Glycoprotein IIb-IIIa-dependent aggregation by glycoprotein Ibα is reinforced by a Src family kinase inhibitor (PP1)-sensitive signalling pathway

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It has been proposed that the receptor for von Willebrand factor (vWF), glycoprotein (GP)Ib-IX-V, signals through the same pathway as the collagen receptor, GPVI, namely via Src kinases, the Fc receptor (FcR) γ-chain and Syk, leading to tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2). The aim of the present study was to assess the functional significance of this pathway in platelet activation by GPIb-IX-V. In washed platelets, vWF/ristocetin and vWF/botrocetin stimulate weak tyrosine phosphorylation of the FcR γ-chain, Syk and PLCγ2, but not the adaptor LAT (linker for activation of T-cells), which is localized to glycolipid-enriched membrane domains. Increases in tyrosine phosphorylation were blocked by the Src family kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-D-3,4-pyrimidine (PP1). Under the same conditions, neither stimulus induced activation of PLCγ2 nor functional responses, such as Ca^{2+} elevation, secretion or GPIIb-IIIa-dependent aggregation. In contrast, in platelet-rich plasma (PRP), threshold concentrations of ristocetin or asialo-vWF stimulated GPIb-dependent biphasic aggregation, in which the second phase was blocked by PP1. Importantly, a significant component of the initial phase and the complete second phase of aggregation was blocked by GPIIb-IIIa receptor antagonists in PRP. Higher concentrations of ristocetin stimulated GPIIb-IIIa-independent agglutination in PRP. These results demonstrate that GPIb-IX-V initiates activation of GPIIb-IIIa in PRP through an undefined pathway that is reinforced by a PP1-sensitive pathway. In contrast, activation of GPIbα in washed platelets does not promote functional responses.

Key words: Src kinases, tyrosine phosphorylation, von Willebrand factor.

INTRODUCTION

The interaction between the platelet glycoprotein (GP)Ib-IX-V receptor complex and its endogenous ligand, von Willebrand factor (vWF), plays a key role in maintaining haemostasis and promoting thrombus formation in pathological conditions [1]. vWF bridges exposed subendothelial collagen to the GPIb-IX-V receptor of circulating platelets, promoting adhesion and supporting activation of GPIIb-IIIa which leads to irreversible platelet aggregation [2,3].

The GPIb-IX-V receptor complex is constitutively expressed on the surface of platelets at a level of 25000 copies/platelet. The receptor complex consists of four distinct gene products of the leucine-rich-repeat superfamily, GPIbα, GPIbβ, GPIX and GPV [4]. The A1 domain of vWF contained within the fragment Leu^{480}–Gly^{718} recognizes and binds to the N-terminus of GPIbα (His^{1}–Glu^{282}) [5]. A conformational change of vWF is required to enable it to bind GPIbα, an effect achieved under physiological conditions by exposure to the subendothelium or high shear stress [6,7]. In the absence of high shear, vWF can also be induced to bind GPIbα in the presence of the viper toxin, botrocetin, the antibiotic, ristocetin, or upon treatment with neuraminidase (also known as asialo-vWF) [8–12]. A number of snake venom toxins have also been reported to bind directly to GPIbα and inhibit vWF-dependent agglutination in washed platelets, including echicetin, and *Crotalus horridus horridus* (CHH) A and B [13,14]. On the other hand, echicitin has recently been reported to promote platelet aggregation in plasma through the interaction with the immunoglobulin, IgMκ [15]. A dimeric (25 kDa) viper venom C-type lectin, alboaggregin-B, also promotes agglutination of platelets through cross-linking of GPIbα [16]. A related tetrameric (50 kDa) C-type lectin, alboaggregin-A, binds to both GPIbα and the collagen receptor, GPVI, promoting powerful GPVI-dependent increases in cytosolic Ca^{2+}, protein kinase C activation, dense granule secretion and GPIIb-IIIa-dependent aggregation [16–19].

The nature of the intracellular signals that lead to cytoskeletal rearrangement and functional responses mediated by binding of vWF to the GPIb-IX-V complex have been poorly characterized, in part because functional responses have not been seen in all studies. For example, a number of groups have reported that the GPIb–vWF interaction promotes Ca^{2+} elevation and secretion, whereas others did not observe these responses [20–23]. A number of signalling pathways have been proposed to underlie these events, including Ca^{2+} elevation, activation of tyrosine kinases, phosphoinositide 3-kinase and cytoskeletal rearrangement.

Recently, GPIbα has been proposed to signal through a pathway analogous to that used by the collagen receptor, GPVI, namely through tyrosine phosphorylation of the Fc receptor (FcR) γ-chain, the tyrosine kinase Syk and phospholipase Cγ2 (PLCγ2) [21]. This study [21] extends two earlier reports, which describe tyrosine phosphorylation and activation of Syk by

Abbreviations used: ACD, acid/citrate/dextrose; FcR, Fc receptor; GEM, glycolipid-enriched membrane domain; GP, glycoprotein; GST, glutathione S-transferase; 5-HT, 5-hydroxytryptamine; LAT, linker for activation of T-cells; mAb, monoclonal antibody; PLCγ2, phospholipase Cγ2; PP1, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-D-3,4-pyrimidine; PRP, platelet-rich plasma; SH, Src homology; SLP-76, SH2-containing leucocyte phosphoprotein of 76 kDa; TBS-T, Tris-buffered saline/Tween 20; vWF, von Willebrand factor.

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vWF/botrocetin and a GPIbα-specific antibody [24,25]. In addition, a recent study demonstrated that a monomeric, soluble fragment of vWF A1 bound GPIbα and induced Syk phosphorylation, but that this was not sufficient for activation of GPIIb-IIIa [26]. The present study was undertaken to address the significance of the FcR γ-chain signalling pathway in platelet activation in response to vWF.

