Glycoproteins VI and Ib-IX-V stimulate tyrosine phosphorylation of tyrosine kinase Syk and phospholipase Cγ2 at distinct sites

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Glycoproteins GPVI and GPIb-IX-V stimulate robust tyrosine phosphorylation of Syk and PLCγ2 (phospholipase Cγ2) in washed platelets, but only the former stimulates pronounced activation of phospholipase. Using phospho-specific antibodies, we demonstrate that GPVI, but not GPIb-IX-V, stimulates significant tyrosine phosphorylation of Syk at the autophosphorylation site pY525/526, a marker of Syk activity. In addition, GPVI stimulates tyrosine phosphorylation of PLCγ2 at Tyr^{753} and Tyr^{759}, whereas GPIb-IX-V only induces significant phosphorylation at Tyr^{753}. Both receptors stimulate tyrosine phosphorylation of Btk at the regulatory Tyr^{223} and Tyr^{551}. Syk and Btk phosphorylate peptides from PLCγ2 containing Tyr^{753} and Tyr^{759} respectively, suggesting that they may stimulate phosphorylation at these sites in phospholipase. Studies using PLCγ2-deficient platelets demonstrated that phospholipase is not required for the activation of integrin αIIbβ3 by GPIb-IX-V. Our results demonstrate fundamental differences between GPVI and GPIb-IX-V in the regulation of tyrosine phosphorylation of Syk and PLCγ2 consistent with the functional impairment of phospholipase in signalling by GPIb-IX-V.

Key words: blood platelet, collagen, glycoprotein VI (GPVI), glycoprotein Ib-IX-V (GPIb-IX-V), phospholipase Cγ2 (PLCγ2), von Willebrand factor (vWF).

INTRODUCTION

PLCγ2 (phospholipase Cγ2) is the major PLCγ isoform expressed in platelets [1]. It is activated by the collagen receptor glycoprotein (GP) VI, which associates with the Fc receptor γ-chain. The Fc receptor γ-chain contains one copy of an ITAM (immunoreceptor tyrosine-based activation motif) in its cytoplasmic tail. PLCγ2 is also activated by platelet integrins αIIbβ3 and α2β1 through a pathway, which is independent of an ITAM [2–4]. Stimulation of PLCγ2 by GPVI and integrin receptors is mediated downstream of the tyrosine kinase Syk through a pathway that involves the adapter SLP-76 (SH2-containing leucocyte phosphoprotein of 76 kDa) [5], although the two signalling cascades are distinct and are believed to take place in distinct regions of the membrane [6]. Syk, SLP-76 and PLCγ2 have been shown to play a critical role in platelet activation by GPVI and integrin receptors [2–4,7,8].

vWF (von Willebrand factor) is a multimeric protein, which mediates platelet tethering to the exposed subendothelium at sites of arteriole injury via the GPIb-IX-V complex. In addition, GPIb generates intracellular signals that lead to weak activation of integrin αIIbβ3. Similar to GPVI and platelet integrins, GPIb-IX-V signals through an Src kinase-dependent pathway that involves tyrosine phosphorylation of Syk, SLP-76 and PLCγ2 [9–13]. Although GPIb-IX-V stimulates robust tyrosine phosphorylation of PLCγ2, it only stimulates, at best, weak activation of phospholipase, which is in marked contrast with the robust activation induced by GPVI and the integrin αIIbβ3 (cf. e.g. [10,11] with [12]).

In the present study, we investigated the molecular basis of the discrepancy between the degree of tyrosine phosphorylation of PLCγ2 by GPVI and GPIb-IX-V and the level of activation of phospholipase. To examine this, we mapped the phosphorylation sites of PLCγ2 and the upstream tyrosine kinases Syk and Btk, using phospho-specific antibodies. Our results demonstrate fundamental differences in the sites of tyrosine phosphorylation of Syk and PLCγ2 between GPVI and GPIb-IX-V, which explains the discrepancy in the magnitude of activation between the two receptors.

EXPERIMENTAL

Antibodies and reagents

Human vWF and botrocetin were donated by Dr M. C. Berndt (Monash University, Melbourne, Vic., Australia). Convulxin was a gift from Dr M. Leduc and Dr C. Bon (Institut Pasteur, Paris, France). Anti-PLCγ2 pAb (polyclonal antibody) and anti-Btk pAb were gifts from Dr M. Tomlinson (DNAX, Palo Alto, CA, U.S.A.). The cDNA for human wild-type PLCγ2 was subcloned into pcDNA3.1 plasmid (myc/his-tagged; Invitrogen, San Diego, CA, U.S.A.). Lotrafiban was a gift from GlaxoSmithKline (King of Prussia, PA, U.S.A.). Mutant PLCγ2, in which Tyr^{759} is replaced by a phenylalanine residue, was generated using the

Abbreviations used: GP, glycoprotein; ITAM, immunoreceptor tyrosine-based activation motif; mAb, monoclonal antibody; pAb, polyclonal antibody; PLCγ2, phospholipase Cγ2; SLP-76, SH2-containing leucocyte phosphoprotein of 76 kDa; vWF, von Willebrand factor.

