Biochemical and Biophysical Research Communications 443 (2014) 580–585

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A critical role for the regulation of Syk from agglutination to aggregation in human platelets

Chun-Ho Shih, Tin-Bin Chiang, Wen-Jeng Wang *

Chang Gung University of Science and Technology, Kwei-Shan, Tao-Yuan, Taiwan

ARTICLE INFO

Article history:
Received 24 November 2013
Available online 8 December 2013

Keywords:
Agglucetin
Convulxin
Glycoprotein Ibα
Integrin αIIbβ3
Syk

ABSTRACT

Agglucetin, a tetrameric glycoprotein (GP) Ibα agonist from Formosan *Agkistrodon acutus* venom, has been characterized as an agglutination inducer in human washed platelets (WPs). In platelet-rich plasma (PRP), agglucetin dramatically elicits a biphasic response of agglutination and subsequent aggregation. For clarifying the intracellular signaling events from agglutination to aggregation in human platelets, we examined the essential signaling molecules involved through the detection of protein tyrosine phosphorylation (PTP). In WPs, an anti-GPIbα monoclonal antibody (mAb) AP1, but not a Src kinase inhibitor PP1, completely inhibited agglucetin-induced agglutination. However, PP1 but not AP1 had a potent suppression on platelet aggregation by a GPVI activator convulxin. The PTP analyses showed agglucetin alone can cause a weak pattern involving sequential phosphorylation of Lyn/Fyn, Syk, SLP-76 and phospholipase Cγ2 (PLCγ2). Furthermore, a Syk-selective kinase inhibitor, piceatannol, significantly suppressed the aggregating response in agglucetin-activated PRP. Analyzed by flow cytometry, the binding capacity of fluorophore-conjugated PAC-1, a mAb recognizing activated integrin αIIbβ3, was shown to increase in agglucetin-stimulated platelets. Again, piceatannol but not PP1 had a concentration-dependent suppression on agglucetin-induced αIIbβ3 exposure. Moreover, the formation of signalosome, including Syk, SLP-76, VAV, adhesion and degranulation promoting adapter protein (ADAP) and PLCγ2, are required for platelet aggregation in agglucetin/fibrinogen-activated platelets. In addition, GPIbα-ligation via agglucetin can substantially promote the interactions between αIIbβ3 and fibrinogen. Therefore, the signal pathway of Lyn/Fyn/Syk/SLP-76/ADAP/VAV/PLCγ2/PKC is sufficient to trigger platelet aggregation in agglucetin/fibrinogen-pretreated platelets. Importantly, Syk may function as a major regulator for the response from GPIbα-initiated agglutination to integrin αIIbβ3-dependent aggregation in human platelets.

1. Introduction

The platelet glycoprotein (GP) Ib-IX-V complex, consisting of four subunits, GPIbα, GPIbβ, GPIX and GPV, plays a pivotal role in vascular biology and is constitutively expressed on platelet membrane with a ratio of 2:2:2:1 [1,2]. The interaction of GPIb-IX-V, a non-integrin adhesion receptor, with multimeric von Willebrand factor (vWf) in the subendothelial matrix mediates platelet adhesion at high shear in the hemostatic response to vessel wall injury. In thrombosis, pathologic shear stress induces binding of platelet GPIb-IX-V to plasma vWf, initiating platelet aggregation. Accumulating evidences showed that vWf binds to at least two sites within the first 282 residues of GPIbα [2]. The venom proteins modulating the GPIbα–vWf interaction have several implications for the elucidation of biological mechanism underlying haemostasis and thrombosis [3]. It was established that platelet–platelet interactions are mediated via GPIbα-mediated agglutination and integrin αIIbβ3 (also known as GPIIb-IIIa complex)-dependent aggregation. Agglutination results from the passive cross-linking of adjacent platelets by the multimeric stimulator, whereas aggregation requires the activation of integrin αIIbβ3 through inside-out signaling. Since GPIbα can signal to integrin αIIbβ3 and then cause platelet activation through distinct signaling pathways. It is well-known that three responses of platelet activation may occur, including agglutination, aggregation, or a combination of agglutination and subsequent aggregation [4].

Currently, a variety of heterodimeric or multimeric C-type lectin-like snake venom proteins have been identified as platelet activators or inhibitors by binding to specific receptors on platelet

Abbreviations: ADAP, adhesion and degranulation promoting adapter protein; FcRγ, Fc receptor γ-chain; FITC, fluorescein isothiocyanate; GP, glycoprotein; LAT, linker for activation of T-cells; mAb, monoclonal antibody; pAb, polyclonal antibody; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLCγ2, phospholipase Cγ2; PRP, platelet-rich plasma; PTP, protein tyrosine phosphorylation; SLP-76, SH2-domain-containing leucocyte protein of 76 kDa; vWf, von Willebrand factor; WPs, washed platelets.

* Corresponding author. Address: Chang Gung University of Science and Technology, No. 261 Wen-Hwa 1st Rd, Kwei-Shan, Tao-Yuan 33303, Taiwan. Fax: +886 3 211 8866.

