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Invited Review

Adhesion-dependent signalling and the initiation of haemostasis and thrombosis

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Summary. Cell-cell and cell-extracellular matrix adhesion are critical aspects of platelet function, regulating interactions between circulating platelets in the bloodstream with the blood vessel wall. In haemostasis, platelets adhere to the subendothelial matrix of a damaged vessel, spread over the surface and recruit additional platelets within a developing platelet aggregate or thrombus. In addition to this normal physiological response, platelet adhesion is critical in the pathological process of thrombosis, where circulating platelets adhere to sclerotic lesions or undergo shear-induced aggregation within vessels occluded by atherosclerotic plaque. Under these circumstances, the resulting thrombus may result in acute myocardial infarction or stroke. Each stage of platelet adhesion and aggregation in haemostasis and thrombosis is regulated by specific cell surface adhesion receptors. Interestingly, most of the adhesive receptors studied in detail have been found not only to regulate contact adhesion, but also to transduce intracellular signals that activate the cell, initiate post-adhesion cellular events, and regulate the adhesive function of other receptors. Platelet activation triggers the cytoskeletal rearrangements that control cell shape change, spreading, secretion, aggregation and contraction. This review will focus on adhesion-dependent signalling induced by the platelet surface receptor, the glycoprotein (GP) Ib-IX-V complex, that initiates thrombus formation in both haemostasis and thrombosis under conditions of high shear blood flow. Emerging evidence suggests GP Ib-IX-V–dependent signalling may involve receptor cross-linking and the cytoplasmic signalling protein, 14-3-3 ζ.

Key words: Glycoprotein Ib-IX-V, von Willebrand Factor, Platelet activation, 14-3-3 protein

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Introduction

Blood platelets play an essential role in normal haemostasis. To prevent excessive blood loss following injury, circulating platelets adhere to the vessel wall, become activated, spread over the surface and form a platelet aggregate or thrombus. Activated platelets promote the coagulation cascade, culminating in stabilization of the thrombus by fibrin. Contractile actin filaments of the platelet cytoskeleton provide the mechanical forces underlying clot contraction. All of these events are regulated, in whole or in part, by specific adhesion receptors on the platelet surface. These receptors mediate interactions with other cells or with adhesive glycoprotein ligands in the subendothelial matrix or plasma. At high shear forces in the bloodstream, the glycoprotein (GP) Ib-IX-V complex, a constitutively expressed receptor on the platelet plasma membrane (~25,000 copies/platelet), mediates initial contact adhesion to subendothelium (reviewed in López, 1994; Andrews et al., 1997; López and Dong, 1997). This involves a specific interaction between GP Ib-IX-V and a multimeric adhesive glycoprotein, von Willebrand Factor (vWF), in the extracellular matrix (Weiss, 1995). Exposure to pathological shear stress also induces binding of soluble plasma vWF to platelet GP Ib-IX-V complex leading to thrombosis (Kroll et al., 1996). The GP Ib-IX-V complex also supports platelet rolling on immobilized vWF under flow conditions *in vitro* (Moroi et al., 1996; Savage et al., 1996). In the Bernard-Soulier syndrome, a rare congenital disease where platelets lack surface expression of functional GP Ib-IX-V complex, there is a bleeding phenotype typically characterized by prolonged bleeding from trauma or minor surgical procedures, frequent epistaxis, gingival oozing and, in women, excessive menorrhagia (reviewed in Dunlop et al., 1997). Bernard-Soulier platelets respond normally to platelet agonists such as ADP and collagen, but show impaired vWF-dependent platelet aggregation, adhere poorly to subendothelium at high shear forces, and aggregate poorly to stimulation by a-thrombin (Dunlop et al., 1998).