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Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. The mutation was verified by sequencing. Anti-phosphotyrosine mAb (monoclonal antibody) 4G10 was purchased from Upstate Biotechnology (TCS Biological, Botolph Claydon, Bucks., U.K.). Anti-phospho PLCγ2 (Tyr^{759}) pAb were generated by Biosource (Calmarillo, CA, U.S.A.). For generating these antibodies, the following peptide was used: NH_2-ERDINSL<u>Y</u>DVSRMpY-VDPSE-CO_2H corresponding to phosphorylated Tyr^{759} in the PLCγ2 linker region. A phospho PLCγ2 (Tyr^{753}) antibody was generated by Dr F. Sekiya and Dr S. G. Rhee (Y. J. Kim, F. Sekiya and S. G. Rhee, unpublished work). Anti-phospho Syk (Tyr^{525}/Tyr^{526}) pAb was generated as described previously [14]. A second anti-phospho Syk (Tyr^{525}/Tyr^{526}) pAb, anti-phospho Syk (Tyr^{352}) pAb, anti-phospho Btk (Tyr^{223} and Tyr^{551}) pAb and anti-myc mAb were obtained from Cell Signalling Technology (Beverly, MA, U.S.A.). Anti-Syk pAb (LR) for immunoprecipitation and anti-Syk mAb (4D10) for Western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Ristocetin was obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture, transient transfection and stimulation of COS-7 cells

Cell culture and transient transfection of COS-7 cells using the calcium chloride method were performed as described previously [15]. Cells were detached by adding trypsin EDTA and incubating for 5 min at 37 °C. After washing with modified Tyrodes buffer (137 mM NaCl/11.9 mM $NaHCO_3$/0.4 mM Na_2HPO_4/2.7 mM KCl/1.1 mM $MgCl_2$/5.6 mM glucose, pH 7.3), they were stimulated by 4 mM H_2O_2 and 1 mM vanadate for 10 min at 30 °C, followed by immunoprecipitation as described below.

Preparation and stimulation of platelets

Platelets were obtained from drug-free volunteers on the day of the experiment and suspended in modified Tyrodes buffer. They were washed as described previously [16]. Washed human platelets (5×10^8/ml, 0.5 ml) were pretreated with integrin αIIbβ3 antagonist, 10 μM lotrafiban, 10 μM indomethacin and 3 units/ml apyrase, followed by stimulation with 10 μg/ml convulxin or 10 μg/ml vWF plus 1 mg/ml ristocetin for the indicated time at 37 °C.

Immunoprecipitation

Stimulations were terminated by the addition of an equal volume of ice-cold lysis buffer [2 % (v/v) Nonidet P40, 20 mM Tris, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 μg/ml pepstatin A, pH 7.3]. Detergent-insoluble debris was removed by centrifugation at 15 000 ***g*** for 10 min and the supernatant precleared with Protein A–Sepharose [50 % (w/v) in Tris-buffered saline (TBS) plus Tween 20 (TBS-T; 20 mM Tris/137 mM NaCl/0.1 % (v/v) Tween 20, pH 7.6)] for 1 h at 4 °C. Specific antibodies and Protein A–Sepharose were added and each sample rotated at 4 °C overnight. Control samples were also set up alongside using isotype-matched antibodies or preimmune serum to demonstrate specificity. The Sepharose pellet was washed sequentially in lysis buffer and TBS-T, before addition of Laemmli sample buffer.

Immunoblotting

Proteins were separated by SDS/PAGE (8 % gels), electrotransferred and blotted as described previously [16].

In vitro kinase assay

Syk and Btk were immunoprecipitated from stimulated or non-stimulated platelets as described above. After washing, the Protein A pellet was washed once in kinase wash buffer (105 mM NaCl/20 mM Hepes/5 mM $MnCl_2$/5 mM $MgCl_2$/100 μM vanadate). Kinase assay buffer (40 μl; kinase wash buffer plus 10 μM ATP, 0.5 μCi [^{32}P]ATP and 50 μM substrate peptide ^+H_3N-ERDINSL<u>Y</u>DVSRM<u>Y</u>VDPSE-CO_2^-, ^+H_3N-ERDINSL<u>F</u>DV-SRM<u>Y</u>VDPSE-CO_2^- or ^+H_3N-ERDINSL<u>Y</u>DVSRM<u>F</u>VDPSE-CO_2^-) was then added to the pellet. Samples were incubated at 37 °C for 10 min, mixing every 2 min, and then spun at 13 000 ***g*** for 2 min. Each sample (30 μl) was then applied to squares of p81 chromatography paper (which bind the peptides via a charge–charge interaction). Each p81 sample was air-dried for 30 s before 3×5 min washes in orthophosphoric acid solution and 3×5 min washes in acetone, before being allowed to dry overnight. The dried pieces of p81 were then placed in scintillation vials and subjected to a 5 min ^{32}P count. Each assay was performed in duplicate.