E-mail address: wjwang@gw.cgust.edu.tw (W.-J. Wang).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.001

membrane, such as GPIb, GPIa/IIa and/or GPVI [3,5]. As described by previous investigations, some components have multiple functions to activate platelets via GPIb as well as GPVI, such as alboaggregin-A [6]. However, some components specifically bind to GPIb and thereby affect the interactions of GPIb with vWf, such as agkistin [7]. Agglucetin, a platelet agglutination inducer from Formosan pit viper, has been identified as a platelet membrane GP Ibα agonist and can directly agglutinate formalin-fixed platelets in the absence of vWf [8]. In washed platelets (WPs), agglucetin has the ability to induce agglutinating response and elicits the surface exposure of integrin αIIbβ3 with a GPIb-dependent manner. Since the enhanced expression of functional αIIbβ3 can diminish by a GPIb-cleaving metalloproteinase crotalin [9] and pretreating platelets with staurosporine or BAPTA-AM can completely suppress the exposure of functional αIIbβ3. Therefore, the intracellular events for the activation of protein kinase C (PKC) and intracellular calcium mobilization are involved in the agglucetin/GPIb/αIIbβ3 signaling cascade [10]. In human platelet-rich plasma (PRP), agglucetin can dramatically elicit a biphasic response of platelet agglutination and subsequent aggregation, implying that other cofactors are required to couple GPIb-mediated agglutination to αIIbβ3-dependent aggregation.

GPIb not only mediates the physical adherence of platelets to the sites of high shear tress, such as the stenotic atherosclerotic arteries, but also initiates signal transduction, leading to the activation of integrin αIIbβ3 and then platelet aggregation [11,12]. However, the molecule mechanism of GPIb-mediated integrin αIIbβ3 activation is still unclear. Recently, a number of hypothetical pathways from GPIb to αIIbβ3 have been proposed. Although the tyrosine kinase-based signaling cascades have not been fully established, those signaling molecules, including Src family kinases [13], phosphoinositide 3-kinase (PI3K) [14] and phospholipase Cγ2 (PLCγ2) [11], may play essential roles in GPIb-mediated platelet activation.

Indeed, GPIb-IX-V complex plays an essential role on platelet adhesion and thrombus formation, whereas the signaling pathways underlying in human platelets remains poorly defined. In this study, we sought to clarify GPIb-related signaling mechanisms. Especially, the signaling events involved from agglutination to aggregation in human platelets via agglucetin-clustering GPIb was further investigated.

2. Materials and methods

2.1. Snake venom proteins and reagents

Agglucetin and convulxin were respectively purified from the crude venoms of Formosan *Agkistrodon acutus* and *Crotalus durissus terrificus* (Latoxan, Valence, France) by the methods described previously [10]. Human fibrinogen, piceatannol, wortmannin, RO-31-7549, N-ethylmaleimide, Na_3VO_4, phenylmethylsulfonyl fluoride (PMSF), Nonidet P-40, leupeptin, aprotinin, pepstatin A and tris (hydroxymethyl) aminomethane (Tris) were obtained commercially from Sigma Chemical (St. Louis, MO). Pyrazolopyrimidine 1 (PP1) [15] was obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Anti-GPIbα monoclonal antibody (mAb) AP1 [16] raised against the N-terminus of GPIbα (residues 1–275) was supplied by Dr. Montgomery (Blood Center of Southeastern Wisconsin, Milwaukee, WI). Fluorescein isothiocyanate (FITC)-PAC-1 mAb [17] was purchased from Becton Dickinson (San Jose, CA). Anti-phosphotyrosine mAb 4G10 and anti Fc receptor γ-chain (FcRγ) polyclonal antibody (pAb) were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Fyn pAb (FYN3), anti-Lyn mAb (H-6), anti- linker for activation of T-cells (LAT) pAb (FL-233), anti-Syk mAb (4D10), anti- SH2-domain-containing leucocyte protein of 76 kDa (SLP-76) mAb (F-7), anti-VAV mAb (D-7), anti-PI-3K (p85α) mAb (B-9), anti-PKC mAb (A-3), anti-PLCγ2 mAb (B-10), anti-adhesion and degranulation promoting adapter protein (ADAP, N-18) pAb and protein A/G plus-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of analytical grade.

2.2. Platelet preparation and agglutination/aggregation assay

Human blood collected in acid citrate dextrose (9:1, v/v) was centrifuged at $100\times g$ at room temperature for 10 min to obtain PRP. Human washed platelet suspensions were prepared according to a previously described method [10]. WPs were suspended in HEPES buffer (20 mM HEPES, 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) and platelet response was then monitored using an aggregometer (Payton Scientific) at 37 °C with stirring (900 rpm). After the addition of platelet activator, the extent of platelet agglutination or aggregation was continually monitored for 6 min by turbidimetry and expressed as increase in light transmission. The effect of various inhibitors on agglucetin- or convulxin-induced platelet activation was tested after 2 min pre-incubation of the inhibitor with WPs.

2.3. Measurement of protein tyrosine phosphorylation

WPs (5×10^8 platelets/ml, 750 μl) in an aggregometer cuvette were stimulated by a platelet inducer (agglucetin or convulxin), without or with human fibrinogen. Aliquots (45 μl) of platelet suspensions were removed at indicated time intervals and lysed by adding the aliquots (5 μl) of HEPES buffer containing 10% SDS, 20 mM N-ethylmaleimide, 20 mM Na_3VO_4, 50 mM EDTA and 10 mM PMSF. Following centrifugation, the supernatants were separated on SDS–PAGE (6–20% acrylamide gradient) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk in Tris-buffered saline plus Tween-20 (TBS-T), tyrosine phosphorylated proteins were detected by a mAb 4G10 followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. Bound antibody was detected by a chemiluminescence system (Millipore, Billerica, MA).