Binding of vWF to platelet GP Ib-IX-V induces signal transduction and platelet activation. One of the consequences of this activation is to elevate intracellular Ca^{2+} leading to activation of the platelet membrane fibrinogen receptor, the αIIbß3 integrin (GP IIb-IIIa) (reviewed in Kroll, 1994). Activated platelets secrete more vWF from α-granules and secrete agonists such as ADP from dense granules, stimulating additional platelets and promoting GP IIb-IIIa-dependent platelet aggregation. Both the adhesion receptors, GP Ib-IX-V and GP IIb-IIIa, as well as vWF multimers and ADP appear to be minimally required for optimal shear-induced platelet aggregation (Kroll, 1994). Under conditions of low shear stress, platelet receptors other than GP Ib-IX-V are more important in mediating platelet adhesion (Staatz et al., 1989; Tandon et al., 1989; Moroi et al., 1996). These include the collagen receptors, GP IV, GP VI and the α2ß1 integrin (GP Ia-IIa; VLA-2). Other integrin receptors on platelets include the vitronectin receptor, αvß3, and the fibronectin and laminin-binding ß1 integrins, α5ß1 (VLA-5) and α6ß1 (VLA-6), all of which potentially contribute to platelet-matrix interactions or aggregation in as yet undefined ways.

Platelets also interact with both leukocytes and endothelial cells during thrombosis or early stages of atherogenesis (Marcus, 1994; Ross, 1995; Frenette et al., 1995). Neutrophils not only adhere to activated platelets under flow conditions, but do so more efficiently than to activated endothelial cells (Kuijper et al., 1996), suggesting activated platelets may promote leukocyte accumulation at sites of injury. Adhesion receptors on platelets such as P-selectin, PECAM-1 and ICAM-2, known to mediate leukocyte-endothelial cell interactions (Springer, 1995; Andrews and Berndt, 1998), are candidates for regulating these interactions of platelets and other vascular cell types. *In vitro*, both P-selectin and ICAM-2 have been demonstrated to effect platelet-leukocyte adhesion (Valles et al., 1993; Diacovo et al., 1994), whereas platelet rolling on activated endothelium involves P-selectin expressed on endothelial cells and an unidentified counter-receptor on platelets (Frenette et al., 1995).

Signal transduction by these adhesion receptors on platelets or other vascular cells regulates the transition of cells from one adhesive phenotype to another, and allows progression through different stages of adhesion, spreading, migration or aggregation. The signalling mechanism of the GP Ib-IX-V complex, the focus of this review, is of particular interest because GP Ib-IX-V–dependent platelet adhesion and activation is critical to the onset and progression of haemostasis and thrombotic disease.

Structure of the platelet membrane GP Ib-IX-V complex

The platelet membrane GP Ib-IX-V complex (Fig. 1) consists of four transmembrane glycoproteins which are all members of the leucine-rich protein family (reviewed in López, 1994; López and Dong, 1997; Andrews et al., 1997). The GP Ib α-chain (~135 kDa) consists of an N-terminal, extracellular domain (His1–Glu282) that contains seven tandem leucine-rich repeats, a highly glycosylated macroglycopeptide domain, a transmembrane domain and a cytoplasmic tail of ~100-residues. In GP Ibα, the leucine-rich repeats, each of ~24-residues, are flanked by distinct N- and C-terminal disulfide-linked sequences. Immediately downstream of the C-terminal flanking sequence are three tyrosine residues (Tyr276, Tyr278 and Tyr279) embedded within a negatively-charged sequence (DLYDYYPEED); all of these tyrosines meet the consensus for sulfation by Golgi tyrosylsulfate sulfotransferase and are sulfated both in the recombinant protein expressed on mammalian cells (Dong et al., 1994; Marchese et al., 1995) and in the native molecule derived from human platelets (Ward et al., 1996). The GP Ibß-chain (~25 kDa), covalently bound to GP Ibα via a disulfide bridge near the extracellular surface of the membrane, contains a single leucine-rich domain and flanking sequences in the extracellular region, a transmembrane domain and a cytoplasmic tail of ~34 residues. GP IX (~22 kDa) is homologous to GP Ibß, but with a much shorter cytoplasmic tail of ~5 residues. GP IX forms a noncovalent 1:1 complex with GP Ib (Du et al., 1987). GP V (~82 kDa) consists of fifteen extracellular leucine-rich repeats and has a cytoplasmic tail of ~16 residues. GP V forms a noncovalent 1:2 complex with GP Ib-IX on the platelet surface (Fig. 1) (Modderman et al., 1992; López, 1994).

