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The Integrin αIIb/β3 in Human Platelet Signal Transduction

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ABSTRACT. Platelets are critical for the maintenance of the integrity of the vascular system and are the first line of defence against haemorrhage. When they encounter a subendothelial matrix exposed by injury to a vessel, platelets adhere, are activated, and become adhesive for other platelets so that they aggregate. αIIb/β3, a platelet-specific integrin, is largely prominent amongst the adhesion receptors and is essential for platelet aggregation. The ligands for αIIb/β3 are the multivalent adhesive proteins fibrinogen and von Willebrand factor. In resting platelets, αIIb/β3 is normally in a low activation state, unable to interact with soluble fibrinogen. Stimulation of platelets with various agonists will induce a conformational change in αIIb/β3 (inside-out signalling), which is then able to bind soluble fibrinogen resulting in the onset of platelet aggregation. However, fibrinogen binding to its membrane receptor is not simply a passive event allowing the formation of intercellular bridges between platelets. Indeed, a complex signalling pathway triggered by integrin ligation and clustering (outside-in signalling) will regulate the extent of irreversible platelet aggregation and clot retraction. Amongst the signalling enzymes activated downstream of αIIb/β3 engagement, phosphoinositide 3-kinase plays an important role in the control of the irreversible phase of aggregation. BIOCHEM PHARMACOL **60**;8:1069–1074, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. human platelets; αIIb/β3 integrin; signal transduction; phosphoinositide 3-kinase; irreversible aggregation; IAP.

Platelets circulate freely in the blood and, for a correct role in haemostasis, they must be activated by sequential and coordinated mechanisms. In pathological situations, a loss of the strict controls of these processes can lead to haemorrhage or to platelet thrombus in blood vessels, possibly causing a complete blockage of blood flow. One of the subtle processes that must be particularly well controlled is the conversion of the major platelet integrin αIIb/β3 (about 80,000 copies per platelet) from a resting to an active conformation, allowing its interaction with soluble fibrinogen (Fig. 1). This mechanism of αIIb/β3 integrin activation is termed inside-out signalling and results in the formation of intercellular bridges between platelets and finally in platelet aggregation. A number of platelet receptors, such as the thrombin receptors, are able to generate inside-out signalling. Glanzmann's thrombasthenia patients, devoid of a functional αIIb/β3, have contributed to discriminate between biochemical events occurring either upstream or downstream of integrin engagement. However, the precise biochemical reactions involved in the inside-out activation of αIIb/β3 remain poorly understood. Conversely, several of the mechanisms initiated by the integrin engagement, termed outside-in signalling, are now well characterised. As soon as a fibrinogen molecule binds to its receptor, a complex intracellular signalling is generated sequentially and in a coordinated manner with integrin-independent signals. The investigations of the outside-in signalling induced by αIIb/β3 have provided significant insights into integrin signalling in general and a better understanding of the mechanisms involved in the control of the irreversible phase of platelet aggregation. Some recent general reviews [1, 2] elegantly describe the different aspects of integrin signalling and provide a number of references concerning this domain. Here, we summarise inside-out signalling and focus on one important pathway of outside-in signalling. The latter implicates PI 3-kinase† as a key element of a positive feedback loop controlling the irreversible phase of platelet aggregation. Finally, we discuss the potential role of IAP, a partner of αIIb/β3.

MECHANISMS INVOLVED IN INSIDE-OUT SIGNALLING

Activation of αIIb/β3 is required for the binding of soluble ligand, and a number of primary agonists are able to achieve

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† *Abbreviations:* PI 3-kinase, phosphoinositide 3-kinase; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; MHC, myosin heavy chain; IAP, integrin-associated protein; PLC, phospholipase C; PAR1, protease-activated receptor 1; TRAP, thrombin receptor-activating peptide; FAK, focal adhesion kinase; TSP1, thrombospondin-1; and SH2, Src homology 2.