2.4. Immunoprecipitation

Platelet stimulations were lysed by adding an equal volume of ice-cold IP buffer (2% (v/v) Nonidet P-40, 20 mM Tris, 300 mM NaCl, 10 mM EDTA, 1 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, pH 7.3).

Detergent-insoluble material was removed by centrifugation and lysates were pre-cleared with protein A/G plus-agarose in TBS-T. Antibodies were subsequently added and samples were rotated overnight at 4 °C. The agarose pellet was washed sequentially in lysis buffer and TBS-T before addition of Laemmli sample buffer. Proteins were separated by SDS–PAGE and electrically transferred onto PVDF membranes. Membranes were washed in TBS-T after each incubation and developed using a chemiluminescence system.

2.5. Flow cytometry analysis

Human WPs (3×10^7/ml) were pre-incubated without (DMSO added) or with protein kinase inhibitor at 37 °C with mixing but without stirring. Following the co-incubation of 10 min, agglucetin was thereafter added to trigger platelet activation and then probed with FITC-PAC-1 mAb, or mouse FITC-nonimmune IgG (as a negative control, 1:50 dilution), for a further 10 min-incubation. Platelets were then fixed with 1% formalin for 10 min. After washing and resuspending in PBS, platelets with a total volume of 100 μl were diluted to 1 ml with PBS and analyzed on a FACSCalibur flow

cytometry (Becton Dickinson, Mountain View, CA) using excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals from 10,000-gated cells were collected to calculate mean fluorescence intensity of single or aggregated platelet.

2.6. Statistics

All data were presented as mean ± SEM (n = 3–6). Student's t-test was used to assess the significant statistical differences.

3. Results and discussion

The increasing evidences for a critical role of Src kinases in GPIb-dependent platelet activation have been proposed [13]. We, therefore, investigated whether platelet agglutination via agglucetin is a Src-dependent response. Using the preparation of human WPs, we firstly compared the mode of action of agglucetin and convulxin, a GPVI activator [1], on platelet activation. As shown in Fig. 1A, GPIb-dependent platelet agglutination by agglucetin could completely abolish by a specific GPIbα mAb AP1 (10 μg/ml), but not a Src kinase inhibitor PP1 (10 μM). Conversely, PP1, but not AP1, was shown to have a potent suppression on GPVI-dependent platelet aggregation by convulxin (Fig. 1B). This result revealed that Src kinase activity is not a prerequisite for agglucetin-induced platelet agglutination. PP1, a tyrosine kinase inhibitor with specificity for Src, exerted a distinct effect on agglucetin- and convulxin-induced platelet activation. Thus, platelet agglutination by agglucetin is a Src-independent process.

During platelet activation by different inducers, numerous proteins become phosphorylated at tyrosine residues. Using immunoblotting with an anti-phosphotyrosine mAb 4G10, we subsequently examined the protein tyrosine phosphorylation (PTP) profiles in human platelets stimulated by agglucetin and convulxin. Fig. 1C shows that only a pattern of weak and few PTP, of molecular masses 155, 76, 72, 59, 55, 30 and 26 kDa, was found under platelet activation by agglucetin. However, convulxin could elicit a substantial increase on PTP with a wide range of signaling proteins (Fig. 1D). Those tyrosine-phosphorylated proteins as presented in Fig. 1C were shown to have more intensive and rapid phosphorylation following convulxin stimulation. Additionally, other PTPs appeared with molecular masses of 95, 85, 80, 36, 28, 25 and 10 kDa were also heavily phosphorylated.

Previously, Falati et al. [18] proposed that Lyn and Fyn, two Src family kinases, play critical roles in the initial step of platelet activation. To identify substrates of Src family kinases that are situated downstream of GP Ibα, tyrosine-phosphorylated proteins from agglucetin-stimulated platelets were examined. Induction of tyrosine phosphorylation in platelet lysates was detectable as soon as 0.5 min upon platelet activation. As shown in Fig. 1C, several protein bands in the range of 70–155 kDa had become phosphorylated during the initial stimulation of 4 min. To confirm those corresponding proteins as molecular weights indicated in Fig. 1C, immunoprecipitation analysis was subsequently performed. 4G10 and specific antibodies recognizing intracellular signaling molecules were used for detecting the level of PTP in immunoprecipitates from agglucetin-activated platelets. As shown in Fig. 2A, those tyrosine-phosphorylated bands are the same blots after stripping

