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Original Article

TNF Receptor-Associated Factor 4 (TRAF4) is a novel binding partner of glycoprotein Ib and glycoprotein VI in human platelets

Running head: TRAF4 binds platelet GPIb and GPVI

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SUMMARY

Background: Reactive oxygen species generation is one consequence of ligand engagement of platelet glycoprotein (GP) receptors GPIb-IX-V and GPVI, which bind VWF/collagen and initiate thrombosis at arterial shear, however the precise molecular mechanism coupling redox pathway activation to engagement of these receptors is unknown. *Objective*: The objective of this study was to identify novel binding partners for GPIb-IX-V and GPVI that could provide a potential link between redox pathways and early platelet signalling events. *Methods and Results*: Using protein array analysis and affinity-binding assays, we demonstrated that the orphan TNF receptor-associated factor (TRAF) family member, TRAF4, selectively binds cytoplasmic sequences of GPIbβ and GPVI. TRAF4, p47^{phox} (of the NADPH oxidase (Nox2) enzyme complex), and other redox relevant signalling proteins such as Hic-5, co-immunoprecipitate with GPIb/GPVI from human platelet lysates whilst MBP-TRAF4 or MBP-p47^{phox} fusion proteins specifically pull-down GPIb/GPVI. GPIb- or GPVI-selective agonists induce phosphorylation of the TRAF4-associated proteins, Hic-5 and Pyk2, with phosphorylation attenuated by Nox2 inhibition. *Conclusion*: These results describe the first direct association of TRAF4 with a receptor, and identify a novel binding partner for GPIb-IX-V and GPVI, providing a potential link between these platelet receptors and downstream TRAF4/Nox2-dependent redox pathways.

Keywords: glycoprotein Ib-IX-V, glycoprotein VI, platelets, NADPH oxidase, redox, TRAF4

INTRODUCTION

The initial adhesion of platelets to exposed vascular matrix at arterial and pathological shear rates is dependent on two unique platelet receptors, glycoprotein (GP)VI, which binds collagen, and GPIb-IX-V, which binds von Willebrand Factor (VWF) and other ligands (reviewed in [1, 2]). GPIb-IX-V and GPVI play a central role in initiating thrombotic diseases such as myocardial infarction or stroke and are implicated in platelet dysfunction associated with bleeding disorders, coagulopathy, immune and non-immune thrombocytopenias, and inflammatory arthritis [3-6] (and references therein). Important roles for these receptors are also emerging in experimental animal models of metastasis [7-9]. Understanding how engagement of these receptors initiates signalling events leading to activation of the platelet integrin, $\alpha_{IIb}\beta_3$, which binds fibrinogen or VWF to mediate platelet aggregation, should provide new avenues for clinical diagnosis or treatment.

In complex with the Fc receptor γ-chain (FcRγ) dimer, GPVI is the major collagen signalling receptor on platelets. Engagement of GPVI activates intracellular signalling events leading to platelet aggregation through $\alpha_{IIb}\beta_3$, and to activation of the collagen-binding integrin, $\alpha_2\beta_1$, which stabilizes the interaction of platelets with fibrillar collagen [2]. The 51 amino acid cytoplasmic tail of GPVI interacts with constitutively-bound Src family kinases, Fyn and Lyn [10, 11], and calmodulin [12]. When GPVI is cross-linked by binding collagen, a GPVI-specific activating collagen-related peptide (CRP), or snake venom-derived GPVI agonists such as convulxin, GPVI forms a disulfide-dependent homodimer [13] and constitutively-bound Src kinases phosphorylate the immunoreceptor tyrosine-based activation motif (ITAM) sequence in FcRγ, allowing the assemblage of Syk and activation of downstream signalling pathways [10].

GPVI is physically and functionally associated, at least in part [14], with the GPIb-IX-V complex consisting of GPIbα (the major ligand-binding subunit) disulfide-linked to GPIbβ

and noncovalently associated with GPIX and GPV [1]. The cytoplasmic tail of GPIb-IX-V includes binding sites for actin-binding filamin, 14-3-3ζ, calmodulin and the p85 regulatory subunit of phosphoinositide 3-kinase (PI3-kinase) [15-17]. Studies using pharmacological inhibitors or genetically-modified mice deficient in individual signalling proteins have defined early signalling events upstream of a secretion-dependent pathway. The most receptor-proximal signalling protein identified is Lyn, which is upstream of Syk, SLP-76, Bruton tyrosine kinase (Btk), PI3-kinase and PLCγ2, leading sequentially to release of agonists such as ADP and TXA_2, which activate platelets in an autocrine fashion and facilitate activation of $\alpha_{IIb}\beta_3$ [18]. A second secretion-independent pathway involving NO/cGMP/PKG upstream of p38 and ERK1/2, also leading to $\alpha_{IIb}\beta_3$ integrin activation, has been proposed [19]. Lyn is also the first identified signalling protein in this pathway [20], upstream of PI3-kinase and Akt [17, 20]. Other workers have also identified key signalling proteins activated downstream of GPVI in human or mouse platelets, including PI3-kinase, Akt and other members of Lyn/Syk-initiated pathways [21-24].