Ligands of the GP Ib-IX-V complex

There are two known physiological ligands for the platelet GP Ib-IX-V complex: the multimeric adhesive

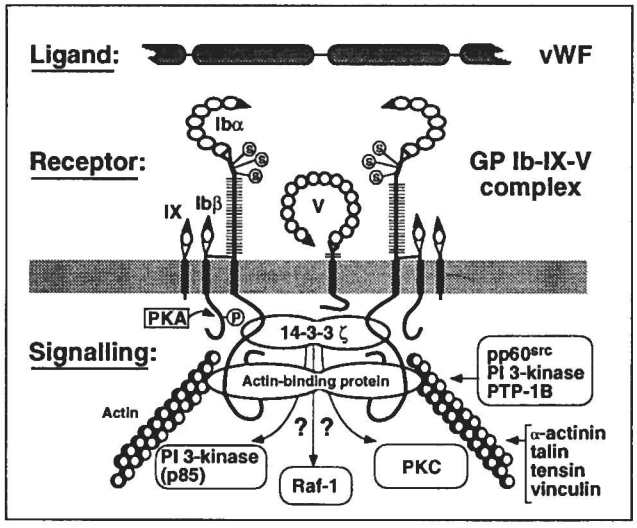

Fig. 1. The platelet membrane GP Ib-IX-V complex and associated cytoskeletal/signalling proteins potentially involved in GP Ib-IX-V-dependent signal transduction.

glycoprotein, von Willebrand Factor (vWF), and α-thrombin. Binding of vWF to platelet GP Ib-IX-V *in vivo* requires association of vWF with the extracellular subendothelial matrix or exposure of platelets and vWF in plasma to high shear forces (reviewed in Andrews et al., 1997). *In vitro*, vWF is activated to bind GP Ib-IX-V in the absence of shear by the non-physiological modulators, the bacterial glycopeptide, ristocetin, or the viper venom proteins, botrocetin or jaracetin. There is accumulating evidence that vWF binds to at least two sites within the first 282 residues of GP Ibα (López, 1994; Ward et al., 1996; Andrews et al., 1997). One of these sites involves the anionic/sulfated tyrosine sequence (Tyr276-Glu282) that is preferentially used by botrocetin, whereas an additional site(s) within the His1–Leu275 sequence is utilized mainly by ristocetin. This is based on functional analysis of (i) proteolytic fragments of GP Ibα generated by cathepsin G (Leu275/Tyr276), mocarhagin (Glu282/Asp283) and trypsin (Arg293/Thr294), (ii) anti-GP Ibα monoclonal antibodies with epitopes involving Tyr276-Arg293 (SZ2, ES85, C34) or His1-Leu275 (AK2, VM16d, AN51, AP1), and (iii) recombinant fragments of GP Ibα with site-directed mutagenesis of charged residues within or near Tyr276-Glu282 (Murata et al., 1990). In addition, blocking sulfation of the three tyrosine residues (Tyr276, Tyr278 and Tyr279) on GP Ibα diminishes ristocetin-dependent vWF binding and has a greater effect on botrocetin-dependent binding (Dong et al., 1994; Marchese et al., 1995). A functional role for the leucine-rich repeats was further suggested by a single amino acid substitution (Leu57/Phe) within the first leucine-rich repeat of GP Ibα associated with a form of Bernard-Soulier syndrome where GP Ibα is expressed in a dysfunctional form that does not bind vWF (Miller et al., 1992). In contrast, two gain-of-function pseudo-von Willebrand's disease substitution mutations (Gly233/Val or Met239/Val) are located within the flanking sequence C-terminal to the leucine-rich repeats (Dunlop et al., 1998). Both of these mutants spontaneously bind vWF in the absence of ristocetin, botrocetin or shear, implying that this domain may have a vWF recognition or regulatory function.

α-Thrombin also binds to the anionic/sulfated tyrosine sequence (Tyr276-Glu282) of GP Ibα (Marchese et al., 1995; Ward et al., 1996). The physiological role for thrombin binding to GP Ib-IX-V is not certain; however, recent evidence suggests that both GP Ib-IX-V and seven-transmembrane protease activated receptors, PAR1 and/or PAR3, may be required for optimal response of platelets at least to low concentrations of α-thrombin (Greco et al., 1997). As mentioned previously, where there is low or absent surface expression of GP Ib-IX-V in Bernard-Soulier syndrome platelets, response to thrombin is markedly impaired. The critical question, however, of whether the GP Ib-IX-V complex signals in response to thrombin binding is unproven.