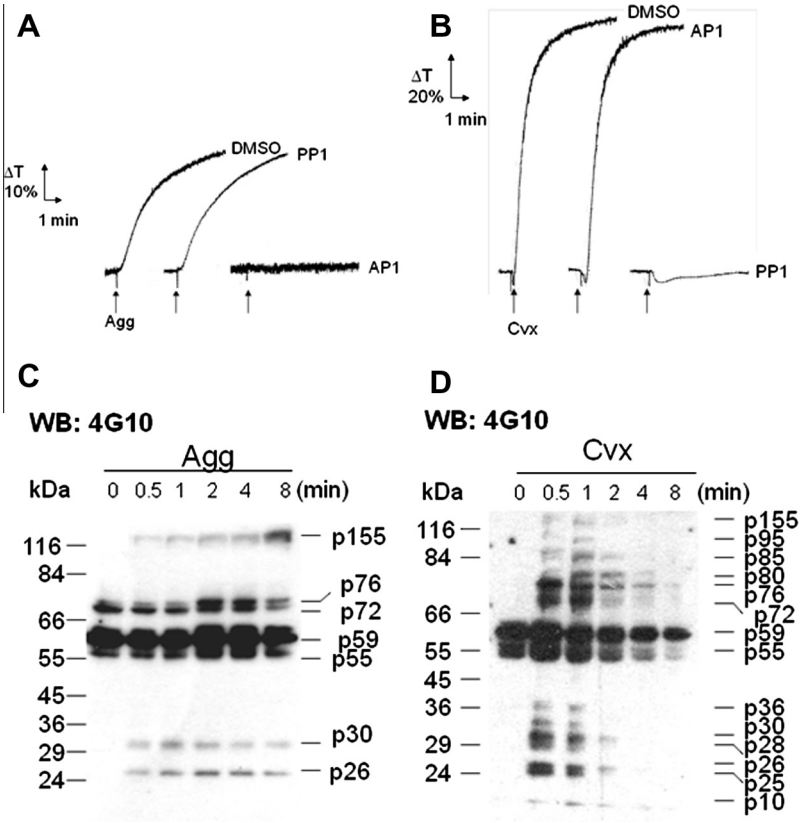

Fig. 1. Distinct effects of agglucetin and convulxin on platelet response and time-dependent PTP. Human WPs (5×10^8/ml, 750 μl) were pre-incubated without (0.1% DMSO added) or with various inhibitors (AP1, 10 μg/ml; PP1, 10 μM) for 2 min prior to the addition of (A) agglucetin (Agg, 10 μg/ml), or (B) convulxin (Cvx, 1 μg/ml). Each inducer was added at the point indicated by an arrow. ΔT indicates a change in light transmission. After platelet activated by (C) agglucetin, or (D) convulxin, aliquots were removed at the times indicated and dissolved in SDS buffer containing inhibitors. After separation by SDS–PAGE gel electrophoresis and transfer to PVDF membranes, proteins were incubated with the anti-phosphotyrosine antibody 4G10 before detection by a peroxidase-linked second antibody and chemiluminescence.

and reblotting with the specific antibodies to confirm that equal amounts of each component were immunoprecipitated from each platelet preparation. Notably, a 72 kDa-signaling protein, identified as Syk, exhibited a heavy phosphorylation during the initial 4 min-stimulation (Figs. 1C and 2A). However, a 155 kDa-protein, identified as PLCγ2 which extensively phosphorylated at 8 min as shown in Fig. 1C, also could be detectable as soon as 0.5 min. Since platelet agglutination via agglucetin was not affected by PP1 (Fig. 1A), suggesting PLCγ2 phosphorylation is a Src-independent process. Moreover, PLCγ2 also could be co-immunoprecipitated with the Src substrate Syk and an adapter protein SLP-76 (data not shown). Thus, PLCγ2 might form a signal complex with Syk and SLP-76. In addition, tyrosine-phosphorylation of PLCγ2 was also not dependent on calcium influx, because it was not inhibited by 5 mM EDTA (data not shown). These results indicate that GP Ib-IX-V ligation can activate Src kinases, presumably including Lyn and Fyn, resulting in subsequent phosphorylation of Syk, SLP-76 and PLCγ2. Thus, Lyn, Fyn, Syk, SLP-76 and PLCγ2 are involved in agglucetin/GPIbα signaling. Previously, Munday et al. [19] proposed that PI3K can form a complex with platelet GPIb-IX-V, thereby regulating the formation phosphoinositide-signaling molecules in subcellular fractions. Furthermore, VAV proteins also have been reported to play a crucial role in regulating some of the earliest events [20]. As shown in Fig. 2A, both signaling molecules PI3K (p85) and VAV (p95) are still inactivated and, therefore, absent at agglucetin/GPIbα-related signaling.