Despite these considerable research efforts, the molecular mechanism by which GPVI- and GPIb-IX-V-associated proteins coordinate the assemblage and activation of Lyn-dependent signalling pathways in human platelets is not understood and roles for additional receptor-associated proteins are feasible. In this study, we used protein-array and affinity-binding approaches to search for new binding partners for the cytoplasmic tails of GPIb-IX-V and GPVI and identified tumor necrosis factor (TNF) receptor-associated factor (TRAF) family member, TRAF4, as a novel binding partner for both receptors. Both GPIb-IX-V and GPVI interacted with TRAF4 as well as TRAF4-associated proteins Hic-5, p47*phox* and Pyk2. These interactions were functional since TRAF4-associated proteins, Hic-5 and Pyk2, could be phosphorylated by selective ligand binding at either GPIb-IX-V or GPVI. Our findings help illustrate how platelet receptor-ligand interactions integrate with Lyn-dependent

signalling pathways and provide the first evidence of association of TRAF4 with mammalian receptors, thus characterising a redox-relevant molecular pathway linking GPIb-IX-V and GPVI activation to reactive oxygen species (ROS)-dependent regulation of platelet function.

MATERIALS AND METHODS

Antibodies

Anti-p47^{phox}, anti-TRAF4, anti-Hic-5, anti-Lyn and anti-Pyk2 monoclonal antibodies were from BD Biosciences (San Jose, CA); anti-Hic-5 polyclonal antibody from Cell Signaling Technology (Boston, MA); anti-phosphotyrosine (4G10) and anti-Syk monoclonal and anti-Pyk2 polyclonal antibodies from Millipore (Upstate; CA, USA); and anti-phosphotyrosine (PY20) was from Zymed Laboratories (San Francisco; CA). Anti-GPVI ectodomain, anti-glycocalicin (GPIbα ectodomain) and anti-GPIbβ tail polyclonal antibodies, and anti-αIIb (CRC54) and anti-GPVI (6B12, 4B8, 12A5) monoclonal antibodies have been previously described [5, 14, 25]. HRP-conjugated anti-mouse or anti-rabbit IgG raised in sheep were from Chemicon (Melbourne, Australia).

GPVI- and GPIb-specific reagents

The GPVI-specific agonist, collagen-related peptide (CRP) was generated as previously described [26]. COS-7 cells expressing the VWF-A1 domain containing an R543W mutation (a gain-of-function mutation found in Type 2B von Willebrand's disease) were used as a selective agonist for GPIb-IX-V [27]. This agonist robustly activates platelets without the requirement for modulators, and a ratio of 1:1200 COS-7 cells:platelets was used in the present study.

Screening of protein array

To identify proteins that specifically bind to the membrane-proximal cytoplasmic sequence of GPIbβ (Arg151–Ala161), a hEx1 human recombinant protein array on PVDF membrane (222

x 222 mm, Millipore) was screened with a biotin-RRLRARARARA peptide, as previously described [28]. The high density protein array is comprised of proteins expressed from cDNA clones from the hEx1 library, a human foetal brain cDNA library subcloned into an *E. coli* strain SCS-1, which permits IPTG-inducible expression of His_6-tagged fusion proteins directly onto a membrane. The polyvinylidene difluoride (PVDF) array contained up to 28,000 spots in duplicate in a distinct 5 x 5 pattern around a guide dot. Bound peptide was detected using an anti-biotin antibody (Amersham Biosciences, Sweden). The identity of positive clones was determined by sequencing and BLAST analysis.

MBP-GPIbβ and MBP-GPVI affinity columns

A maltose-binding protein (MBP)-GPVI cytoplasmic tail (Glu266–Ser316) fusion protein was prepared as previously described [10]. MBP-GPIbβ cytoplasmic tail (Arg149–Ser181) was prepared using similar methods. Proteins (~0.1 mg) were coupled to a 1:1 mixture of Affigel-10/15 resin (Bio-Rad, Richmond, CA; ~10-mL bed volume) using published methods [29]. Detergent-free human platelet lysates were prepared as previously described [30], then separated on MBP-GPIbβ or MBP-GPVI affinity columns. Flow-through or guanidine-HCl eluted fractions (1 mL) were dotted onto nitrocellulose membranes and immunoblotted for specific signalling proteins.

MBP-TRAF4 MATH domain and MBP-p47*phox* SH3 domain fusion proteins

Human TRAF4 cDNA and human p47*phox* cDNA were purchased from OriGene (Rockville, MD). The cDNA corresponding to TRAF4-MATH (meprin and traf homology) domain (amino acid sequence 307-462) cDNA was synthesized by PCR with a BamHI overhang forward primer (5'CGC GGA TCC GAT GGC GTG CTC ATC TGG AAG ATT G 3') and HindIII overhang reverse primer (5' CCC AAG CTT TCA AAC AGC AGC ACG GAT GAA GAC TGC ATC). Human p47*phox* cDNA corresponding to tandem SH3 domains (amino acid sequence 151-292) was synthesized by PCR with an EcoRI overhang forward primer (CCG

GAA TCC GAC ATC ACC GGA CCC ATC ATC CAG) and SalI overhang reverse primer (ACGC GTC GAC TCA GCG TTG GGC CTG GGA CAC). PCR products of TRAF4-MATH domain and p47^{phox}-SH3 domains were digested with the corresponding restriction enzymes and cloned into pMAL-C2X vector (New England BioLabs, MA). Both TRAF4-MATH and p47^{phox}-SH3 constructs were verified by sequencing.