EXPERIMENTAL

Materials

vWF, prepared from freeze-dried Factor VIII concentrates as described previously [27,28], was kindly provided by Dr M. C. Berndt (Baker Medical Research Institute, Melbourne, Australia). This preparation of vWF has previously been shown to contain high molecular mass multimers (approx. 1×10^6 to 1×10^7 Da) as determined by electrophoretic analysis [29,30], giving an average molecular mass of 4.3×10^6 Da based on laser densitometry and integration of the SDS/agarose electrophoretic pattern. Asialo-vWF was prepared as previously described [8]. Botrocetin was purified from the venom of *Bothrops jararaca* according to published procedures [28,31]. Alboaggregin-B was purified from the venom of *Trimeresurus albolabris* as described previously [17]. Convulxin, purified from the venom of *Crotalus durissus terrificus*, was kindly donated by Dr M. Leduc and Dr C. Bon (Unite des Venens, Institut Pasteur, Paris, France) [32]. Ristocetin was purchased from Sigma (Poole, Dorset, U.K.). Anti-GPIbα monoclonal antibody (mAb) 6D1 was a gift from Dr B. Coller (Mount Sinai Medical Centre, New York, NY, U.S.A.). Anti-(SLP-76) [where SLP-76 corresponds to Src homology (SH)2-containing leucocyte phosphoprotein of 76 kDa] sheep polyclonal serum was generously provided by Dr G. Koretzky (Abramson Family Cancer Center, University of Pennsylvania, PA, U.S.A.). Anti-phosphotyrosine mAb 4G10 and anti-LAT (where LAT corresponds to linker for activation of T-cells) were purchased from Upstate Biotechnology (TCS Biologicals Ltd., Botolph Claydon, Buckingham, Bucks., U.K.). The anti-PLCγ2 and anti-Syk mAbs were gifts from Dr M. G. Tomlinson (DNAX Research Institute, Palo Alto, CA, U.S.A.). The fluorophore-labelled anti-(P-selectin) antibody was obtained from Becton Dickinson (Oxford, U.K.). 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-D-3,4-pyrimidine (PP1) was purchased from Calbiochem–Novabiochem (Nottingham, U.K.). The enhanced chemiluminescence, ECL®, reagents were obtained from Amersham International (Cardiff, Wales, U.K.). [^{32}P]Ortho-phosphoric acid and [^{3}H]5-hydroxytryptamine (5-HT) were from NEN Life Science Products (Boston, MA, U.S.A.), and Fura 2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, U.S.A.). Other reagents were from previously described sources.

Preparation of human platelets

Blood from drug-free volunteers was taken into tri-sodium citrate [3.2% (w/v)] for platelet-rich plasma (PRP) studies, and into acid/citrate/dextrose (ACD; 42 mM citric acid, 85 mM sodium citrate and 67 mM glucose) for washed platelet studies. In both instances, a ratio of anticoagulant to whole blood of 1:9 was used. PRP was isolated from whole blood by centrifugation at 200 ***g*** for 20 min. PRP containing 3.2% (w/v) tri-sodium citrate was used in aggregation studies. Platelets were isolated from PRP containing ACD by centrifugation at 620 ***g*** for 20 min in the presence of prostacyclin (0.1 μg/ml), apyrase (5 units/ml) and indomethacin (2.8 μM). Platelets were then resuspended in ACD containing apyrase (5 units/ml) and indomethacin (2.8 μM), and centrifuged at 620 ***g*** for 20 min and resuspended at a concentration of 5×10^8 cell/ml in a modified Hepes buffer (140 mM NaCl, 5 mM KCl, 15 mM Hepes, 5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) containing fibrinogen (1 mg/ml). Platelets were also prepared under a variety of other conditions, which differed from the above protocol in the use of centrifugation speeds, and in the presence or absence of Hepes (15 mM), apyrase (5 units/ml), indomethacin (10 μM), Ca^{2+} (1 mM) and fibrinogen (1 mg/ml) either through the isolation procedure or in the final resuspension [33,34]. Stimulations were performed at 37 °C for 90 s in the presence of EGTA (1 mM), unless otherwise stated, with continuous stirring at 1200 rev./min. EGTA was absent in aggregation studies.

Immunoprecipitation studies

Platelet stimulations were stopped by the addition of an equal volume of ice-cold lysis buffer (300 mM NaCl, 20 mM Tris/HCl, 2 mM EGTA, 2 mM EDTA, 2 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotonin and 1 μg/ml pepstatin A, pH 7.3) containing the non-ionic detergent Nonidet P40 [2% (v/v)]. Detergent-insoluble material was removed by centrifugation at 15000 ***g*** for 10 min and lysates were pre-cleared with 30 μl of a 50% (w/v) suspension of Protein A–Sepharose in Tris-buffered saline/Tween 20 [TBS-T; 20 mM Tris/HCl, 137 mM NaCl and 0.1% (v/v) Tween 20, pH 7.3] for 1 h at 4 °C. Proteins were immunoprecipitated with a specific antibody and 30 μl of Protein A–Sepharose for 2 h at 4 °C. The Protein A–Sepharose pellet was then washed once in lysis buffer and three times in TBS-T before the addition of Laemmli sample buffer [4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 50 mM Tris/HCl, pH 6.8], and was subsequently boiled for 10 min.

Immunoblotting

Proteins were separated by SDS/PAGE on 8–18% gradient gel slabs and transferred on to PVDF membranes using a semi-dry transfer system (Trans-blot SD; Bio-Rad). Membranes were blocked by incubation in 10% (w/v) BSA in TBS-T for 1 h to prevent the non-specific binding of antibodies. Primary and secondary antibodies were incubated with the PVDF membranes for 1 h. Membranes were washed in TBS-T following each incubation with antibodies, and then analysed using an ECL® detection system.

Fusion protein precipitation studies

A glutathione S-transferase (GST) fusion protein containing the tandem SH2 domains of Syk was prepared and bound to GSH-coated agarose beads as previously described [35]. Precipitation of platelet proteins with the fusion construct was performed as previously described [36].

Glycolipid-enriched membrane domain (GEM) preparation

Platelet stimulations were stopped using ice-cold lysis buffer containing the non-ionic detergent Brij 58 [2% (v/v)]. The lysate was mixed with an equal volume of 80% (w/v) sucrose, and overlaid with a 25% (w/v) sucrose solution and lysis buffer, both containing Brij 58 [1% (v/v)]. The samples were centrifuged at 200000 ***g*** for 2.5 h at 4 °C, and seven equal fractions were obtained from the top of the gradient. The visible light scattering band (corresponding to fractions 2 and 3, the 'GEM fraction') and an equal volume of fractions 6 and 7, the 'soluble fraction', were obtained for immunoprecipitation studies as described above.