In addition to vWF and α-thrombin, a family of viper venom proteins has been described that bind to the same region of GP Ibα (His1-Glu282) as the physiological ligands (Peng et al., 1992; Andrews et al., 1996 and references therein). The venom proteins are ~25-kDa heterodimers of the C-type lectin family, and are functional in that they inhibit vWF binding to GP Ib-IX-V. A number of these structurally-related venom proteins, including alboaggregin, echicetin and the rattlesnake proteins, CHH-A and CHH-B, bind to overlapping but not identical sites within the N-terminal domain of GP Ibα suggesting the possibility that they recognize different leucine-rich repeats (Andrews et al., 1996).

A receptor cross-linking mechanism for GP Ib-IX-V-dependent signalling?

Signalling by adhesion receptors on other cell types may be initiated by ligand-induced receptor cross-linking. For example, PECAM-1–dependent signalling on leukocytes in response to binding of anti-PECAM-1 monoclonal antibodies is augmented using polyvalent cross-linked Fab fragments or antibody complexes (Berman and Muller, 1995) and ICAM-3-dependent signalling on T cells also involves receptor cross-linking (Juan et al., 1994). In another example, dimerization of platelet GP IIb-IIIa induces signalling, as evidenced by tyrosine phosphorylation of the signalling protein, $pp72^{syk}$ (Du and Ginsberg, 1997). Although not definitive, there is indirect evidence that GP Ib-IX-V-dependent signalling on platelets also involves receptor cross-linking by a multivalent ligand. Firstly, a monomeric 39/34-kDa proteolytic fragment of vWF is able to bind to the GP Ib-IX-V complex and inhibit binding of multimeric native vWF, but does not activate platelets (Andrews et al., 1989). Secondly, GP Ibα is arranged on the cell surface as part of a larger receptor complex, with two or more molecules forming a cluster with the other glycoproteins of the complex (López, 1994), and is associated via the cytoplasmic region of the complex with actin-binding protein and 14-3-3 ζ protein (see below), both of which form noncovalent dimers. Finally, a 50-kDa presumably multivalent viper venom alboaggregin induces platelet activation, whereas structurally related monomeric 25-kDa venom proteins bind to the same domain on GP Ibα, but do not induce platelet activation (Andrews et al., 1996).

Signalling events associated with the GP Ib-IX-V complex

The signalling events induced by vWF binding to GP Ib-IX-V in the presence of shear, ristocetin or botrocetin include elevation of cytosolic Ca^{2+}, activation of protein kinase C (PKC) and activation of tyrosine kinase(s) (Kroll, 1994; Razdan et al., 1994; Ozaki et al., 1995; Kroll et al., 1996). Two major protein kinase substrates, the 47-kDa protein, pleckstrin, and 20-kDa myosin light chain are rapidly phosphorylated when vWF binds to platelets. A major tyrosine kinase substrate has a molecular mass of ~76 kDa, but

unknown identity (Kroll, 1994; Kroll et al., 1996). Interestingly, both the ~76-kDa species and an ~36-kDa species that are tyrosine phosphorylated on shear-induced vWF binding to platelets, are also phosphorylated in response to the viper venom protein, 50-kDa alboaggregin, that activates platelets coincident with its binding to the GP Ib-IX-V complex (Andrews et al., 1996). GP Ib-IX-V-dependent platelet activation also induces redistribution and cytoskeletal association of activated phosphatidylinositol 3-kinase (PI 3-kinase) and $pp60^{src}$ (Jackson et al., 1994). PI 3-kinase and PKC have both been implicated in activating the ligand-binding function of GP IIb-IIIa (Du and Ginsberg, 1997), a consequence of vWF binding to GP Ib-IX-V. Binding of vWF to GP Ib-IX-V also induces breakdown of phophatidylinositol 4,5-bisphosphate, phosphatidic acid generation, activation of phospholipase A2, and arachidonic acid and thromboxane A2 synthesis (Kroll, 1994). The key question, however, is how does GP Ib-IX-V initiate signal transduction, and what cytoplasmic messengers are involved in regulating these responses?