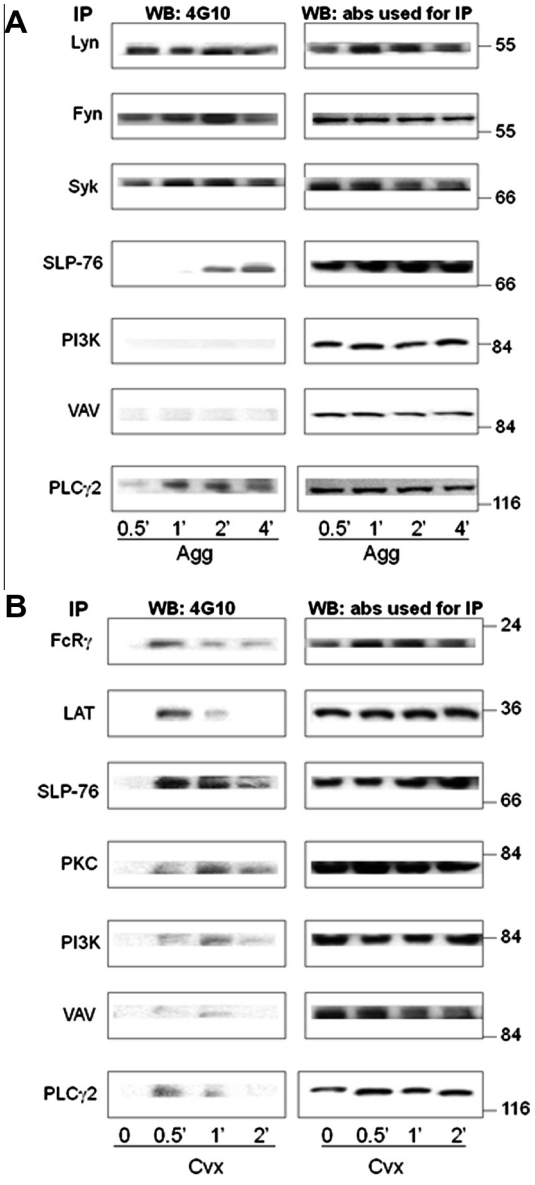

Fig. 2. Comparison of PTP profiles of signaling molecules downstream GPIbα and GPVI in human platelets. WPs were stimulated by (A) agglucetin (10 μg/ml), or (B) convulxin (1 μg/ml), respectively. Aliquots were removed at the times indicated and reactions were terminated by the addition of an equal volume of lysis buffer. Platelet lysates were precleared and detergent-insoluble debris was removed by centrifugation. Intracellular signaling molecules were isolated from lysates by immunoprecipitation with the indicated antibodies, respectively. The resultant supernatants were incubated with protein A/G plus-agarose overnight. Proteins were then separated by SDS/PAGE and Western blotted for phosphotyrosine (left panels). Blots were subsequently stripped and re-probed with the indicated antibodies to check the loading amounts (right panels).

The precise details underlying GPVI signaling have been described elsewhere [21]. Initially, collagen binding to GPVI results in the clustering of associated FcRγ. The tyrosine-based activation motifs within the clustered FcR γ are phosphorylated by the Src kinases, Fyn and Lyn, providing binding sites for Syk. Subsequent recruitment of the adaptor proteins LAT [22] and SLP-76 [23] may promote accumulation of numerous other proteins, including VAV, PI3K and PKC. Ultimately, this signaling cascade leads to the phosphorylation and activation of PLCγ2. Convulxin, a tetrameric platelet activator, has the ability to crosslink four GPVI receptors and acts as a powerful GPVI agonist [21]. As shown in Figs. 1D and 2B, convulxin extensively and rapidly elicited tyrosine kinase-based signaling cascade leading to the activation of PLCγ2. Especially, SLP-76 exhibited a rapid activation and could be detected at 0.5 min upon platelet activation by convulxin. Our data also indicate that other signaling proteins including FcRγ (10 kDa), LAT (36 kDa), PKC (80 kDa), PI3K and VAV are phosphorylated and involved in convulxin/GPVI-activated signaling. Taken together, these results demonstrate that agglucetin can stimulate tyrosine phosphorylation of those established signaling proteins, such as Syk, SLP-76 and PLCγ2, but not FcRγ, LAT, PKC, PI3K and VAV in human platelets. Apparently, PLCγ2-phosphorylation of agglucetin is distinct from that of convulxin, indicating assembly into distinct signalosome [20], a stable signaling complex that have been proposed to regulate the level of intracellular calcium and subsequent downstream events.

In human plasma, agglucetin dramatically induced a biphasic response of agglutination and subsequent aggregation (the left panel of Fig. 3A). However, piceatannol (10 μg/ml), a Syk-selective kinase inhibitor [24], significantly blocked the aggregating response and had no effect on the agglutinating response. In contrast, other kinase inhibitors, PP1, a PI3K inhibitor wortmannin and a PKC inhibitor Ro-31-7549 all failed to inhibit agglucetin-induced aggregation. Therefore, this result implied that Syk plays a central role from GPIbα-initiated agglutination to aggregation. Imaginally, agglucetin might interact with unknown factors in human plasma, leading to platelet aggregation.

For clarifying the suppressive effect of piceatannol on platelet aggregation, we thereby examined whether piceatannol can influence the conformation of αIIbβ3. Flow cytometry analysis was subsequently carried out and a mAb PAC-1was used as a probe for detection. Since PAC-1 can recognize the activated αIIbβ3 on the surface of unfixed platelets [25], FITC-conjugated PAC-1 was added prior to platelet activation. Platelet fixation was then performed following the addition of agglucetin. As expected, agglucetin stimulation could increase the surface expression of activated αIIbβ3 and PP1 also exhibited no effect on integrin exposure. However, pre-treating platelets with piceatannol led to a concentration-dependent inhibition on αIIbβ3 activation (Fig. 3B). Therefore, Syk regulation is required and may play an essential role from agglutination to aggregation.