Preparation of murine platelets

PLCγ2-deficient mice and integrin αIIb-deficient mice were used as described previously [2,7]. Both sets of mice were bred from heterozygotes on a B6 background and genotyped by PCR as described in [8]. Wild-type littermates were used as controls. Murine blood was drawn from CO_2 terminally-anaesthetized mice by cardiac puncture and taken into 100 μl of ACD (aged citric acid/sodium citrate/dextrose) and washed platelets were prepared as described previously [17]. Murine platelets were resuspended in modified Tyrodes buffer at a cell density of 2×10^8/ml. Washed murine platelets were pretreated with vehicle solution or 10 μM lotrafiban for 5 min before stimulation with 5 μg/ml botrocetin in the presence of 10 μg/ml human vWF. Platelet aggregation was monitored using BioData PAP-4 (Alpha Laboratories, Eastleigh, Hants, U.K.).

Statistical evaluation

Statistical analysis was performed using the unpaired Student's *t* test with $P < 0.05$ taken as significant.

RESULTS

Distinct sites of tyrosine phosphorylation of PLCγ2 by GPIb and GPVI

The GPVI-specific snake toxin convulxin induces powerful tyrosine phosphorylation and activation of PLCγ2 in platelets [18]. In contrast, despite marked tyrosine phosphorylation of PLCγ2, ristocetin/vWF induces minimal activation of phospholipase [10–12]. To investigate whether this discrepancy is due to differences in the sites of phosphorylation of PLCγ2 by the two GP receptors, we raised phospho-specific antibodies to the two major regulatory sites Tyr^{753} and Tyr^{759} [19,20]. The specificity of the 759 antibody was demonstrated by transient transfection of myc-tagged wild-type PLCγ2 or T759F (Tyr^{759} $\rightarrow$ Phe) mutant PLCγ2 in COS-7 cells. After stimulation with H_2O_2, we immunoprecipitated PLCγ2 with an anti-myc antibody and blotted with the anti-Tyr^{759} antibody. The only new band recognized by phospho-specific 759 antibody was wild-type PLCγ2 in stimulated cells, demonstrating its specificity (Figure 1). Several minor bands were seen after longer exposures, but did not change after activation. The same approach was used to recognize an antibody to Tyr^{753}. A full report of the characteristics of this

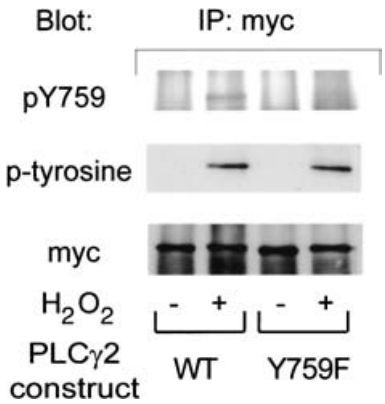

Figure 1 Specificity of the anti-phospho PLC$\gamma 2$ (Tyr^{759}) antibody

COS-7 cells transiently expressing wild-type PLC$\gamma 2$ (WT) or mutant PLC$\gamma 2$ (Y759F) were stimulated without or with 4 mM H_2O_2 plus 1 mM vanadate. The cells were then lysed and the expressed PLC$\gamma 2$ was immunoprecipitated with anti-myc antibody. Phosphorylated Tyr^{759} and total phosphotyrosine in transfected PLC$\gamma 2$ were detected by immunoblotting with anti-phospho PLC$\gamma 2$ Tyr^{759} antibody (pY759) or anti-phospho tyrosine antibody 4G10 (p-tyrosine) respectively. Precipitation of similar levels of transfected PLC$\gamma 2$ proteins was confirmed by immunoblotting with anti-myc antibody (myc). The results are representative of three experiments.

antibody will be reported elsewhere (Y. J. Kim, F. Sekiya and S. G. Rhee, unpublished work).