The most extensively studied cell surface signalling receptors are the family of growth factor receptors that contain functional tyrosine kinase domain(s), and the multiple membrane-spanning receptors that are coupled to cytoplasmic G-proteins. Platelets lack any known tyrosine kinase receptors, while G-protein coupled receptors on platelets include the seven-transmembrane receptors to agonists such as thrombin, epinephrine, vasopressin, platelet-activating factor (PAF) and thromboxane A_2 (reviewed in Kroll, 1994). In contrast, adhesion receptors on platelets and other cells commonly transmit signals by forming clusters with other signalling receptors, or by coupling to cytosolic kinases. Examples of adhesion receptors that signal by a functional association with the FcγRII receptor include PECAM-1 on monocytes and the neutrophil αMß2 integrin (Chen et al., 1994; Zhou and Brown, 1994). On platelets, there is some evidence that GP IIb-IIIa, GP Ib-IX-V and GP VI may all be associated with FcγRII (Berndt et al., 1993; López, 1994; Tsuji et al., 1997), although any functional significance for these interactions remains to be firmly established. A number of platelet adhesion receptors have been shown to be linked to cytosolic kinases. These include the αIIbß3 integrin, GP IIb-IIIa, that is associated with the focal adhesion kinase ($pp125^{fak}$), $p72^{syk}$ and the adaptor proteins, grb2 and shc (Du and Ginsberg, 1997). Grb2 and shc are associated with phosphotyrosine sequences in the ß3 cytoplasmic domain. The α2ß1 integrin, GP Ia-IIa, is also associated with $pp125^{fak}$ and $p72^{syk}$ (Shattil and Brugge, 1991; Lipfert et al., 1992; Asazuma et al., 1996); GP IV is linked to the src-related tyrosine kinases, $pp60^{fyn}$, $pp62^{yes}$ and $pp54/58^{lyn}$ (Huang et al., 1991; Bull et al., 1994); GP VI is associated with $pp60^{src}$ and $p72^{syk}$ (Ichinohe et al., 1995); and PECAM-1 is linked to src homology-2 (SH2) domains of shp-2 or src (Jackson et al., 1997; Lu et al., 1997). The GP Ib-IX-V complex has not been found to associate with any cytosolic kinases directly, but is directly associated with two cytoplasmic proteins, actin-binding protein and 14-3-3 ζ, that may provide a critical linkage to other signalling proteins, as discussed in the following sections.

Interaction of GP Ib-IX-V with actin-binding protein and the platelet cytoskeleton

The GP Ib-IX-V complex is the major membrane adhesion receptor linked to the platelet submembranous actin network, and vWF binding to GP Ib-IX-V induces extensive cytoskeletal rearrangements (Fox and Meyer, 1998). The platelet cytoskeleton is comprised of a network of short submembranous actin filaments (the membrane skeleton), long actin filaments extending into the cytoplasm, and an array of attached cytoskeletal proteins such as actin-binding protein, α-actinin, vinculin, tensin, talin, tropomyosin, VASP and caldesmon (Hartwig and DeSisto, 1991; Cunningham et al., 1996; Fox and Meyer, 1998). A further group of cytoskeletal-associated proteins bind to either polymerized or monomeric actin and regulate its polymerization; 30-40% of actin is filamentous in resting platelets and this level increases to 60-70% in activated platelets (Fox and Meyer, 1998). Proteins such as CAPZ and tensin cap actin polymers blocking further polymerization, whereas proteins such as thymosin $ß_4$, ASP-56 and profilin bind monomeric actin. Another regulator of cytoskeletal structure is WASP, a 62-kDa protein that binds to the signalling complex scaffold protein, $p47^{nck}$ (Dunlop et al., 1998). WASP also interacts with a member of the Rho family of GTPases, cdc42, which is involved in regulating actin polymerization. The WASP gene is the target of a rare X-linked congenital defect of platelets and T lymphocytes (Wiskott-Aldrich syndrome) associated with a cytoskeletal abnormality and characterized by accelerated platelet destruction.