Measurement of phosphatidic acid and pleckstrin phosphorylation

Platelets were labelled with [^{32}P]orthophosphoric acid (0.5 mCi/ml) for 1 h at 37 °C. Stimulations were performed for 5 min in the presence of $CaCl_2$ (1 mM) with continuous stirring at 1200 rev./min. Samples for pleckstrin phosphorylation analysis were taken into Laemmli sample buffer and resolved by SDS/PAGE as described above. Samples for phosphatidic acid analysis were terminated by the addition of an equal volume of chloroform/methanol (1:1, v/v). Phospholipids were extracted from the sample by centrifugation at 1000 ***g*** for 5 min at 4 °C in the presence of HCl/EDTA [42% (v/v) 10 M HCl and 58 mM EDTA]. The lower phase was collected from each sample and resolved by TLC. Lipids were detected by autoradiography and analysed by densitometry.

Measurement of [^{3}H]5-HT secretion

Platelets were labelled with [^{3}H]5-HT (0.5 μCi/ml) at 37 °C for 1 h. Stimulations were performed for 5 min in the presence of apyrase (5 units/ml) with continuous stirring at 1200 rev./min, and were terminated by the addition of an equal volume of 6% (v/v) glutaraldehyde. Supernatants were analysed for [^{3}H]5-HT content by scintillation spectrometry. [^{3}H]5-HT concentration is expressed as a percentage of total tissue content.

Measurement of cytosolic Ca^{2+}

Washed platelets were incubated with fura 2 acetoxymethyl ester (2 μM) for 1 h at 37 °C. Stimulations were conducted for 5 min in the absence or presence of $CaCl_2$ (1 mM), with stirring, at 37 °C in a PerkinElmer LS50B spectrofluorimeter. Fluorescence excitation was conducted at 340 and 380 nm, and emission was measured at 510 nm. Data is presented as the internal Ca^{2+} concentration (nM), as calculated by the software program FL Winlab (PerkinElmer).

Flow cytometry

Washed platelets (5×10^6 cells/ml) were stimulated for 10 min under non-stirring conditions at room temperature (~ 22 °C) in the presence of $CaCl_2$ (1 mM) and saturating amounts of fluorophore-labelled P-selectin antibody. After a 5-fold dilution in Tyrodes-Hepes (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM Hepes, 5 mM glucose and 1 mM $MgCl_2$, pH 7.3), samples were analysed using a Facscalibur flow cytometer (Becton Dickinson). The light scatter and the fluorescence signals were set in logarithmic gain and 1×10^4 ungated events were acquired from each sample. Results were analysed in the form of a histogram plot (cell count versus fluorescence intensity in channel 1).

Analysis of data

All experiments were performed at least three times and data are shown as means ± S.E.M. Statistical analysis was conducted using Student's paired *t* test.

RESULTS

GPIbα promotes weak protein tyrosine phosphorylation in washed platelets

We initially investigated the effect of a number of ligands specific to the GPIb-IX-V receptor complex on tyrosine phosphorylation using washed platelets. We did not observe major changes throughout the length of the gel in response to vWF (10 μg/ml) in the presence of either botrocetin (10 μg/ml) or ristocetin (1 mg/ml), the GPIbα-specific snake venom toxin, alboaggregin-B (1 μg/ml), asialo-vWF (100 μg/ml) or upon crosslinking of the receptor complex with specific antibodies (Figure 1A, and results not shown). In contrast, the GPVI-specific snake venom toxin, convulxin (1 μg/ml), stimulated a substantial increase in tyrosine phosphorylation of a wide spectrum of proteins (Figure 1A).

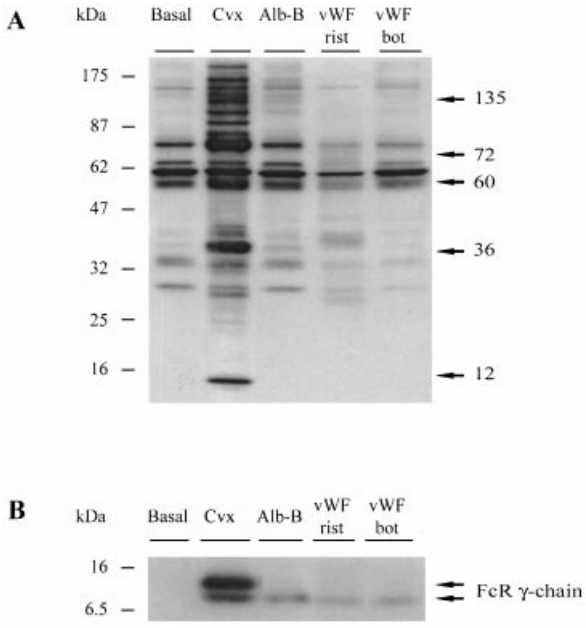

Figure 1 GPIbα promotes minimal protein tyrosine phosphorylation in washed platelets

Human washed platelets (5×10^8/ml) were stimulated by convulxin (Cvx; 1 μg/ml), alboaggregin-B (Alb-B; 1 μg/ml), vWF/ristocetin (vWF-rist; 10 μg/ml vWF and 1 mg/ml ristocetin) or vWF/botrocetin (vWF-bot; 10 μg/ml vWF and 10 μg/ml botrocetin). (**A**) Reactions were stopped by the addition of an equal volume of Laemmli sample buffer. (**B**) Experiments were stopped by the addition of an equal volume of cold lysis buffer, and the FcR γ-chain was precipitated from lysates by addition of the tandem SH2 domains of Syk expressed as a GST fusion protein. Proteins were separated by SDS/PAGE (8–18% gradient gel) and Western blotted for phosphotyrosine with mAb 4G10. Blots are representative of five experiments.

It is possible that increases in phosphorylation of individual proteins in response to activation of the GPIb-IX-V receptor complex may not have been apparent in whole-cell lysates. To address this, proteins were precipitated from platelet lysates using specific antibodies or GST fusion proteins and analysed for phosphotyrosine by Western blotting using the mAb 4G10. Consistent with previous reports, stimulation of platelets with vWF/ristocetin and vWF/botrocetin resulted in weak tyrosine phosphorylation of the FcR γ-chain and Syk. Tyrosine phosphorylation of the FcR γ-chain was demonstrated following its precipitation using the tandem SH2 domains of the tyrosine kinase Syk (Figure 1B), and by the presence of a characteristic doublet in Syk immunoprecipitates (Figure 2A) [21,24,25]. In contrast, we did not observe tyrosine phosphorylation of the low-affinity immunoreceptor, FcγRIIA, which is readily observed on a one-dimensional gel because of its migration away from other phosphorylated proteins, under either experimental condition (Figure 2A, and results not shown). Alboaggregin-B also stimulated weak tyrosine phosphorylation of the FcR γ-chain, but did not induce a significant increase in phosphorylation