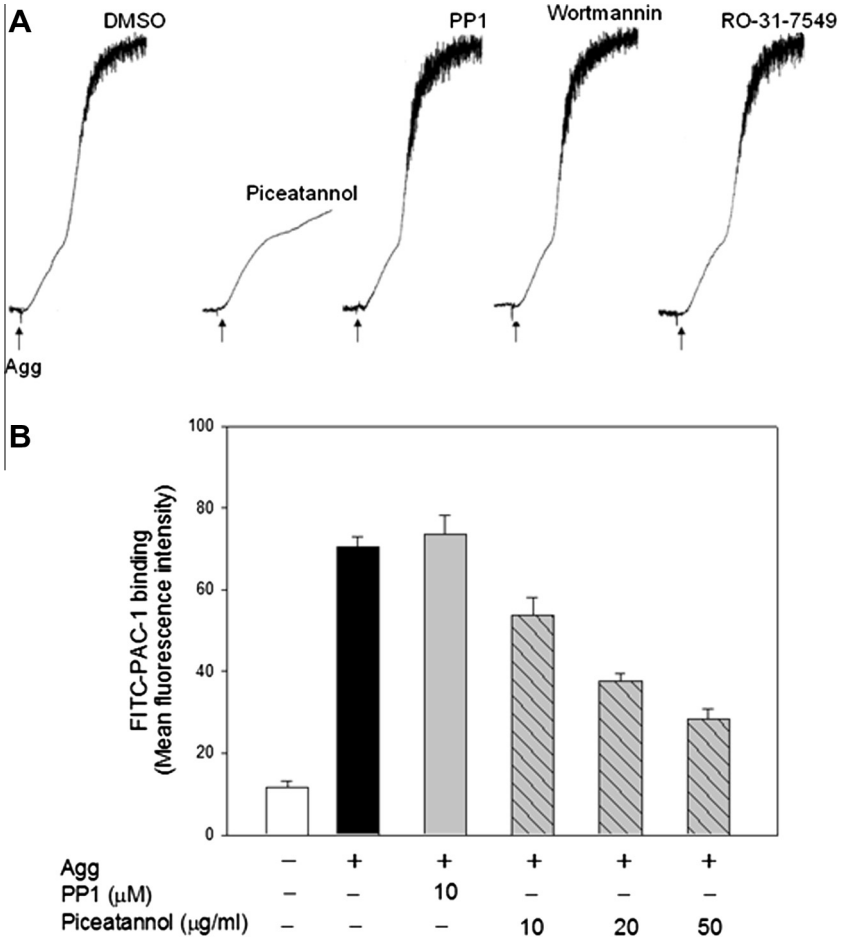

Fig. 3. Effects of various kinase inhibitors on agglucetin-induced platelet activation by agglutination/aggregation assay and flow cytometry analysis. (A) PRP was preincubated without or with various kinase inhibitors (piceatannol, 10 μg/ml; PP1, 10 μM; wortmannin, 0.1 μM; RO-31-7549, 10 μM) at 37 °C for 5 min, and then agglucetin (10 μg/ml) was added to trigger platelet activation. The left tracing shows the control response. Results are representative of 3 experiments. (B) WPs were preincubated without or with various protein kinase inhibitors (PP1, 10 μM; piceatannol, 10, 20, 50 μg/ml) for 15 min and then stimulated without or with agglucetin (10 μg/ml). The binding capacity of FITC-PAC-1 against αIIbβ3 was determined by flow cytometry analysis. The results shown are the mean ± SEM from three separate experiments.

Integrins acting as adhesion receptors can regulate cell–cell and cell–matrix interactions [26]. Often an integrin cannot bind the extracellular ligand until its conformational change that increases its affinity for ligand. In other cases, signals cause an integrin to cluster, thus increasing its avidity for ligand [27]. Integrin αIIbβ3 was thought to have an exclusive role in mediating platelet–platelet interactions. The dimeric nature of fibrinogen enables it to cross-link adjacent activated αIIbβ3 leading to stable platelet aggregation. To investigate the molecular mechanism of platelet aggregation following agglucetin stimulation, the turbidimetry assay and Western blotting analysis were used. Using the subthreshold concentration of 250 μg/ml for a 10 min-incubation, human fibrinogen alone was not sufficient to elicit platelet aggregating response (data not shown). Dramatically, agglucetin (10 μg/ml) added after 2-min incubation of fibrinogen with platelet suspension, could elicit platelet aggregation (as shown in Fig. 4A). It is suggested the activation of integrin αIIbβ3 by agglucetin–GPIbα interaction may convert it from a low- to a high-affinity receptor capable of binding soluble fibrinogen. Therefore, this result demonstrated fibrinogen is an essential cofactor for agglucetin-induced platelet aggregation and agglucetin–GPIbα interactions may promote the binding of fibrinogen to αIIbβ.

Following fibrinogen binding to αIIbβ3, a series of complex intracellular signaling events are initiated, including phosphorylation of the β3 tail by the Src kinase, Lyn and Fyn. Syk, SLP-76 and VAV also associate at the cytoplasmic tail of ligated integrin. Downstream activation of PLCγ2 results in calcium mobilization [28]. As shown in Fig. 4B, platelet activation by co-incubation of agglucetin and fibrinogen resulted in tyrosine-phosphorylation of p55 (Lyn), p59 (Fyn), p72 (Syk), p76 (SLP-76), p95 (VAV), p155 (PLCγ2) and an unknown band p130 in WPs, which rapidly phosphorylated at about 2 min after platelet activation. This unknown protein was subsequently identified by immunoprecipitation as ADAP (data not shown). Previously, Kasirer-Friede et al. [29] reported that GPIb-IX-V itself can signal to activate integrin αIIbβ3 independently of other receptors. More recently, Kasirer-Friede et al. [30] further proposed that ADAP as a component of inside-out signaling pathways coupling GPIb to αIIbβ3 activation. Therefore, the formation of signalosome consisting of Syk, SLP-76, VAV, ADAP and PLCγ2 are necessary for platelet aggregation in agglucetin/fibrinogen-activated platelets. Taken together, our results indicate that αIIbβ3 activation can be regulated via GPIbα–agglucetin interactions.