An increasing number of proteins involved in cell signalling are also found to associate with the cytoskeleton, including PI 3-kinase, src-related tyrosine kinases ($pp60^{src}$, $pp60^{fyn}$, $pp62^{yes}$, $pp54/58^{lyn}$ and $pp72^{syk}$), the focal adhesion kinase $pp125^{fak}$, $p21^{ras}$ GTPase-activating protein (GAP), and tyrosine phosphatases such as PTP-1B and SHPTP1 (Jackson et al., 1994; Yuan et al., 1997; Fox and Meyer, 1998). Finally, a number of transmembrane proteins are also attached to the cytoskeleton in resting platelets, including the GP Ib-IX-V complex and the α2ß1 integrin, GP Ia-IIa (Fox and Meyer, 1998). The interaction of GP Ib-IX-V with the submembranous actin network is mediated by actin-binding protein binding to the cytoplasmic domain of GP Ibα (Andrews and Fox, 1992; Cunningham et al, 1996). The region of actin-binding protein that binds GP Ibα has been localized to internal ß sheet repeats 17-19 spanning residues 1850-2136 (Meyer et al., 1997), proximal to a cAMP-dependent protein kinase A (PKA) phosphorylation site that potentially regulates the interaction of GP Ibα and actin-binding protein. This linkage brings

into proximity the cytoplasmic domain of the GP Ib-IX-V complex and signalling proteins attached to the cytoskeleton (see above and Fig. 1). Further, the GP Ib-IX-V complex is known to regulate cytoskeletal organization in several crucial ways. Firstly, cAMP-dependent phosphorylation by PKA at Ser166 of GP Ibß is known to inhibit cytoskeletal rearrangement by inhibiting actin polymerization in response to platelet activation (Fox and Berndt, 1989). Platelet antagonists that elevate cAMP, such as prostaglandin I_2 or D_2, activate PKA and inhibit platelet activation. Secondly, vWF binding to GP Ib-IX-V on platelets leads to activation of the Ca^{2+}-dependent protease, calpain, that cleaves and dissociates a number of cytoskeletal-associated proteins including actin-binding protein and talin, and regulates recruitment of signalling proteins, PI 3-kinase, $pp60^{src}$, $pp125^{fak}$ and the protein tyrosine phosphatase, PTP-1B (Fujitani et al., 1997; Yuan et al., 1997). Finally, elevated cytosolic Ca^{2+} induced by vWF- or α-thrombin-dependent platelet activation activates both calmodulin-dependent myosin light chain kinase, enabling the phosphorylation-dependent interaction of myosin and actin that modulates actin contractility, and gelsolin (Fox and Meyer, 1998). Activation of gelsolin shears actin filaments into small fragments which act as the template for subsequent actin filament organization and polymerization during platelet activation. GP Ib-IX-V remains associated with actin-binding protein and the sheared actin filaments and becomes incorporated into the activated platelet cytoskeleton (Kovacsovics and Hartwig, 1996).

The cytoskeleton, therefore, is a complex network of both structural and signalling proteins that regulates its own organization and rearrangements in response to extracellular stimuli, and in turn, regulates the function of surface adhesion receptors to which it is attached. In addition to actin-binding protein, another protein directly associated with the cytoplasmic domain of the GP Ib-IX-V complex is 14-3-3 ζ. The following section discusses whether 14-3-3 protein may be involved in assembly of signalling complexes related to GP Ib-IX-V-dependent signal transduction.

Interaction of the GP Ib-IX-V complex with 14-3-3 protein

Du et al. (1994) first demonstrated the physical association of the GP Ib-IX-V complex with the zeta isoform of the signalling protein, 14-3-3, by its co-isolation with GP Ib-IX on immunoaffinity chromatography of platelet detergent extracts. This provided a major candidate for an early messenger in GP Ib-IX-V-dependent signal transduction. 14-3-3 proteins regulate the activity and assemblage of key signalling molecules that in turn regulate such diverse processes as mitogenesis, cell cycling, vesicular transport and apoptosis (reviewed in Aitken, 1996). Proteins reported to bind 14-3-3 include the cell death agonist BAD, Raf-1, bcr, cbl, PKCε, PKCγ, cdc25a and cdc25b phosphatases, the p85 subunit of PI 3-kinase, tyrosine hydroxylase, tryptophan hydroxylase and ADP ribosyl-transferase. The 14-3-3 protein family consists of a number of closely related, highly conserved isoforms with subunit molecular weights of ~30 kDa which form highly stable homo- and hetero-dimers (Aitken, 1996). This latter property allows them to bridge and assemble cytoplasmic proteins containing 14-3-3 recognition motifs. The 14-3-3 isoform most commonly identified as binding signalling molecules is 14-3-3 ζ.