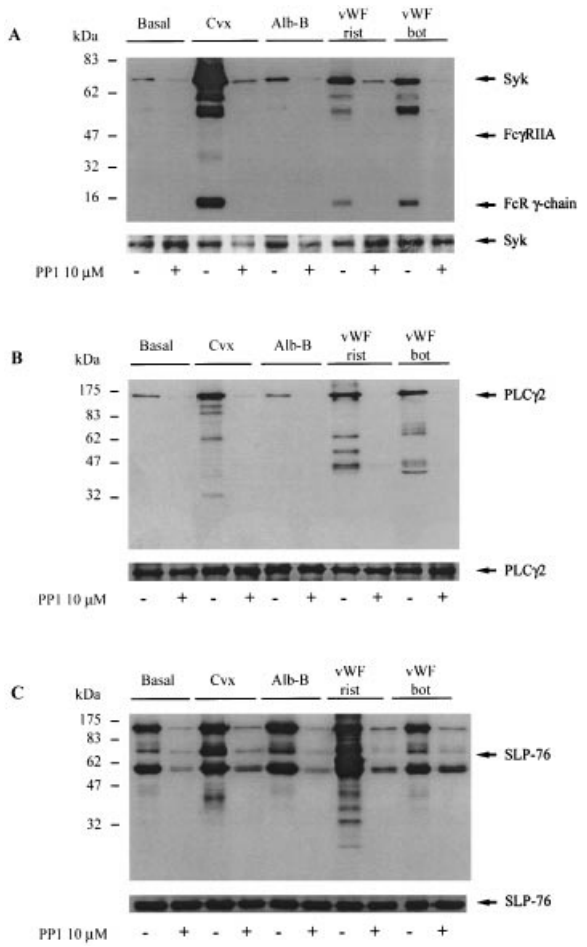

Figure 2 GPIbα promotes phosphorylation of Syk, SLP 76 and PLCγ2 in washed platelets

Human washed platelets (5×10^8/ml) were preincubated for 5 min with either vehicle [0.1% (v/v) DMSO] or the Src family kinase inhibitor, PP1 (10 μM). This was followed by stimulation with convulxin (Cvx; 1 μg/ml), alboaggregin-B (Alb-B; 1 μg/ml), vWF/ristocetin (vWF-rist; 10 μg/ml vWF and 1 mg/ml ristocetin) or vWF/botrocetin (vWF-bot; 10 μg/ml vWF and 10 μg/ml botrocetin). Experiments were stopped by the addition of an equal volume of cold lysis buffer. (**A**) Syk, (**B**) PLCγ2 or (**C**) SLP-76 were isolated from lysates by immunoprecipitation with anti-Syk, anti-PLCγ2 or anti-(SLP-76) antibodies respectively. Proteins were separated by SDS/PAGE (8–18% gradient gel) and Western blotted for phosphotyrosine with mAb 4G10. Blots were subsequently stripped and re-probed with the anti-Syk, anti-PLCγ2 and anti-(SLP-76) antibodies to check that equal amounts of protein had been loaded. Blots are representative of three experiments.

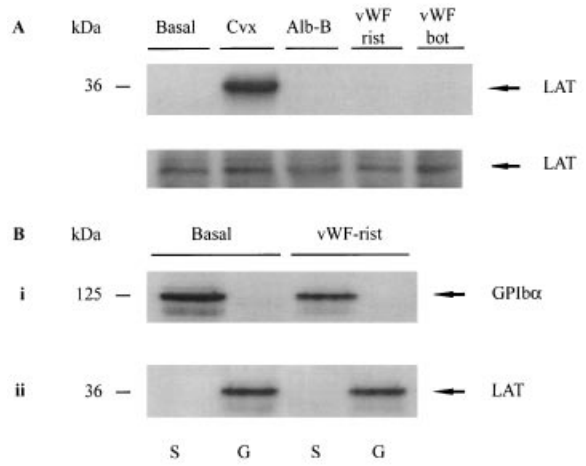

Figure 3 GPIbα does not stimulate phosphorylation of LAT in washed platelets

(**A**) Human washed platelets (5×10^8/ml) were stimulated by convulxin (Cvx; 1 μg/ml), alboaggregin-B (Alb-B; 1 μg/ml), vWF/ristocetin (vWF-rist; 10 μg/ml vWF and 1 mg/ml ristocetin) or vWF/botrocetin (vWF-bot; 10 μg/ml vWF and 10 μg/ml botrocetin). Experiments were stopped by the addition of an equal volume of cold lysis buffer and LAT was isolated from lysates by immunoprecipitation with an anti-LAT antibody. Proteins were separated by SDS/PAGE (8–18% gradient gel) and Western blotted for phosphotyrosine with mAb 4G10. Blots were subsequently stripped and re-probed with the anti-LAT antibody to check equal amounts of protein had been loaded. (**B**) Human washed platelets (2×10^9/ml) were stimulated with vWF/ristocetin (10 μg/ml vWF and 1 mg/ml ristocetin). Experiments were stopped by the addition of an equal volume of cold lysis buffer containing 1% (v/v) Brij 58. Samples were centrifuged at 200 000 ***g*** in a sucrose gradient to separate platelet GEM (G) and soluble (S) fractions. GPIbα and LAT were isolated from both fractions by immunoprecipitation with anti-GPIbα and anti-LAT antibodies respectively. Proteins were separated by SDS/PAGE (8–18% gradient gel) and Western blotted for GPIbα and LAT. Blots are representative of three experiments.

of Syk (Figures 1B and 2A), whereas asialo-vWF had no effect on tyrosine phosphorylation of either protein (results not shown). Phosphorylation of the FcR γ-chain and Syk in response to the GPVI-specific snake venom toxin, convulxin, was significantly stronger than that to vWF/ristocetin, vWF/botrocetin and alboaggregin B (Figures 1B and 2A). vWF/ristocetin and vWF/botrocetin, but not alboaggregin-B, also stimulated tyrosine phosphorylation of PLCγ2, which approached 50% of the response to convulxin (Figure 2B). Interestingly, the pattern of proteins co-precipitating with PLCγ2 in response to vWF/ristocetin, vWF/botrocetin and convulxin were distinct from each another (Figure 2B), and included unidentified tyrosine phosphorylated proteins of 45, 50 and 60 kDa. Tyrosine phosphorylation of FcR γ-chain, Syk and PLCγ2 in response to convulxin and GPIbα receptor ligands was inhibited in the presence of the Src family kinase inhibitor PP1 (Figures 2A and 2B).