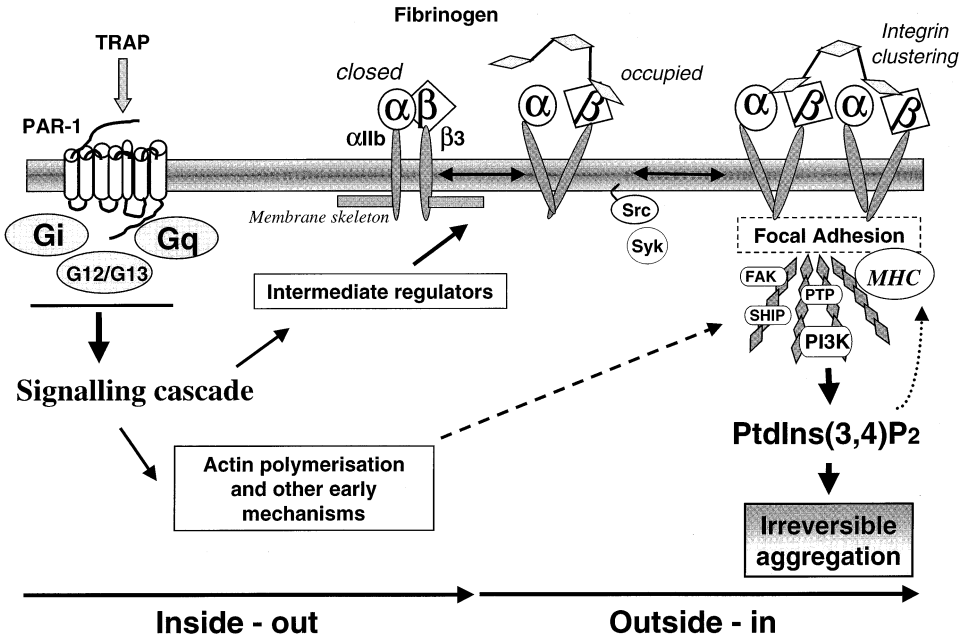

FIG. 1. αIIb/β3 signalling and irreversible platelet aggregation in TRAP-stimulated platelets. The exact function of most of the molecules involved in αIIb/β3 signalling is still poorly known, but it is possible to summarise the main sequences of the process as in this cartoon. PTP, phosphotyrosine phosphatase.

this process [1, 2]. Thrombin, ADP, collagen, thromboxane A2, thrombospondin, or immune complexes through FcγRIIa are amongst the platelet activators able to induce an efficient inside-out signalling. The receptors for these agonists act either through heterotrimeric G-proteins or by activation of non-receptor tyrosine kinases such as Src and Syk. The intermediate signalling molecules will induce a conformational change in αIIb/β3, leading to an increased affinity for soluble fibrinogen [2]. The clustering of αIIb/β3 will then increase the avidity for the ligand [1, 3]. One of the common features of receptors for excitatory agonists is the rapid stimulation of phosphoinositide-specific PLC, resulting in the production of the two second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). This classical pathway clearly contributes to αIIb/β3 activation [4–6]. Receptors coupled to the heterotrimeric Gq protein such as the thrombin receptor PAR1 or the thromboxane A2 receptor activate PLCβ, whereas receptors activating the non-receptor tyrosine kinase pathway such as FcγRIIa or the collagen receptor GpVI will preferentially activate PLCγ2. In this respect, it is interesting to note that the stimulation of PLCγ2 by FcγRIIa or GpVI requires PI 3-kinase activation [7, 8]. Therefore, in these cases, PI 3-kinase can be an indirect upstream regulator of inside-out signalling. IP_3 supports a Ca^{2+} mobilisation that is required but not sufficient for activation of αIIb/β3 [4]. Moreover, stimulation of conventional PKC by DAG is thought to play an important direct or indirect role in inside-out signalling. Interestingly, the β3 cytoplasmic tail becomes phosphorylated on serine and threonine residues in response to thrombin stimulation [6, 9]. However, whether PKC directly phosphorylates αIIb/β3 has not been proved and another serine–threonine kinase, the integrin-linked kinase (ILK), is able to interact with the β tail of integrins. Moreover, it is noteworthy that a patient with a point mutation in the β3 chain (S752 to P752) exhibits a defect in the activation of αIIb/β3 [10]. Altogether, these results suggest that the phosphorylation of αIIb/β3 might be an important element in its activation.