As previously reported, the GPVI-specific snake toxin convulxin stimulates rapid and robust tyrosine phosphorylation of PLC$\gamma 2$ which peaks within 15 s and decreases slightly after 5 min (Figure 2A). Ristocetin/vWF also stimulated robust tyrosine phosphorylation of PLC$\gamma 2$ within 15 s, although this increased further by 5 min (Figure 2A). Interestingly, both convulxin and vWF-ristocetin stimulated marked phosphorylation of Tyr^{753}, whereas only convulxin induced significant phosphorylation of Tyr^{759} when measured at the time of peak tyrosine phosphorylation of PLC$\gamma 2$ for each stimulus (Figure 2B). Similar results were also seen at other times (results not shown). The absence of detectable tyrosine phosphorylation of Tyr^{759} provides a molecular explanation for the relatively weak activation of PLC$\gamma 2$ by vWF-ristocetin.

vWF-ristocetin does not induce detectable tyrosine phosphorylation of Syk at the autophosphorylation site

Studies in Syk-deficient mouse platelets have demonstrated a key role for the kinase in the regulation of PLC$\gamma 2$ by GPVI [21]. Syk is phosphorylated on multiple sites downstream of ITAM-coupled receptors [22]. Of these, autophosphorylation of Tyr^{525} and Tyr^{526}

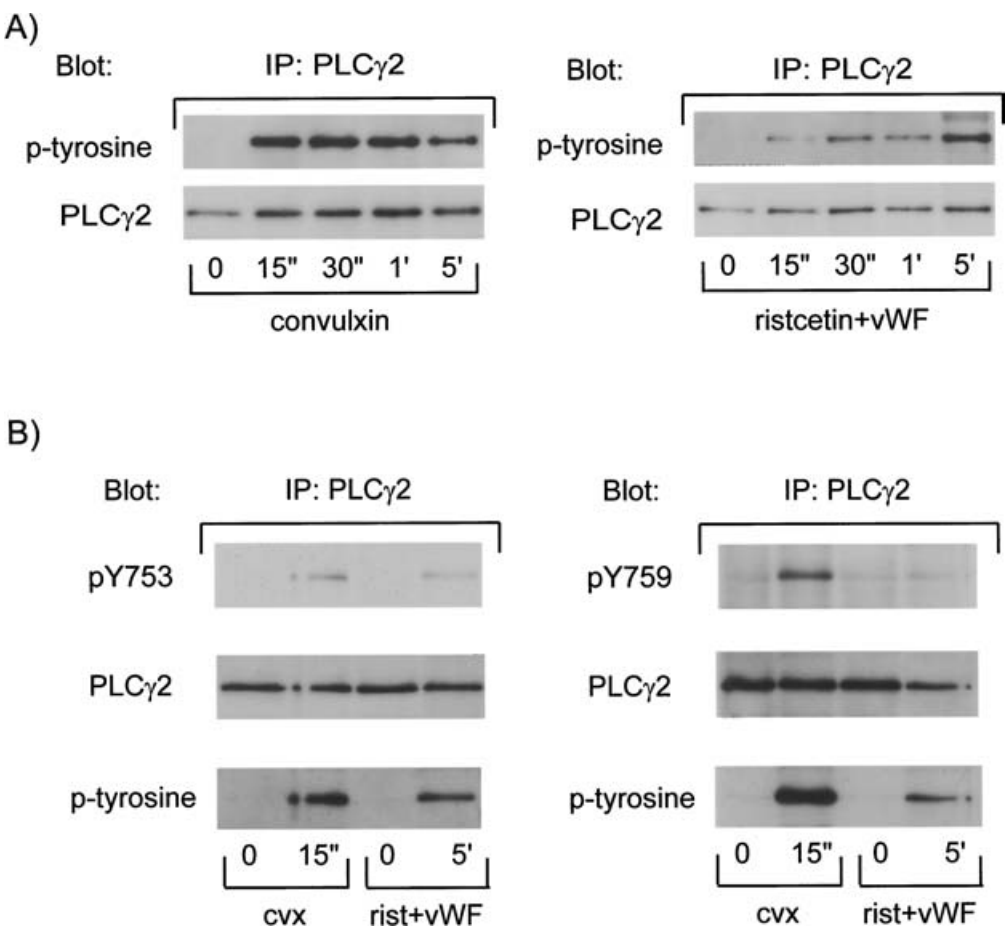

Figure 2 Phosphorylation of regulatory tyrosine residues in PLC$\gamma 2$ by convulxin and vWF-ristocetin

Washed platelets stimulated with 10 μg/ml convulxin or 1 mg/ml ristocetin plus 10 μg/ml vWF for the indicated times were lysed with lysis buffer. Proteins precipitated with the anti-PLC$\gamma 2$ antibody were resolved by SDS/PAGE (8 % gel) and transferred on to PVDF membranes. Membranes were immunoblotted with antibodies against phosphotyrosine (p-tyrosine) (**A**, **B**), phospho Tyr^{753} (pY753) (**B**, left panel), or phospho Tyr^{759} (pY759) (**B**, right panel). Precipitation of similar levels of proteins was confirmed by blotting with anti-PLC$\gamma 2$ (PLC$\gamma 2$) antibody (**A**, **B**). The results are representative of four experiments.