The adapter molecules SLP-76 and LAT have been shown to play a critical role in platelet activation by GPVI [34,37]. We were therefore interested to determine if they also play a role in the response to activation of GPIbα. vWF/ristocetin was observed to promote an increase in SLP-76 tyrosine phosphorylation, whereas there was minimal change in response to vWF/botrocetin and alboaggregin-B (Figure 2C). Convulxin induced a much greater level of tyrosine phosphorylation of SLP-76 than seen with vWF/ristocetin. In all cases, SLP-76 was observed to co-precipitate with a 130 kDa phosphorylated protein, which has previously been reported to be the adaptor SLP-76-associated phosphoprotein of 130 kDa ('SLAP-130')/Fyb [37]. The Src kinase inhibitor PP1 completely inhibited phosphorylation of SLP-76 by vWF/ristocetin, suggesting a role for Src kinases and possibly Syk in this response. In contrast, vWF/ristocetin, vWF/botrocetin and alboaggregin-B had no significant effect on tyrosine phosphorylation of the membrane adapter LAT, whereas convulxin promoted a marked response (Figure 3A).

The GPIb-IX-V receptor complex exists outside of GEMs

GPVI signalling occurs in specific regions of the platelet membrane known as GEMs (P. Wonerow and S. P. Watson, unpublished work). These regions are characterized by their re-

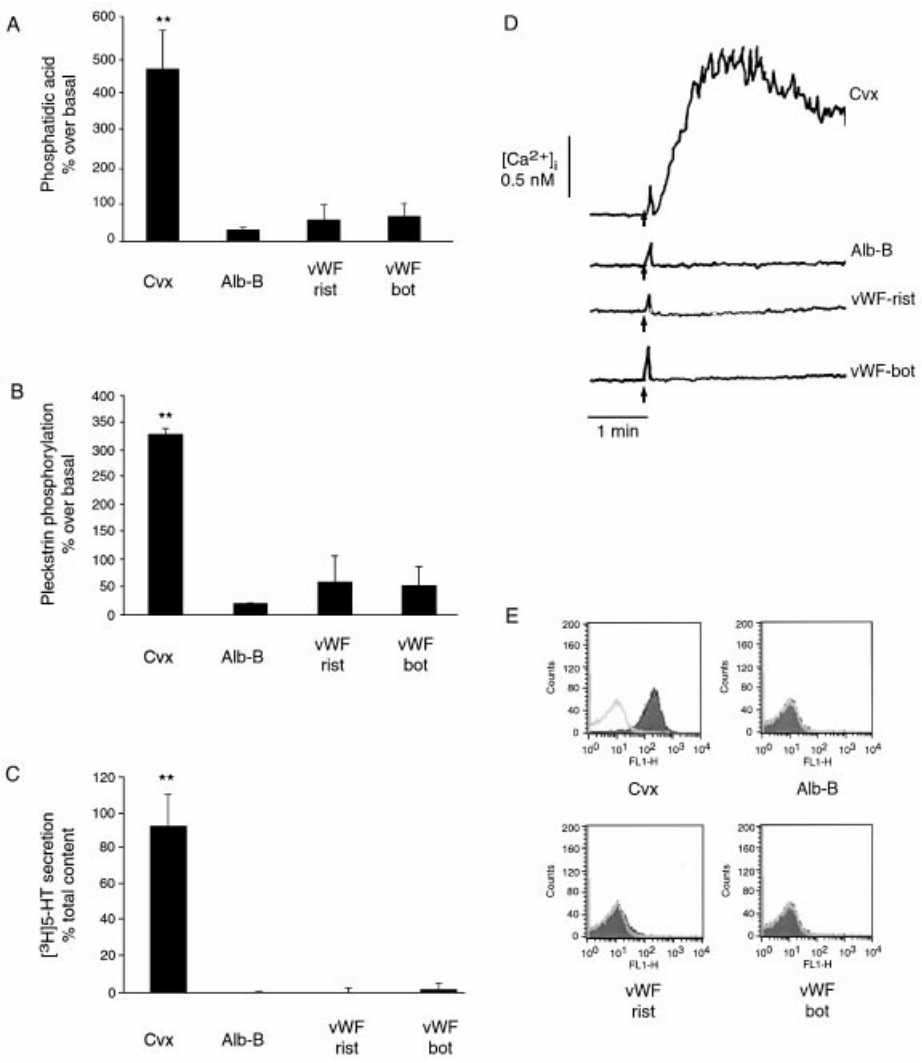

Figure 4 GPIbα activation does not promote functional responses in human washed platelets

Human washed platelets (1×10^9/ml) labelled with [^{32}P]orthophosphoric acid or [^{3}H]5-HT were stimulated with convulxin (Cvx; 1 μg/ml), alboaggregin-B (Alb-B; 1 μg/ml), vWF/ristocetin (vWF-rist; 10 μg/ml vWF and 1 mg/ml ristocetin) or vWF/botrocetin (vWF-Bot; 10 μg/ml vWF and 10 μg/ml botrocetin) for 5 min. (**A**) Phosphatidic acid formation. Reactions were stopped by addition of an equal volume of chloroform/methanol (1:1, v/v). Phospholipids were separated by TLC and analysed by autoradiography. (**B**) Pleckstrin phosphorylation. Lysates were separated by SDS/PAGE and analysed by autoradiography. Results are presented as percentage increases over basal (means ± S.E.M.). (**C**) 5-HT secretion. Reactions were stopped by the addition of an equal volume of 6% (v/v) glutaraldehyde solution and analysed by scintillation counting. Results are presented as percentage release of total content (means ± S.E.M.). (**D**) Fura 2-loaded human platelets (1×10^8/ml) were stimulated by convulxin (1 μg/ml), alboaggregin-B (1 μg/ml), vWF/ristocetin (10 μg/ml vWF and 1 mg/ml ristocetin) and vWF/botrocetin (10 μg/ml vWF and 10 μg/ml botrocetin) in the presence of $CaCl_2$ (1 mM) in a spectrofluorimeter cuvette with dual excitation at 340/380 nm and emission at 510 nm. Intracellular Ca^{2+} concentration was calculated using the software FL Winlab (PerkinElmer). Arrows indicate the time of agonist addition. (**E**) Human platelets (5×10^7/ml) were incubated with fluorescently-labelled anti-(P-selectin) antibody during stimulation by convulxin (1 μg/ml), alboaggregin-B (1 μg/ml), vWF/ristocetin (10 μg/ml vWF and 1 mg/ml ristocetin) or vWF/botrocetin (10 μg/ml vWF and 10 μg/ml botrocetin). P-selectin binding was determined by FACS analysis. Results are shown as measurements of fluorescent intensity (shaded areas). Basal levels are shown as solid grey lines. Results are representative of four experiments performed. FL1-H, fluorescence intensity in channel 1.