Another interesting hypothesis is that inside-out signalling releases the cytoplasmic tails of αIIb/β3 from an inhibitory state. Whether this release reaction is due to intramolecular reorganisation of the interaction of αIIb with β3 or involves other intracellular binding proteins is not known. *In vitro*, several proteins have been shown to physically interact with the cytoplasmic tail of the integrin. Amongst them, β3-endonexin, which is present in platelets, may play a role in the process of activation of αIIb/β3 [11]. The membrane skeleton has also been proposed to play a role in maintaining a constraint to the integrin. Indeed, a population of αIIb/β3 interacts with this cytoskeleton in resting platelets [12]. However, the major actin polymerisation occurring rapidly upon platelet activation is not involved in the initial inside-out signalling, but rather plays a role in the irreversible binding of fibrinogen to αIIb/β3 [13]. Small GTPases of the Rho family and some of their guanine nucleotide exchange factors such as Vav are also thought to contribute to the activation of αIIb/β3 through the control of cytoskeleton organisation [1]. However, their precise function is still difficult to assess.

Finally, it is important to note that several signalling pathways have also been shown to inhibit αIIb/β3. For instance, activation of protein kinase A (PKA) by elevation of cyclic AMP production or stimulation of protein kinase G (PKG) by elevation of cyclic GMP are two efficient ways to inhibit platelet activation and aggregation

[14, 15]. The exact mechanisms by which these kinases affect inside-out signalling are still unknown.

αIIb/β3-MEDIATED SIGNALLING

αIIb/β3-mediated signalling actually starts as soon as a fibrinogen molecule binds to the integrin, allowing the recruitment of signalling proteins at the vicinity of its cytoplasmic tail independently of actin polymerisation [16]. This initial phase of outside-in signalling will contribute to further activate the integrin. The clustering of αIIb/β3 and the achievement of a complex network of signalling and structural cytoskeletal proteins, allowing a strong interaction with the newly polymerised actin filaments, will lead to a full outside-in signalling. The recruitment of a number of key enzymes and docking proteins at the proximity of integrins parallels the formation of focal adhesions [1]. These complexes, strongly associated with the reorganised actin cytoskeleton during platelet aggregation, co-sediment with the Triton X-100 insoluble material at 12,000 × *g* [12]. αIIb/β3 outside-in signalling results in calcium mobilisation, tyrosine phosphorylation of a number of proteins, activation of the phosphoinositide metabolism, and cytoskeleton reorganisation. Various protein kinases such as the tyrosine kinases of the Src family [17, 18], of the Tec family [19, 20] and FAK [17], lipid kinases such as PI 3-kinase [17], as well as protein and lipid phosphatases [21, 22] are relocated to these structures upon platelet aggregation (Fig. 1). Recently, the critical role of two tyrosine residues of the cytoplasmic tail of β3 in platelet physiology was demonstrated *in vivo* [23]. Their mutation leads to bleeding disorders and strongly affects clot–retraction responses *in vitro*. These tyrosine residues are indeed phosphorylated upon platelet aggregation and one of them is in the context of NPXY, and this NPXY domain, once phosphorylated, is potentially able to interact with phosphotyrosine-binding domains [24]. These results highlight the critical role of αIIb/β3 outside-in signalling in platelet function *in vivo*. However, the tyrosine kinase involved in the phosphorylation of the β3 chain upon aggregation is not known. The remarkable tyrosine kinase FAK could be involved directly or indirectly, since it is recruited to the focal adhesions, phosphorylated, and activated upon platelet aggregation [17, 25]. This kinase interacts with several cytoskeletal and signalling proteins and can potentially bind to integrins. One of the signalling proteins that directly interact with FAK upon platelet aggregation is PI 3-kinase α [17]. The phosphorylation of Y397 of FAK seems to be required for its binding to the C-terminal SH2 domain of the regulatory subunit (p85α) of PI 3-kinase [26]. In addition, the SH3 domain of p85α can interact with a proline-rich sequence of FAK [17]. Interestingly, PI 3-kinase plays an important role in strengthening platelet aggregation during the irreversible phase of aggregation [27, 28].