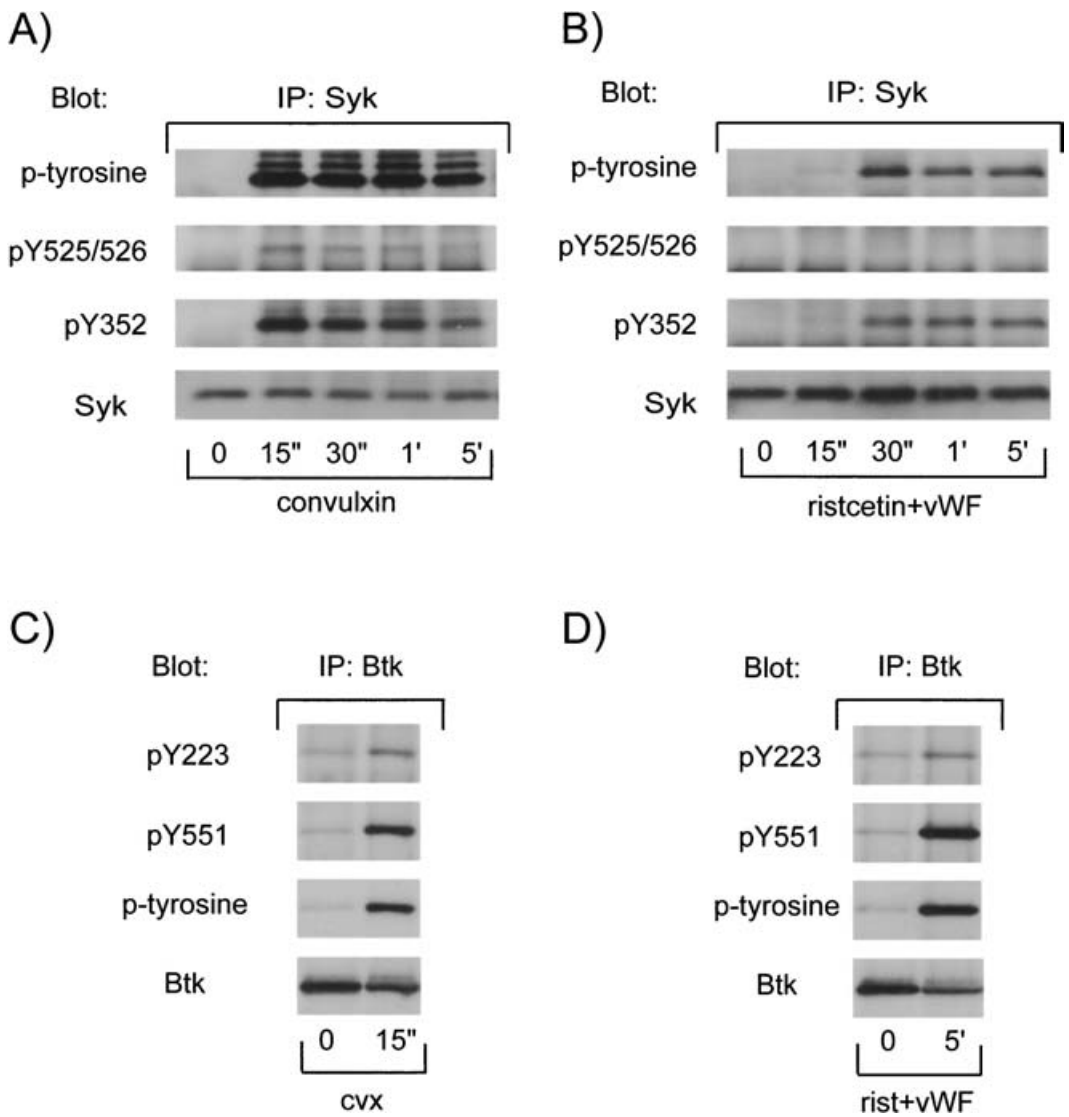

Figure 3 vWF-ristocetin activates Btk, but not Syk

Washed platelets stimulated with 10 μg/ml convulxin (**A**, **C**) or 1 mg/ml ristocetin plus 10 μg/ml vWF (**B**, **D**) for the indicated times were lysed with lysis buffer. Proteins, precipitated with anti-Syk (**A**, **B**) or Btk pAb (**C**, **D**), were resolved by SDS/PAGE (8 % gel), transferred on to PVDF membranes, and immunoblotted with antibodies against phosphotyrosine (p-tyrosine), phospho Syk Tyr^{525}/Tyr^{526} (pY525/526), phospho Syk Tyr^{352} (pY352), phospho Btk Tyr^{223} (pY223) or phospho Btk Tyr^{551} (pY551). Precipitation of similar levels of proteins was confirmed by blotting with anti-Syk mAb (Syk) or anti-Btk pAb (Btk). The results are representative of three experiments.

in the activation loop of Syk is essential for full activation and can be used as a marker of activity [14]. In addition, phosphorylation of Tyr^{352} has been shown to mediate association of Syk with PLCγ1 [23]. We used phospho-specific antibodies to these two sites to investigate their phosphorylation downstream of GPVI and GPIb-IX-V. The two receptors stimulated robust phosphorylation of Tyr^{352}, whereas only convulxin stimulated phosphorylation of Tyr^{525}/Tyr^{526} (Figures 3A and 3B). These observations were confirmed using a second phospho-specific antibody to Tyr^{525}/Tyr^{526} ([14]; results not shown). Therefore there is a marked discrepancy in the degree of phosphorylation at Tyr^{525}/Tyr^{526} relative to Tyr^{352} between GPIb-IX-V and GPVI, with only the latter stimulating significant phosphorylation at the regulatory site Tyr^{525}/Tyr^{526}.