4. Conclusion

In this report, we had investigated the molecular basis for a biphasic response of agglutination and subsequent aggregation in agglucetin-activated human PRP. Our data established that

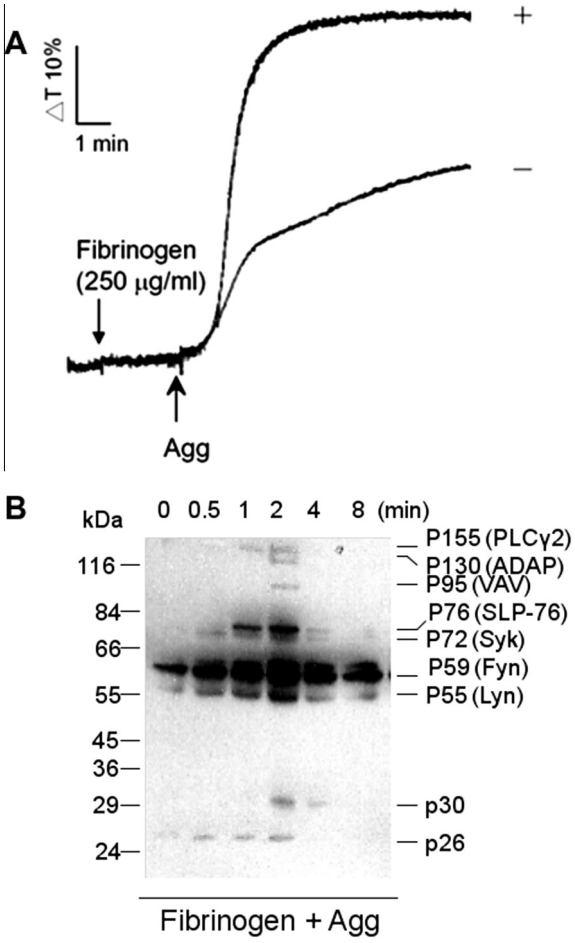

Fig. 4. Functional analyses of platelet aggregation and time-dependent PTP in agglucetin-stimulated platelets with and without fibrinogen. (A) WPs were stirred in the absence (−; PBS added) or presence (+) of human fibrinogen (250 μg/ml). After 2-min incubation, agglucetin (10 μg/ml) was then added to elicit platelet activation. (B) Aliquots were removed at the times indicated and dissolved in lysis buffer. After separation by SDS–PAGE gel electrophoresis and transfer to PVDF membranes, proteins were incubated with 4G10 before detection by a peroxidase-linked second antibody and chemiluminescence.

cross-linking of GPIbα by agglucetin is sufficient to induce platelet aggregation under the presence of fibrinogen in human WPs. The signaling pathway of GPIbα/Lyn/Fyn/Syk/SLP-76/VAV/ADAP/PLCγ2/PKC/αIIbβ3 may involve in agglucetin-activated platelet agglutination and subsequent aggregation. The present study adds to growing body of evidence for a critical role of Syk kinase, but not Src kinases, in the regulation of platelets by GPIbα and further reveals Syk as an essential target for development of novel antithrombotic agents.

Acknowledgment

This work was financially supported by grants from Chang-Gung Medical Research Foundation (CMRPF19001-1∼3).