sistance to mild detergents and relatively high proportion of signalling molecules. Specific markers of GEMs include the adapter molecule LAT and the Src kinases Fyn and Lyn [38,39]. In light of the inability of GPIbα to phosphorylate LAT, we set out to determine whether the GPIbα receptor is present within these domains. The presence of GPIbα in soluble and GEM fractions was investigated by immunoprecipitation and subsequent immunoblotting. GPIbα was localized to the soluble fraction, but was absent from the GEM fraction (Figure 3B); furthermore, GPIbα did not translocate to the GEM fraction upon activation by vWF/ristocetin. In contrast, the adapter LAT was present within the GEM fraction as assessed by immunoprecipitation followed by immunoblotting with a LAT-specific antibody (Figure 3B).

GPIbα activation does not promote PLCγ2 activity and functional responses

In order to clarify the functional consequence of PLCγ2 phosphorylation induced by GPIbα activation, we investigated increases in phosphatidic acid and pleckstrin phosphorylation as indirect measurements of PLC activity. vWF/ristocetin, vWF/botrocetin and alboaggregin-B did not promote a significant increase in either phosphatidic acid or pleckstrin phosphorylation

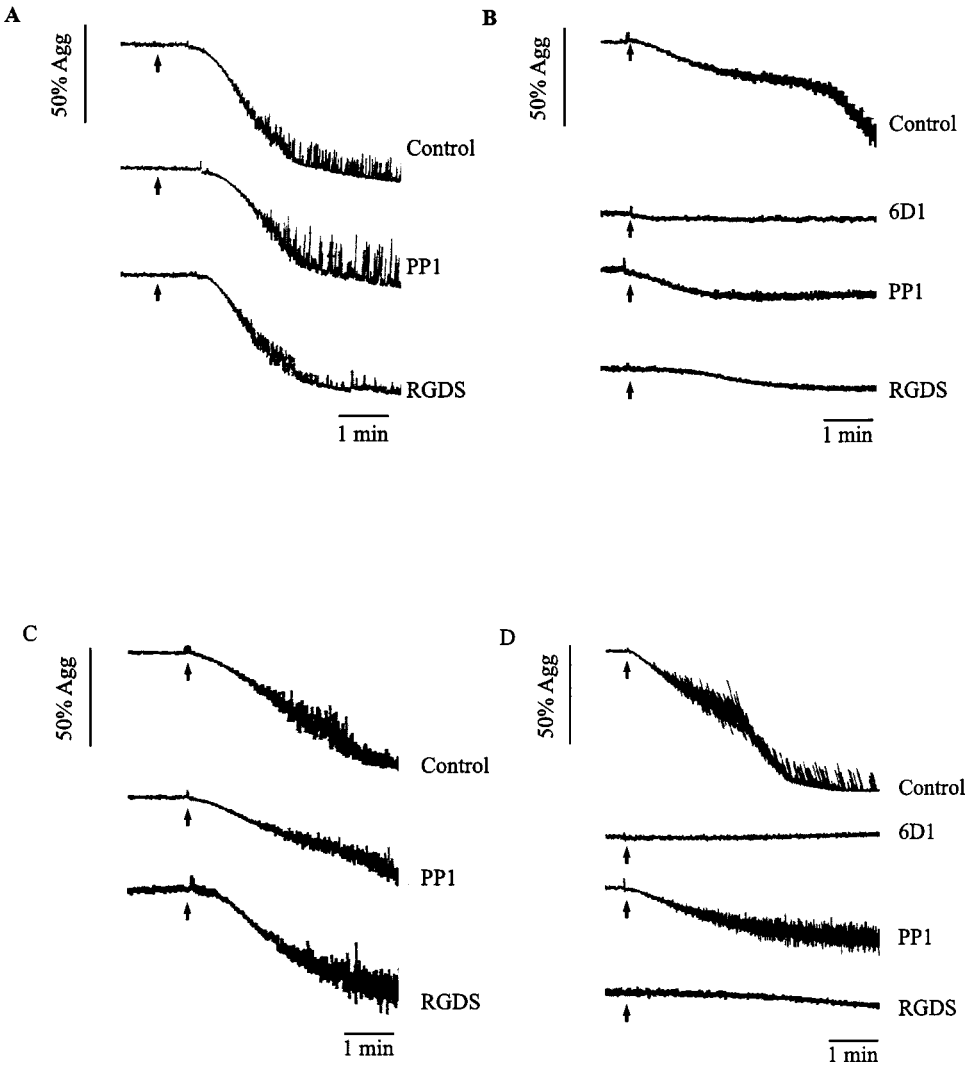

Figure 5 Comparison of the GPIb–vWF response between washed platelets and PRP

Human washed platelets (3×10^8 platelets/ml) (**A**) and human PRP (3×10^8 platelets/ml) (**B**–**D**) were preincubated with vehicle [0.1% (v/v) DMSO], Src family kinase inhibitor PP1 (10 μM), RGDS peptide (1 mM) or anti-GPIbα mAb 6D1 (10 μg/ml) followed by stimulation with vWF/ristocetin (10 μg/ml vWF and 1 mg/ml ristocetin) (**A**), ristocetin (1.5 mg/ml) (**B**), ristocetin (2 mg/ml) (**C**) or asialo-vWF (10 μg/ml) (**D**). Experiments were conducted in aggregometer cuvettes with stirring at 37 °C. Arrows indicate the time of agonist addition. Results are representative of five experiments.

over basal levels, whereas strong increases were seen in response to convulxin (Figures 4A and 4B). This suggests that GPIb–vWF-dependent PLCγ2 phosphorylation is not sufficient to promote activation of the enzyme.