The Tec family kinase Btk has been shown to mediate partially tyrosine phosphorylation of PLCγ2 by GPVI [24]. Btk is regulated by transphosphorylation of Tyr^{551} in the activation loop by an Src family kinase leading to autophosphorylation at Tyr^{223} within the Src homology 3 domain [25,26]. Phosphorylation of both sites is necessary for full activation and can be used as a marker of Btk activity [26]. vWF-ristocetin and convulxin stimulate robust tyrosine phosphorylation of Btk at both Tyr^{223} and Tyr^{551} (Figures 3C and 3D). These results indicate that agonists stimulate robust activation of Btk in platelets in contrast with the observations made for Syk.

Evidence that Syk and Btk preferentially phosphorylate Tyr^{759} and Tyr^{753} in PLCγ2 respectively

The observation that convulxin induces robust phosphorylation of Tyr^{525}/Tyr^{526} in Syk and Tyr^{759} in PLCγ2, whereas neither of these sites shows detectable increases in response to vWF-ristocetin,

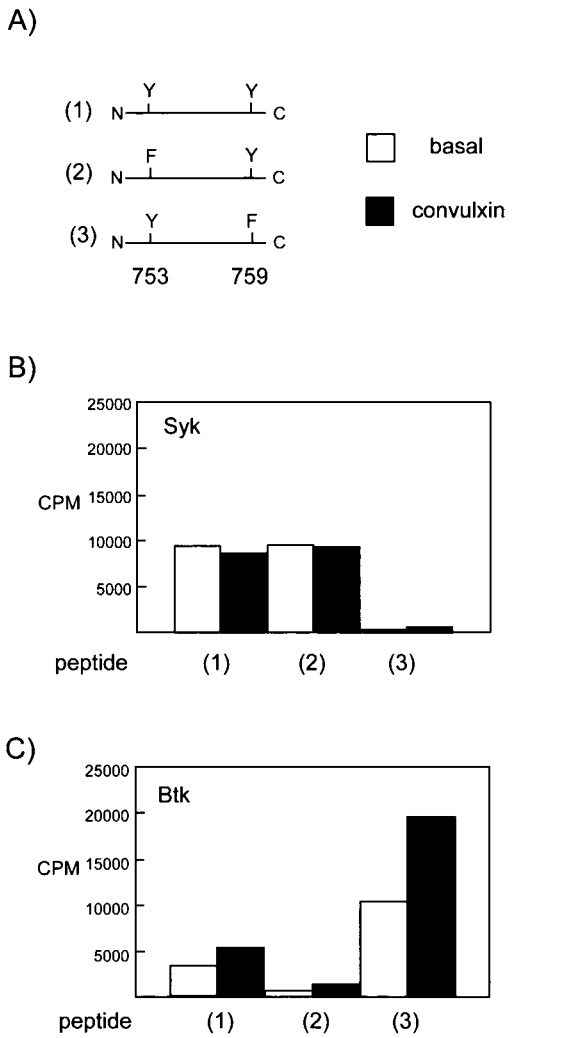

Figure 4 ***In vitro*** **kinase phosphorylation of phenylpeptides containing Tyr[759] and/or Tyr[753] of PLCγ2**

Syk (**B**) and Btk (**C**) immunoprecipitated from stimulated or non-stimulated platelets were incubated with kinase assay buffer containing ATP, [^{32}P]ATP, and one of the substrate peptides described in (**A**) [(1) $^{+}H_3N$-ERDINSLYDVSRMYVDPSE-CO_2^-, (2) $^{+}H_3N$-ERDINSLFDVSRMYVDPSE-CO_2^- and (3) $^{+}H_3N$-ERDINSLYDVSRMFVDPSE-CO_2^-]. The samples were incubated at 37 °C for 10 min and then spun at 13000 ***g*** for 2 min. Each sample (30 μl) was applied to squares of p81 chromatography paper, washed, and dried overnight as described in the Experimental section. The dried pieces of p81 were then analysed by scintillation counting. Each assay was performed in duplicate.

identifies Syk as a candidate kinase for mediating phosphorylation of Tyr[759]. Additionally, the ability of convulxin and vWF-ristocetin to induce activation of Btk and phosphorylation of Tyr[753] in PLCγ2 suggests that the Tec family may mediate phosphorylation of phospholipase at this site.