Recent analysis of 14-3-3 binding to Raf-1 has identified two discontinuous amino acid sequences that bind 14-3-3, overlapping phosphorylation sites at Ser259 and Ser621 (Muslin et al., 1996). PKA-dependent phosphorylation of Raf-1 enhances 14-3-3 binding, while phosphatase treatment of either Raf-1 or the phosphorylated synthetic peptide containing Ser259 markedly decreases binding of 14-3-3. Both Ser259 and Ser621 are encompassed by a conserved RSXSXP motif, and this consensus sequence is contained in a number of other 14-3-3-binding proteins, including PKCε, cdc25b, bcr and BAD (Aitken, 1996). Phosphorylation of BAD also enhances 14-3-3 binding, attenuating the ability of BAD to form a complex with its physiological ligand, a homologue of the cell survival factor, bcl (Zha et al., 1996). Sequences other than RSXSXP have been reported to mediate 14-3-3 recognition by cbl (Liu et al., 1997) and the insulin-like growth factor-1 (IGF-1) receptor (Craparo et al., 1997). In the case of cbl, it was proposed that two $RX_{1\text{-}2}SX_{2\text{-}3}S$ consensus sequences may represent novel recognition motifs for 14-3-3 τ binding (Liu et al., 1997), analogous to, but distinct from the conserved RSXSXP sequence identified in Raf-1 (Muslin et al., 1996).

The GP Ib-IX-V complex and the IGF-1 receptor represent the first examples of transmembrane receptors that bind 14-3-3 (Du et al., 1994; Craparo et al., 1997). The platelet GP Ib-IX-V complex cytoplasmic domain, comprised of cytoplasmic sequences of GP Ibα (~100 residues), GP Ibß (~34 residues), GP IX (~5 residues) and GP V (~16 residues), does not contain an RSXSXP motif like that of Raf-1 and BAD, or an $RX_{1\text{-}2}SX_{2\text{-}3}S$ consensus sequence like that identified in cbl. Du et al. (1996) identified the C-terminal sequence Ser606-Leu610 (SGHSL) of GP Ibα as a potential binding site for 14-3-3, since C-terminal truncation of recombinant GP Ibα after Tyr-605 abolished 14-3-3 ζ binding and a synthetic peptide based on the sequence Gly596-Leu610 was shown to bind 14-3-3 ζ. Subsequently, our laboratory analyzed synthetic peptides corresponding to cytoplasmic sequences of GP Ibα, GP Ibß, GP IX and GP V for the ability to bind 14-3-3 ζ (Andrews et al., 1998), and identified additional sites that may participate in binding 14-3-3 ζ. These include the central region of the GP Ibα cytoplasmic domain (Arg557-Gly575) and the cytoplasmic tail of GP V. A further binding site for 14-3-3 ζ encompassed the PKA phosphorylation site in GP Ibß and serine phosphorylation of this peptide increased its affinity for 14-3-3 ζ. This effect of phosphorylation on a 14-3-3 ζ-binding sequence in GP Ibß suggests an additional effect of