References

[1] R.K. Andrews, E.E. Gardiner, Y. Shen, M.C. Berndt, Platelet interactions in thrombosis, IUBMB Life 56 (2004) 13–18.
[2] R.K. Andrews, E.E. Gardiner, Y. Shen, J.C. Whisstock, M.C. Berndt, Glycoprotein Ib-IX-V, Int. J. Biochem. Cell Biol. 35 (2003) 1170–1174.
[3] T. Morita, Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities, Toxicon 45 (2005) 1099–1114.
[4] N. Asazuma, S.J. Marshall, O. Berlanga, D. Snell, A.W. Poole, M.C. Berndt, R.K. Andrews, S.P. Watson, The snake venom toxin alboaggregin-A activates glycoprotein VI, Blood 97 (2001) 3989–3991.
[5] Q. Lu, A. Navdaev, J.M. Clemetson, K.J. Clemetson, Snake venom C-type lectins interacting with platelet receptors. Structure-function relationships and effects on haemostasis, Toxicon 45 (2005) 1089–1098.
[6] D. Dormann, J.M. Clemetson, A. Navdaev, B.E. Kehrel, K.J. Clemetson, Alboaggregin A activates platelets by a mechanism involving glycoprotein VI as well as glycoprotein Ib, Blood 97 (2001) 929–936.
[7] C.H. Yeh, W.C. Wang, T.T. Hsieh, T.F. Huang, Agkistin, a snake venom-derived glycoprotein Ib antagonist, disrupts von Willebrand factor-endothelial cell interaction and inhibits angiogenesis, J. Biol. Chem. 275 (2000) 18615–18618.
[8] W.J. Wang, T.F. Huang, A novel tetrameric venom protein, agglucetin from *Agkistrodon acutus*, acts as a glycoprotein Ib agonist, Thromb. Haemost. 86 (2001) 1077–1086.
[9] W.B. Wu, H.C. Peng, T.F. Huang, Crotalin, a vWF and GP Ib cleaving metalloproteinase from venom of *Crotalus atrox*, Thromb. Haemost. 86 (2001) 1501–1511.
[10] W.J. Wang, Q.D. Ling, M.Y. Liau, T.F. Huang, A tetrameric glycoprotein Ib-binding protein, agglucetin, from Formosan pit viper: structure and interaction with human platelets, Thromb. Haemost. 90 (2003) 465–475.
[11] Y. Ozaki, N. Asazuma, K. Suzuki-Inoue, M.C. Berndt, Platelet GPIb-IX-V-dependent signaling, J. Thromb. Haemost. 3 (2005) 1745–1751.
[12] S.P. Jackson, W.S. Nesbitt, S. Kulkarni, Signaling events underlying thrombus formation, J. Thromb. Haemost. 1 (2003) 1602–1612.
[13] S.J. Marshall, Y.A. Senis, J.M. Auger, R. Feil, F. Hofmann, G. Salmon, J.T. Peterson, F. Burslem, S.P. Watson, GPIb-dependent platelet activation is dependent on Src kinases but not MAP kinase or cGMP-dependent kinase, Blood 103 (2004) 2601–2609.
[14] Y. Wu, N. Asazuma, K. Satoh, Y. Yatomi, T. Takafuta, M.C. Berndt, Y. Ozaki, Interaction between von Willebrand factor and glycoprotein Ib activates Src kinase in human platelets: role of phosphoinositide 3-kinase, Blood 101 (2003) 3469–3476.
[15] J. Bain, H. McLauchlan, M. Elliott, P. Cohen, The specificities of protein kinase inhibitors: an update, Biochem. J. 371 (2003) 199–204.
[16] R.K. Andrews, M.C. Berndt, Adhesion-dependent signalling and the initiation of haemostasis and thrombosis, Histol. Histopathol. 13 (1998) 837–844.
[17] S. Kao, N.A. Turner, J.L. Moake, L.V. McIntire, A novel flow cytometric analysis for platelet activation on immobilized von Willebrand factor or fibrillar collagen, J. Thromb. Haemost. 1 (2003) 347–354.
[18] S. Falati, C.E. Edmead, A.W. Poole, Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor γ-chain, Fyn, and Lyn to activate human platelets, Blood 94 (1999) 1648–1656.
[19] A.D. Munday, M.C. Berndt, C.A. Mitchell, Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14–3-3zeta, Blood 96 (2000) 577–584.
[20] M. Turner, D.D. Billadeau, VAV proteins as signal integrators for multi-subunit immune-recognition receptors, Nat. Rev. Immunol. 2 (2002) 476–486.
[21] S.P. Watson, J.M. Auger, O.J. McCarty, A.C. Pearce, GPVI and integrin αIIbβ3 signaling in platelets, J. Thromb. Haemost. 3 (2005) 1752–1762.
[22] J.M. Pasquet, B. Gross, L. Quek, N. Asazuma, W. Zhang, C.L. Sommers, E. Schweighoffer, V. Tybulewicz, B. Judd, J.R. Lee, G. Koretzky, P.E. Love, L.E. Samelson, S.P. Watson, LAT is required for tyrosine phosphorylation of phospholipase Cγ2 and platelet activation by the collagen receptor GPVI, Mol. Cell Biol. 19 (1999) 8326–8334.
[23] B.S. Gross, S.K. Melford, S.P. Watson, Evidence that phospholipase C-γ2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor γ-chain after stimulation of the collagen receptor glycoprotein VI in human platelets, Eur. J. Biochem. 263 (1999) 612–623.
[24] D.A. Law, L. Nannizzi-Alaimo, K. Ministri, P.E. Hughes, J. Forsyth, M. Turner, S.J. Shattil, M.H. Ginsberg, V.L. Tybulewicz, D.R. Phillips, Genetic and pharmacological analyses of Syk function in αIIbβ3 signaling in platelets, Blood 93 (1999) 2645–2652.
[25] T.J. Kovacsovics, C. Bachelot, A. Toker, C.J. Vlahos, B. Duckworth, L.C. Cantley, J.H. Hartwig, Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation, J. Biol. Chem. 270 (1995) 11358–11366.
[26] S.J. Shattil, P.J. Newman, Integrins: dynamic scaffolds for adhesion and signaling in platelets, Blood 104 (2004) 1606–1615.
[27] P.E. Hughes, M. Pfaff, Integrin affinity modulation, Trends Cell Biol. 8 (1998) 359–364.
[28] P. Wonerow, A.C. Pearce, D.J. Vaux, S.P. Watson, A critical role for phospholipase Cγ2 in αIIbβ3-mediated platelet spreading, J. Biol. Chem. 278 (2003) 37520–37529.
[29] A. Kasirer-Friede, M.R. Cozzi, M. Mazzucato, L. De Marco, Z.M. Ruggeri, S.J. Shattil, Signaling through GP Ib-IX-V activates αIIbβ3 independently of other receptors, Blood 103 (2004) 3403–3411.
[30] A. Kasirer-Friede, B. Moran, J. Nagrampa-Orje, K. Swanson, Z.M. Ruggeri, B. Schraven, B.G. Neel, G. Koretzky, S.J. Shattil, ADAP is required for normal αIIbβ3 activation by VWF/GP Ib-IX-V and other agonists, Blood 109 (2007) 1018–1025.