We also examined the ability of GPIb–vWF to promote increases in cytosolic Ca^{2+} concentration and dense granule secretion. Convulxin stimulated a significant release of [^{3}H]5-HT ($P < 0.01$), a marker of dense granules, whereas vWF/ristocetin, vWF/botrocetin and alboaggregin-B had no effect (Figure 4C). Convulxin also stimulated a rapid increase in cytosolic Ca^{2+} above basal ($P < 0.01$) (Figure 4D), whereas vWF/ristocetin, vWF/botrocetin and alboaggregin-B again had no effect either in the absence or presence of the extracellular cation (Figure 4D). These results suggest that increases in tyrosine phosphorylation of the above proteins in response to GPIbα do not promote functional events.

We proceeded to use flow cytometry to assess whether GPIbα might activate a subpopulation of platelets. Fluorophore-labelled antibodies were used to measure P-selectin surface expression in response to GPIbα agonists. Convulxin promoted marked P-selectin binding, a marker of platelet α-granule secretion (Figure 4E), whereas alboaggregin-B, vWF/ristocetin and vWF/botrocetin did not promote an increase in binding. These results suggest that GPIbα activation does not promote α-granule secretion in washed platelets and provides evidence against a subpopulation of platelets which are sensitive to GPIbα.

We proceeded to investigate the nature of the aggregation response to GPIbα activation. The increase in light transmission induced by all concentratins of vWF/ristocetin, vWF/botrocetin and alboaggregin-B was reduced by less than 10% in the presence of the GPIIb-IIIa antagonist RGDS (1 mM) or the Src family kinase inhibitor PP1 (10 μM) as shown in Figure 5(A). This was a constant finding under a wide range of experimental conditions used in the preparation of washed platelets, including varying parameters such as the anticoagulant, centrifugal forces and presence of fibrinogen, apyrase, indomethacin and Ca^{2+}. Fur-

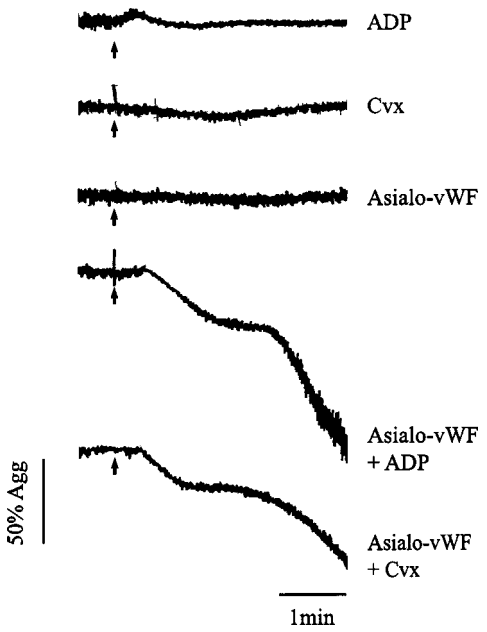

Figure 6 Asialo-vWF synergizes with convulxin and ADP

Human PRP (3×10^8 platelets/ml) was stimulated with threshold concentrations of ADP (0.5 μM), convulxin (Cvx; 0.01 μg/ml), asialo-vWF (AvWF; 3 μg/ml), asialo-vWF and ADP in combination, and asialo-vWF and convulxin in combination. Experiments were conducted in aggregometer cuvettes with stirring at 37 °C. Arrows indicate the time of agonist addition. Results are representative of five experiments.

thermore, aggregation was completely inhibited by mAb 6D1 (10 μg/ml), raised against GPIbα. These results demonstrate that, in washed platelets, the increase in light transmission in an aggregometer is primarily due to GPIbα-mediated agglutination, a process mediated by the passive crosslinking of adjacent GPIb-IX-V receptor complexes by vWF.

GPIbα promotes GPIIb-IIIa-dependent aggregation in PRP

Following the absence of functional responses in washed platelets, we conducted studies in the more physiological setting of plasma. Studies were conducted with the antibiotic ristocetin, as this was the most powerful of the GPIbα ligands in inducing tyrosine phosphorylation under washed conditions. This may be because of the ability of vWF/ristocetin to cluster a greater number of GPIbα receptors than either alboaggregin-B or vWF/botrocetin. Low concentrations of ristocetin (1.5 mg/ml) promoted weak, biphasic aggregation in agreement with previous studies [40,41]. Furthermore, in contrast with the results in washed platelets, the response to ristocetin was blocked by up to 90% by the GPIIb-IIIa antagonists RGDS (1 mM) and lotrafiban (10 μM) demonstrating that it was primarily GPIIb-IIIa-dependent (Figure 5B, and results not shown). At a slightly higher concentration of ristocetin (2 mg/ml) the response consisted of only one phase, which was insensitive to inhibitors of GPIIb-IIIa and the Src family kinase PP1 (Figure 5C), mimicking the agglutination response in washed platelets (Figure 5A). Asialo-vWF, which does not require a modulator such as ristocetin or botrocetin to bind GPIbα, has previously been reported to stimulate powerful GPIIb-IIIa-dependent aggregation in PRP [8,9]. In agreement with this, asialo-vWF (10 μg/ml) stimulated a more pronounced biphasic, GPIIb-IIIa-dependent aggregation in PRP than observed with ristocetin (Figure 5D). The response to all GPIbα agonists was completely inhibited by the anti-GPIbα mAb 6D1 in plasma (Figures 5B and 5D).

The ability of ristocetin and asialo-vWF to stimulate GPIIb-IIIa-dependent aggregation in plasma provided the opportunity to use the Src kinase inhibitor, PP1, to assess the functional significance of tyrosine phosphorylation in this response. PP1 inhibited only the second phase of aggregation to ristocetin and asialo-vWF (Figures 5B and 5D) at a concentration that completely inhibited the response to collagen in PRP (results not shown). This demonstrates that activation of Src kinases is not required for initiation of GPIIb-IIIa activation by GPIbα, but may play a role in the second phase of the response.

GPIbα synergizes with other platelet receptors

Following the observation that GPIbα promotes GPIIb-IIIa-dependent aggregation in PRP and tyrosine phosphorylation of FcR γ-chain, Syk and PLCγ2 in washed platelets, we went on to examine the possibility that GPIbα interacts with other platelet receptors to promote aggregation and associated functional responses in PRP and washed platelets. Concentrations of asialo-vWF that were below threshold for platelet aggregation in PRP were added in combination with threshold concentrations of the GPVI-specific snake venom toxin, convulxin, or ADP. Asialo-vWF (3 μg/ml), in combination with ADP (0.5 μM) or convulxin (0.01 μg/ml), promoted biphasic aggregation which reached a maximum after 4 min (Figure 6). Threshold concentrations of ristocetin, in combination with ADP and convulxin, were also observed to promote platelet aggregation, albeit at a lower level than that of asialo-vWF (results not shown). In contrast, experiments conducted in washed platelets using vWF/ristocetin or asialo-vWF in combination with threshold concentrations of ADP and convulxin did not result in platelet aggregation (results not shown).