A ROLE FOR PI 3-KINASE IN THE IRREVERSIBLE PHASE OF PLATELET AGGREGATION INDUCED BY THE THROMBIN RECEPTOR (PAR1)-ACTIVATING PEPTIDE

The PI 3-kinases are a family of enzymes that phosphorylate the D3 hydroxyl group of phosphoinositides. These lipid kinases have been implicated in multiple biological responses such as cytoskeletal rearrangements, cellular migration, cell proliferation, protection against apoptosis, or insulin-dependent metabolic processes [29]. On the basis of structural characteristics, substrate specificity, and mechanism of regulation, PI 3-kinases have been divided into three main classes [30]. The D3-phosphoinositides are now considered as intracellular second messengers. They can specifically bind functional protein modules such as pleckstrin homology (PH), SH2, or FYVE domains [29] and are able to spatially and temporally regulate membrane targeting of some critical signalling proteins. Several PI 3-kinases are present in human blood platelet [31], and some of them play an important role in their activation process. In platelets stimulated by thrombin or by the PAR1-activating peptide (TRAP), the synthesis of PtdIns(3,4,5)P_3 is rapid and transient, whereas PtdIns(3,4)P_2 accumulates upon increasing stimulation times [17, 32, 33]. Interestingly, using platelets from Glanzmann thrombasthenic patients or RGDS-treated control platelets, we have demonstrated that the synthesis of a major part of PtdIns(3,4)P_2 is dependent upon the engagement of the αIIb/β3 integrin [17, 34]. Thus, the synthesis and the accumulation of this phosphoinositide is part of the αIIb/β3 outside-in signalling. Recently, we found that ADP, secreted during platelet activation, was specifically involved as an important cofactor of TRAP for the production of this particular lipid [28]. ADP may allow a sufficient level of αIIb/β3 activation that is not obtained by these two agonists individually. The P2 family of ADP receptors is composed of two classes, namely the P2X receptors, which are ligand-gated ion channels, and the P2Y receptors, which belong to the serpentine G-protein-coupled receptor family [35]. Using selective antagonists and inhibitors, we obtained pharmacological evidence that amongst the three different platelet ADP receptors, the P2 receptor negatively coupled to adenylyl cyclase plays a key role in the αIIb/β3-dependent accumulation of PtdIns(3,4)P_2 in TRAP-stimulated platelets [28]. The intracellular machinery involved in this process is currently under investigation. Several possibilities have been suggested: i) the activation of a type I PI 3-kinase generating PtdIns(3,4,5)P_3, rapidly transformed by a 5-phosphatase like the SH2 domain-containing inositol 5-phosphatase (SHIP1) possibly regulated through integrin engagement [21, 22]; ii) inhibition of a PtdIns(3,4)P_2 4-phosphatase [36]; or iii) activation of a C2 domain-containing PI 3-kinase producing PtdIns(3)P, which is then phosphorylated by a PtdIns(3)P 4-kinase [37]. However, the precise nature of the enzymes involved in the regulation of the level of PtdIns(3,4)P_2 is still unclear.