To explore this in further detail, we performed *in vitro* kinase assays on Btk and Syk immunoprecipitates using peptides from the PLCγ2 linker region that contained Tyr[753] and/or Tyr[759] (Figure 4A). Immunoprecipitates of Btk and Syk promoted incorporation of ^{32}P into a peptide containing Tyr[753] and Tyr[759] (Figures 4B and 4C). Interestingly, Syk immunoprecipitate from basal and convulxin-stimulated platelets induced a similar level of phosphorylation of the peptide, whereas Btk immunoprecipitate from stimulated platelets induced a greater level of phosphorylation (Figures 4B and 4C). The absence of an increase in phosphorylation by Syk is consistent with our previous observation that autophosphorylation of the kinase is also similar in kinase assays performed *in vitro* on basal and stimulated platelets. This suggests that autophosphorylation, which regulates the kinase activity of Syk, is not rate-limiting in these experiments.

To investigate the specificity of phosphorylation by Syk and Btk, we designed two more peptides in which Tyr[753] and Tyr[759] were replaced by a Phe residue. Interestingly, the peptide containing Tyr[759] and Phe[753] was phosphorylated only by Syk, whereas the converse was the case for the peptide containing Tyr[753] and Phe[759], with phosphorylation being induced only by Btk (Figures 4B and 4C). Btk also induced a much greater level of tyrosine phosphorylation of the peptide containing Phe[759] demonstrating that phosphorylation of Tyr[753] is modulated by the presence of tyrosine or phenylalanine at position 759 (Figure 4C). To investigate this further, we prepared a new peptide which was phosphorylated at Tyr[759]. This modification severely inhibited phosphorylation of Tyr[753] by Btk (results not shown), demonstrating that phosphorylation of Tyr[759] inhibits phosphorylation of Tyr[753]. This has important implications for the order of phosphorylation of Tyr[753] and Tyr[759] in stimulated platelets.

These results demonstrate that Syk and Btk are able to stimulate phosphorylation of Tyr[759] and Tyr[753] respectively, identifying them as candidates for mediating phosphorylation of these sites after receptor engagement.

PLCγ2 does not play a role in aggregation induced by vWF-ristocetin

We used murine platelets to investigate the functional role of PLCγ2 in GPIb-dependent activation of integrin αIIbβ3. We used botrocetin/vWF in these studies because ristocetin/vWF is not effective in murine platelets. Botracetin/vWF promoted a slow increase in light transmission that was partially inhibited in the presence of the αIIbβ3 receptor antagonist, lotrafiban (Figures 5A and 5C), indicating that it is made up of GPIb-dependent agglutination (which is insensitive to lotrafiban) and αIIbβ3-dependent aggregation. This was confirmed by the demonstration that lotrafiban had no effect on the increase in light transmission induced by botracetin/vWF in murine platelets deficient in αIIb (results not shown). Interestingly, however, lotrafiban caused a similar degree of inhibition of the increase in light transmission in PLCγ2-deficient platelets induced by vWF/botrocetin to that seen in controls (Figures 5B and 5C), demonstrating that activation of αIIbβ3 is not dependent on PLCγ2.

DISCUSSION

In the present study, we have investigated the discrepancy between the extent of tyrosine phosphorylation of PLCγ2 by GPVI and GPIb-IX-V and the degree of activation of phospholipase [10,11]. We demonstrate that this is due to distinct profiles of tyrosine phosphorylation of PLCγ2 by the two receptors. GPVI stimulates tyrosine phosphorylation at the regulatory Tyr[753] and Tyr[759] in PLCγ2, whereas GPIb-IX-V induces significant tyrosine phosphorylation of phospholipase at position 753. These observations are explained by a differential regulation of the upstream regulator Syk, with only GPVI inducing a significant tyrosine phosphorylation at its autophosphorylation site.

In the light of these results, it is interesting that both receptors stimulate a similar degree of tyrosine phosphorylation of the Tec family kinase, Btk, at its two regulatory sites. This demonstrates that tyrosine phosphorylation of Btk by GPIb-IX-V is mediated through a pathway distinct from that involved in the regulation