PKA-dependent phosphorylation on regulating platelet activation. Since GP Ibß phosphorylation specifically inhibits actin polymerization, the increased avidity for 14-3-3 ζ is consistent with a role for this protein in the control of actin polymerization. In Raf-1, 14-3-3 binding is dependent upon phosphorylation of serine in the +3 position relative to the initial arginine in the RSXSXP motif. In the case of the RXSX(S/T)XP motif in GP Ibß, serine phosphorylation is in the +2 position. These phosphorylation events mediated by PKA, and potentially other serine protein kinases such as PKC, may be a general mechanism for regulating 14-3-3 binding in other proteins. It is interesting to note that actin-binding protein contains a potential PKA phosphorylation site, RAPSVAN (Gorlin et al., 1990) proximal to the GP Ibα-binding region (Meyer et al., 1997). This raises the possibility that actin-binding protein may interact with 14-3-3 ζ and that this interaction may stabilize GP Ib-IX-V-cytoskeleton interactions. In this regard, Dong and coworkers (1997) have recently shown that truncation of the C-terminal five residues from GP Ibα significantly affects the membrane motility of recombinant GP Ib-IX complex expressed on CHO cells, although other cytoplasmic sequences are also important for cytoskeletal attachment (Andrews and Fox, 1992; Cunningham et al., 1996). In a congenital defect associated with a variant form of Bernard-Soulier syndrome, a dinucleotide deletion in the codon for Tyr508 (TAT) of GP Ibα causes a frameshift within the transmembrane domain and truncation of the cytoplasmic tail (Kenny et al., 1997). This abnormal cytoplasmic domain results in a form of the receptor that does not anchor to the cytoskeleton.

In addition to the phosphorylated 14-3-3-binding sequences on Raf-1 and other proteins, both phosphorylated and non-phosphorylated non-consensus sequences in GP Ibα, cbl and the insulin-like growth factor receptor and insulin receptor substrate-1 (IRS-1) can also bind 14-3-3 (Du et al., 1996; Craparo et al., 1997; Liu et al., 1997; Ogihara et al., 1997). Interestingly, analogous sequences to the C-terminal SGHSL motif in GP Ibα were conserved in a number of other 14-3-3-binding proteins including Raf-1 (QHSLP) and cbl (RHSLP). In these latter examples, the postulated 14-3-3-binding residues were not at the C-terminus, but adjacent to a conserved turn-inducing proline immediately C-terminal to the GHSL-like sequence. Further, the GHSL-like sequence in cbl encompassing Ser619 (RHSLP) overlapped the $RX_{1-2}SX_{2-3}S$ consensus 14-3-3-binding sequence, RHSLPFS (Liu et al., 1997).

Finally, how might 14-3-3 ζ associated with GP Ib-IX-V be involved in transmitting intracellular signals? Since 14-3-3 proteins are dimeric, they have the capacity to simultaneously bind to two or more ligands, such as GP Ib-IX-V and other signalling proteins like Raf-1, thereby regulating at least some of the signalling events downstream of the receptor (Fig. 1). In addition, 14-3-3 ζ has been shown to bind and, in some circumstances, activate PKC (Aitken, 1996); as discussed above, activation of PKC resulting in phosphorylation of pleckstrin (p47) is induced by the GP Ibα ligands, vWF, α-thrombin and 50-kDa alboaggregin (Kroll, 1994; Andrews et al., 1996). Further, since 14-3-3 ζ associates with PI 3-kinase (Aitken, 1996), it may be involved in PI 3-kinase activation and redistribution induced by vWF binding to platelet GP Ib-IX-V. This linkage would allow for interaction with $pp60^{src}$ and $pp60^{fyn}$ which in turn associate with PI 3-kinase in platelets (Gutkind et al., 1990). In summary, therefore, the ability of 14-3-3 ζ to bind to a number of diverse proteins, apparently through different amino acid sequence recognition sites within Raf-1, cbl and other proteins as well as GP Ib-IX-V, suggests that 14-3-3 protein may be involved in the assembly/regulation of GP Ib-IX-V-related signalling complexes.

Final comments

In developing potential anti-thrombotic agents that target the key receptor initiating shear-dependent platelet adhesion and activation, the GP Ib-IX-V complex, it is vital to consider both its ligand binding and signalling functions. Binding of multimeric vWF to the GP Ib-IX-V complex triggers the cytoskeletal rearrangements and other post-adhesion events associated with the secondary stages of adhesion, spreading, secretion, aggregation and contraction. Two key questions concerning GP Ib-IX-V-dependent platelet activation are how binding of extracellular ligand induces the receptor to transmit signals, and what are the specific cytoplasmic messengers that control early stages of the signalling pathway? Recent findings at both levels, including identification of 14-3-3 ζ as one of the molecules potentially involved in assemblage of GP Ib-IX-V–related signalling complexes, have significantly advanced our overall understanding of the initiation and progression of haemostasis and thrombosis.

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