Using two structurally distinct inhibitors of PI 3-kinase (wortmannin or LY294002), Kovacsovics *et al.* [27] suggested that PI 3-kinases may be involved in the irreversible phase of platelet aggregation induced by TRAP. A parallel between the extent of aggregation and PtdIns(3,4)P_2 labelling was also observed in thrombin-stimulated platelets [17]. However, based on these results alone, it was difficult to know whether the accumulation of this lipid is a cause or a consequence of the irreversible aggregation. Recently, we have shown that PI 3-kinase inhibitors, added when aggregation amplitude and PtdIns(3,4)P_2 levels are maximum, i.e. after 2 min of TRAP stimulation, are able to induce a very rapid and dramatic decrease in the level of PtdIns(3,4)P_2, followed by a disaggregation of platelets [28]. Since after 2 min of TRAP stimulation, PtdIns(3,4,5)P_3 has already returned to its basal level, these results strongly suggest a role for the late accumulation of PtdIns(3,4)P_2 in strengthening aggregation. The particularly active turnover of this phosphoinositide indicates that its accumulation results from a sustained PI 3-kinase activation rather than an inhibition of PtdIns(3,4)P_2 hydrolysis. The platelet disaggregation induced by PI 3-kinase inhibitors is accompanied by a rapid destabilisation of the signalling complexes associated with the cytoskeleton and, amongst the structural proteins, a specific release of MHC [28]. Thus, PtdIns(3,4)P_2 appears as a central player in a positive feedback loop, since a certain level of integrin engagement is required for its production and, in turn, this lipid influences the strengthening and the irreversibility of aggregation. It is noteworthy that secreted ADP, which is required for the accumulation of PtdIns(3,4)P_2 in TRAP-stimulated platelets as discussed above, is also required for the irreversible phase of aggregation. ADP scavengers strongly affect the synthesis of PtdIns(3,4)P_2 and transform the irreversible aggregation induced by TRAP into a reversible aggregation [28]. PtdIns(3,4)P_2 seems to play a role in the maturation of focal adhesions and is somehow upstream of the stabilisation of the actomyosin complexes. Indeed, we have observed that PI 3-kinase does not significantly influence the phosphorylation of myosin light chain in TRAP-stimulated platelets, but is involved in the control of the sustained association of MHC with the cytoskeleton.* Under these experimental conditions, PI 3-kinase inhibition induces a rapid release of MHC from the cytoskeleton followed by the disaggregation mechanism without significantly affecting the level of F-actin [28].* The mechanism by which PI 3-kinase and its product PtdIns(3,4)P_2 play this role is currently under investigation. The induction of actin–myosin contractility might be important for integrin clustering and formation of mature adhesion plaques that are required for irreversible aggregation. Moreover, myosin has been shown to interact with the tyrosine phosphorylated β3 tail of αIIb/β3 *in vitro*. As previously discussed, these tyrosine residues are required for the outside-in signalling, leading to stable platelet aggregation *in vivo* and clot retraction [23], possibly by controlling the extent of integrin clustering.

THE ROLE OF INTEGRIN-ASSOCIATED PROTEIN IN PLATELET SIGNALLING

It is becoming clear that fibrinogen binding to αIIb/β3 per se is necessary but not always sufficient to obtain the full set of responses occurring downstream of this integrin. Interestingly, it has recently been shown that integrins are able to form complexes with other membrane receptors on the same cell [38]. These integrin partners can influence their signalling. A 50-kDa protein physically associated with αIIbβ3 and αvβ3 was isolated some years ago [39]. This protein, called IAP or CD47, is a receptor for the C-terminal domain of TSP1, a large adhesive molecule [40]. IAP is composed of an extracellular immunoglobulin superfamily domain sufficient for the non-covalent interaction with integrins, five transmembrane spanning regions, and a short cytoplasmic tail. IAP is largely present in human platelets and appears to be mainly associated with αIIb/β3 and α2β1 in this model [41, 42]. However, based on the number of αIIb/β3, IAP, and α2β1 copies (about 80,000, 50,000, and 2,500, respectively), one can speculate that most of the IAP associates with αIIb/β3 in platelets. Moreover, TSP1 is abundantly secreted during activation and is thought to play a role in the irreversible phase of platelet aggregation. TSP1 can also interact with integrin-bound fibrinogen, which may further increase clustering between IAP and αIIb/β3.

Interestingly, IAP appears to be functionally coupled to heterotrimeric Gi-proteins [43], which is unusual since heterotrimeric G-proteins are normally coupled to heptahelical receptors. Based on the results obtained with pertussis toxin treatment, it is concluded that this coupling is crucial for IAP to play its role as co-stimulatory receptor of integrins, suggesting the attractive hypothesis that IAP may couple integrins to Gi-dependent pathways (Fig. 2). Moreover, triggering of IAP by the peptide RFYVVMWK (RFY), derived from the TSP1-binding sequence, also induces a rapid and strong activation of non-receptor tyrosine kinases [41].† Besides its role as an integrin partner, IAP can also signal and activate platelets independently of αIIb/β3 engagement. Indeed, we have recently observed that the antagonists of fibrinogen binding to αIIb/β3 have only a partial inhibitory effect on RFY-induced platelet aggregation. Accordingly, IAP triggering leads to a significant Glanzmann type I thrombasthenic (patients devoid of αIIb/β3) platelet aggregation.† The precise nature of the interactions allowing this novel and significant αIIb/β3-independent platelet aggregation is currently under investigation. Due to the extracellular immunoglobulin-rich domain of IAP, intercellular homophilic or heterophilic protein interactions may explain this surprising observation. Moreover, our results indicate that RFY induces, in the absence of αIIb/β3, a very rapid tyrosine phosphoryla-