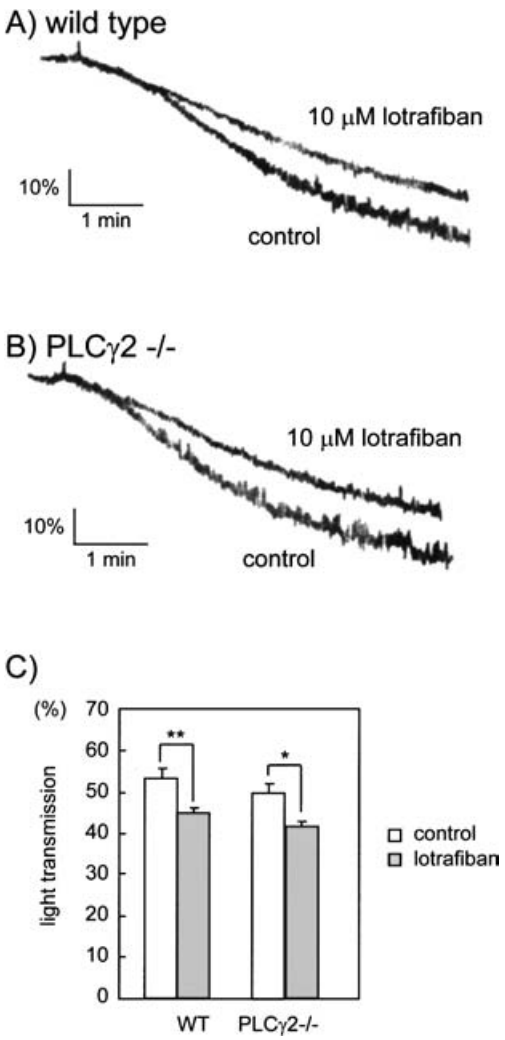

Figure 5 αIIbβ3-dependent aggregation after vWF-botrocetin stimulation

Washed platelets from wild-type (**A**, **C**: WT) and PLCγ2-deficient mice (**B**, **C**: PLCγ2−/−) were pretreated with vehicle solution, 10 μM lotrafiban for 5 min. Platelets were stimulated with 5 μg/ml botrocetin plus 10 μg/ml vWF for 5 min. The results in (**A**) are representative of ten experiments and in (**B**) of three experiments. (**C**) Light transmission at 5 min expresses means ± S.E.M. ($n = 3$–10). $^*P < 0.05$; $^{**}P < 0.01$.

of PLCγ2. Additionally, these results raise the possibility that tyrosine phosphorylation of PLCγ2 at positions 753 and 759 is mediated via Btk and Syk respectively. This is supported in the present study by phosphorylation studies using peptides from the linker region of PLCγ2 and also by the report that a Y753F substitution in the PLCγ2 fragment, PLCγ2-639-770, markedly reduces phosphorylation by Btk *in vitro* [20]. Interestingly, the results also show that phosphorylation of Tyr^{759} inhibits phosphorylation of Tyr^{753} by Btk, demonstrating that the chronology of phosphorylation of these two sites in stimulated platelets influences the activation of PLCγ2.

The distinct profile of tyrosine phosphorylation of Syk and PLCγ2 by GPIb-IX-V relative to that induced by GPVI may be explained by our previous observation that the vWF receptor is excluded from cholesterol-rich lipid-rich domains, known as rafts, which play an essential role in signalling by GPVI [6]. Activation of PLCγ2 by GPVI is critically dependent on tyrosine phosphorylation of the raft-associated membrane adapter LAT (linker for activation of T-cells), whereas the adapter is only minimally phosphorylated in response to activation of GPIb-IX-V. Further, GPVI signals via the related Src family kinases, Fyn and Lyn, which are located in these membrane domains [6,27], whereas GPIb-IX-V signals via the tyrosine kinase Src, which is located outside of membrane rafts [28]. Therefore GPIb-IX-V stimulates assembly of a unique signalling complex similar to that used by GPVI, which leads to tyrosine phosphorylation of Syk and PLCγ2 at distinct sites.

A recent study has reported that up to 15 % of GPIb-IX-V may be associated with membrane rafts [29]. This probably reflects methodological differences with our own study, bearing in mind that there is no established way to isolate membrane rafts. Both studies, however, agree that the major portion of GPIb-IX-V lies outside of membrane rafts [6]. It is interesting to consider whether the small proportion of membrane-raft-associated GPIb-IX-V is able to signal in the same way as GPVI. This scenario may explain, e.g. a recent study [12], which demonstrates a partial role for PLCγ2 in mediating the weak increase in intracellular Ca^{2+} that is induced by GPIb-IX-V engagement in murine platelets. Thus, we speculate that the minor pool of raft-associated GPIb-IX-V is capable of activating PLCγ2 in a way similar to GPVI, whereas the much larger pool of non-raft-associated receptors stimulates tyrosine phosphorylation of phospholipase through a distinct signalling cascade. Our inability to detect tyrosine phosphorylation of Syk and PLCγ2 by GPIb-IX-V at key regulatory sites, in the present study, by the raft-associated pool may reflect the limitations in sensitivity of the available phospho-specific antibodies and the weak nature of the signal.

In conclusion, we have shown that the discrepancy between the level of tyrosine phosphorylation of PLCγ2 and the degree of activation between GPIb-IX-V and GPVI can be explained by differences in the extent of phosphorylation of key regulatory sites in Syk and PLCγ2. This demonstrates the need for caution in equating tyrosine phosphorylation as measured using a pan phosphotyrosine antibody with function, most notably for receptors that signal through distinct signalling cascades.

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