molecular masses of 50–68 kDa, 140 kDa, and the protein tyrosine kinases pp72^{syk} and pp125^{FAK} have been identified (123, 124) (Fig. 5). Agonist-induced tyrosine phosphorylation modulated by GPIIb/IIIa occur in at least two different waves (124, 125). In the first wave, fibrinogen binding to GPIIb/IIIa, receptor oligomerization, and actin polymerization are required for tyrosine phosphorylation of the 50–68 kDa and 140 kDa proteins as well as pp72^{syk}, and these events precede platelet aggregation and phosphorylation of proteins of 95/97 kDa as well as pp125^{FAK} (second wave) (124).

The exact sequence of interactions that result in tyrosyne phosphorylation of pp125^{FAK} is currently unclear. Nevertheless, it has been reported that autonomously expressed β_3, β_1, or β_5 cytoplasmic domains in a single-subunit chimera (consisting of the extracellular and transmembrane regions of the nonsignaling subunit of the human interleukin-2 receptor), are able to constitutively activate tyrosine phosphorylation of pp125^{FAK} in cultured human fibroblasts (126, 127). However, a constructs carrying either α subunit intracellular domains or a variant of the β_3 cytoplasmic tail (called β_{3B}), where the last 21 C-terminal residues of GPIIIa: ^{742}ANNPLYKEATSTFTNITYRGT^{762} are replaced by the polypeptide stretch ^{742}VRDGAGRFLK-SLV^{753}, was unable to induce phosphorylation of pp125^{FAK} (126). Since the β_{3B} variant lacks most of the essential residues required for focal contact localization (see above), it appears that the cytoplasmic tail of GPIIIa may contain all the structural information for both targeting the integrin to focal contacts (see above) and for coupling transmembrane signalling through GPIIb/IIIa into the cytoplasmic transduction cascade leading to tyrosine phosphorylation of pp125^{FAK}. This latter kinase plays a key role in the response of diverse cells to the action of neuropeptides, integrins, and oncogenes (33), suggesting that GPIIb/IIIa may share a signal transduction mechanism with a diverse group of receptors in different cells.

Though the organization of this macromolecular assembly and the events downstream pp125^{FAK} activation remain to be elucidated, it is suggestive that almost all the intracellular moieties involved in its arrangement contain SH2 and SH3 domains, which mediate recognition of specific phosphotyrosine-containing sequences and protein-protein interactions with cytoskeletal components, respectively. In addition to mediating physical association, the interactions of SH2 domains with specific phosphorylated sites allosterically regulates the catalytic activity of the carrier enzymes. Thus, the combined action of SH2 and SH3 domains may be responsible for the compartmentalization and local activation of enzymes involved in the cascade of signals initiated by extracellular ligand-integrin binding. Recent evidence indicates that this mechanism of cytoplasmic signal transduction is highly conserved among multicellular organisms (reviewed in Ref. 128).

In thrombin-stimulated platelets, PI3K interacts with both pp60$^{c\text{-}src}$ and Src^{Fyn}. This latter tyrosine kinase has been found associated with platelet membrane glycoprotein IV, the receptor for thrombospondin, and with a p21^{ras} GTPase-activating protein (GAP). In addition, thrombin promotes the formation of complexes between p21^{ras} GAP and pp60$^{c\text{-}src}$ and between p21^{ras} GAP, p21^{rap1B} and phospholipase Cγ (35 and references cited). As fibrinogen forms stable complexes with thrombospondin during platelet aggregation (129), associations on both sides of the plasma membrane (i.e., fibrinogen-thrombospondin and PI3K-Src^{Fyn}-GAP-Rap^{1B}) could play a role in both the assembly of the integrin-mediated multiprotein transduction unit (schematically depicted in Fig. 5) and the time-dependent irreversibility of the fibrinogen-GPIIb/IIIa interaction (3).

Conclusion

Glycoprotein IIb/IIIa is a two-way signaling integrin receptor. Platelet-specific elements initiate at the cytoplasmic domains of GPIIb/IIIa a signal which is transmitted to the extracellular domains (inside-out signaling) and causes a conformational change allowing the exposure of the cryptic integrin's ligand binding pocket. This active surface is made up from regions of GPIIb and GPIIIa located at the heterodimer interface. Following receptor occupancy, further structural changes trigger receptor clustering and platelet aggregation, and a signal flows back to the cytoplasmic domains of GPIIb/IIIa (outside-in signaling), which play then an essential role in anchoring GPIIb/IIIa to the cytoskeleton and in regulating tyrosine phosphorylation of a number of proteins and the subsequent assembly of a multiprotein complex involved in further downstream signaling. This appears to be a common theme along the signal transduction pathway through a variety of integrin receptors. Major challenges now include the elucidation of the 3-dimen-

sional structure of integrins, particularly their ligand-binding domain topology, and to unravel the molecular details of the two-way signal transduction pathway, including the nature of the conformational changes and the precise architecture and functioning of the cytoskeleton-associated complex.

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