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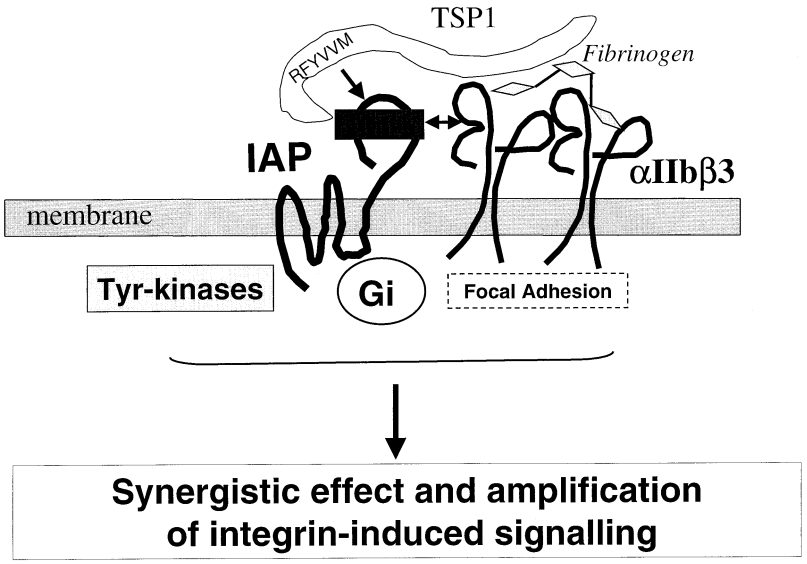

FIG. 2. The thrombospondin receptor IAP is a partner of αIIb/β3 and can modulate both its inside-out and outside-in signalling. The immunoglobulin-like domain of IAP is involved in the association with the extracellular domain of integrins. Thrombospondin may also interact, through its amino-terminal part, with αIIb/β3-bound fibrinogen. The complex thrombospondin-IAP–αIIb/β3-fibrinogen may be very efficient for the activation of signalling pathways leading to irreversible platelet aggregation.

tion of a set of proteins, including Syk and PLCγ2, activation of PI 3-kinase, and calcium signalling.* Altogether, these results provide the first molecular basis of IAP-mediated signalling. Clearly, this protein on its own or co-clustered with αIIb/β3 or α2β1 can recruit and activate several signalling molecules. IAP can influence the αIIb/β3 activation process [41] and co-activate αIIb/β3-mediated signalling pathways [38]. Based on recent results, one can envisage that IAP may synergise with αIIb/β3 for an efficient activation of PI 3-kinase, accumulation of PtdIns(3,4)P_2, and irreversible platelet activation.

CONCLUSIONS

Platelets provide an interesting model to investigate integrin signalling. Experiments dealing with adhesion of platelets to immobilised fibrinogen, binding of soluble fibrinogen induced by anti-LIBS 6 antibody, inhibition of fibrinogen binding by specific antagonists, and stimulation of platelets from patients with Glanzmann's thrombasthenia have largely contributed to our knowledge concerning αIIb/β3 signalling. Based on the critical role of αIIb/β3 in platelet functions, pharmacological inhibitors of ligand binding to αIIb/β3 have been developed, leading to the generation of active compounds now available for use to prevent thrombosis. However, a better understanding of the inside-out and outside-in αIIb/β3 signalling mechanisms should lead to the discovery of new pharmacological targets allowing the modulation of platelet-irreversible aggregation rather than its inhibition. In this respect, proteins involved in the signalling pathways controlling the irreversible aggregation, such as the PI 3-kinase pathway, but also new receptors, such as IAP, could be potential targets for new drugs.

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