DISCUSSION

In the present study we have confirmed the observations of Falati et al. [21] that GPIbα activates the FcR γ-chain pathway in washed platelets, but demonstrate that this does not give rise to functional responses in washed platelets. In contrast, ristocetin and asialo-vWF were shown to induce biphasic GPIIb-IIIa-dependent aggregation in PRP, in which the second phase was inhibited by the Src family kinase inhibitor PP1. It is unclear whether the inhibitory effect of PP1 is mediated through inhibition of events regulated downstream of the GPIbα receptor or a second receptor, such as GPIIb-IIIa. While the physiological significance of the present observations are unclear, bearing in mind that shear stress is the usual physiological modulator, a recent report provides evidence that the mechanism of vWF–GPIbα binding by ristocetin and shear are similar, suggesting that some vWF modulators may be more physiological in nature than first thought [42].

A number of explanations for the paradoxical increase in tyrosine phosphorylation but absence of functional response in washed platelets should be considered. It is notable that although the magnitude of the increase in tyrosine phosphorylation of the FcR γ-chain, Syk and PLCγ2 varies between GPIbα receptor ligands, it is always less than that seen in response to activation of the collagen receptor, GPVI. Furthermore, there are also no major changes in the pattern of tyrosine phosphorylation in whole-cell lysates in response to GPIbα, suggesting that the results may reflect an increased susceptibility of phosphorylated proteins to be immunoprecipitated rather than a relative increase in phosphorylation. In either case, there are approxi-

mately five times more receptors for GPIbα than for GPVI on the platelet surface, and these results suggest that GPIbα must be poorly coupled to the FcR γ-chain pathway or that only a subpopulation of receptors mediate the increase in tyrosine phosphorylation. In the former case, it is possible that the generation of intracellular signals at multiple sites across the platelet surface is insufficient to reach a threshold to promote activation, despite the fact that the net sum of these gives rise to measurable phosphorylation. The signalling by GPIbα may therefore be subliminal, occurring as a consequence of minor changes in the positioning of kinases and phosphatases within the membrane. If, on the other hand, only a subpopulation of receptors gives rise to the increase in phosphorylation it becomes less clear why this does not generate a functional response. One possible explanation is provided by the observation that the adapter LAT, which is present in specialized signalling structures known as GEMs, does not undergo a significant increase in tyrosine phosphorylation in response to activation of GPIbα. LAT has been shown to play a critical role in signalling through GPVI by facilitating its recruitment to GEMs (P. Wonerow and S. P. Watson, unpublished work). Furthermore, a large portion of GPIb-IX-V is bound to the cytoskeleton by filamin, leaving a very low number of receptors for interaction with GEMs [43]. It is also noteworthy that the pattern of co-precipitating proteins with PLCγ2 induced by GPIbα ligands is distinct from that induced by convulxin, indicating assembly into distinct signalling complexes. This may also account for the lack of functional response.

Although we were unable to induce functional responses upon stimulation of GPIbα in washed platelets with vWF/ristocetin and vWF/botrocetin, there are a number of reports in the literature of responses induced through the receptor complex [3,12,22,23,44–46]. It was therefore important to consider the experimental conditions used. Despite the use of a variety of isoforms of vWF and its modulators, and conditions for the preparation of washed platelets, such as the use of different anticoagulants, physiological buffers or the presence of Ca^{2+}, fibrinogen, apyrase and indomethacin, we were unable to detect functional responses. Furthermore, there is no clear difference in the experimental conditions used by other groups to see functional events in washed platelets, to those used in our study [9,12,20,21,23,44–46]. It is therefore possible that a difference in vWF multimer size may account for these differences, bearing in mind that monomeric vWF has no biological activity [2,47]. We consider this to be an unlikely explanation for the inability to obtain functional results in the present study as the preparation of vWF has been well characterized and used in many studies over the years. The mode of vWF presentation is another important consideration, since the extracellular portion of GPIbα to which vWF binds is dependent on the modulator used [2,48]. However, we obtained similar results using two distinct modulators, ristocetin and botracetin, a snake toxin, alboaggregin B, and also with cross-linking with F(ab′)2 fragments of various antibodies (results not shown).

In contrast with the results in washed platelets, ristocetin and asialo-vWF stimulated biphasic aggregation in PRP [8,9]. This suggests that plasma cofactors may enhance other GPIbα ligands to promote aggregation in washed platelet preparations. The situation, therefore, may be analogous to that reported by Navdaev et al. [15], where the GPIbα-specific snake venom toxin, echicetin, requires IgMκ for induction of GPIIb-IIIa-dependent aggregation.

It was of particular interest that the Src family kinase inhibitor PP1 only blocked the second phase of aggregation to asialo-vWF and ristocetin in PRP, demonstrating that phosphorylation of FcR γ-chain, Syk and PLCγ2, all of which were shown to be Src kinase-dependent in washed platelets, is not required for the initial response. In this case, however, it is unclear whether this is due to an effect on signalling by GPIbα or an alternative receptor, possibly GPIIb-IIIa, which also signals through Src kinases. Threshold concentrations of ristocetin and asialo-vWF were also observed to synergize with threshold concentrations of ADP and convulxin in PRP, whereas there was no interaction between these stimuli in washed platelets. The latter observation further suggests that the increase in tyrosine phosphorylation of Syk and PLCγ2 observed in response to GPIbα stimulation in washed platelets is of little functional significance. The mechanism underlying the synergy in PRP requires further investigation, but it may have profound physiological relevance in the normal sequence of events underyling platelet regulation in damaged vessels.

In conclusion, the present study has confirmed earlier reports that GPIbα stimulates tyrosine phosphorylation of FcR γ-chain, Syk and PLCγ2 through a Src kinase-dependent pathway, but has shown that this is unable to induce GPIIb-IIIa-dependent aggregation in washed platelets. On the other hand, GPIbα promotes biphasic aggregation in PRP in which the second phase of aggregation is blocked by PP1. We speculate that GPIbα plays a critical initiating role in the activation of platelets *in vitro* when exposed to vWF in plasma. Experiments under flow conditions are required to investigate this further.

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