MBP-TRAF4-MATH domain fusion protein and MBP-p47^{phox}-SH3 domain fusion protein were expressed in *E.coli* BL-21(DE3) with 1 mM IPTG induction at 37°C for 3 h. MBP-p47^{phox}-SH3 was purified according to the manufacturer's instruction (New England BioLabs). Since MBP-TRAF4-MATH was insoluble, transfected *E.coli* cells were resuspended in column buffer (20 mmol/L Tris-HCl, 200 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) containing 1 mg/mL lysozyme and Complete protease inhibitor cocktail and rocked at room temperature for 15 min. The lysate was made 0.25% (v/v) sarcosyl detergent and sonicated. Insoluble material was pelleted by centrifugation (16,000*g*; 30 min; 4°C) and supernatant made 1% (v/v) Triton X-100 and loaded onto amylose resin (New England BioLabs). Bound protein was eluted with column buffer containing 10 mmol/L maltose, 0.1% (v/v) sarcosyl and 0.5% (v/v) Triton X-100, and dialysed into TS buffer (10 mmol/L Tris, 150 mmol/L NaCl, pH.7.4) containing 0.08% (v/v) sarcosyl.

GPIb- and GPVI-dependent platelet activation

Human platelets were isolated from venous blood using acid citrate dextrose as the anticoagulant, washed and resuspended at 5 x 10^{8}/mL as previously described [16]. Platelet aggregation was induced by addition of (final concentrations) CRP (2.5–10 μg/mL) or VWF-A1/R543W cells (cell-to-platelet ratio of 1:1200), with stirring at 900 rpm in a Lumiaggregometer (Chronolog, Havertown, PA) at 37°C. In some experiments, platelets were preincubated with the Nox inhibitor apocynin (100 μmol/L; Calbiochem, San Diego) or redox inhibitors quercetin (100 μmol/L; Sigma) or diphenylene iodonium (DPI, 10 μmol/L;

Sigma) for 10 min prior to adding agonist. In other experiments, platelets were pre-treated with an RGD-containing peptide (GRGDSP; Auspep, Melbourne, Australia) at 1 mmol/L final concentration to inhibit ligand binding to $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\beta_3$-dependent platelet aggregation. Bands corresponding to GPVI homodimer were scanned and optical density (O.D.) quantified using ImageJ software (NIH, Bethesda, MA; http://rsb.info.nih.gov/ij/). Data were expressed as mean O.D ± SEM and statistical difference was calculated using a one-way ANOVA with Newman-Keuls post-hoc test.

Immunoprecipitation and MBP-fusion protein pull-down assay

Immunoprecipitation from Triton-soluble supernatants was performed by addition of 4–10 μg antibody to pre-cleared platelet lysates and incubation overnight at 4°C [14]. For immunoprecipitations involving TRAF4 and Pyk2, the lysis buffer also contained 10 mmol/L *N*-ethylmaleimide to dissociate GPIb from the cytoskeleton [14, 17], ensuring that association with GPIb was not due to mutual cytoskeletal attachment. Protein G sepharose was used in TRAF4 immunoprecipitations due to a non-specific band of a similar molecular weight to TRAF4 in protein A sepharose immunoprecipitations. Platelet lysates were also precipitated with MBP-TRAF4-MATH or MBP-p47^{phox}-SH3 (20–40 μg/mL). Immunoprecipitates and pull-downs were resolved by SDS-5–20% polyacrylamide gel and immunoblotted as described elsewhere [12, 17]. Blots were visualized using HRP-conjugated secondary antibodies and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

RESULTS

Identification of novel binding partners of GPIbβ and GPVI

We previously showed that calmodulin dynamically interacts with analogous membrane-proximal positively-charged intracellular sequences of GPIbβ (RRLRARARARA) and GPVI (KRLRHRGRAVQ) [12, 16]. To investigate whether other proteins could interact with these

sequences (potentially regulated by calmodulin binding), a protein array displaying 28,000 recombinant, human proteins was probed with the GPIbβ-based peptide (biotin-RRLRARARARA). This screen identified the assemblage and signalling protein, TRAF4, as one of two potential GPIbβ-binding proteins. To confirm the protein array data, a 100,000*g* non-detergent lysate of platelets was chromatographed on an MBP-GPIbβ cytoplasmic tail affinity column and bound proteins were eluted with guanidinium-HCl. TRAF4 was detected in the eluted fractions, supporting an association with GPIbβ (Figure 1). An MBP-GPVI cytoplasmic tail affinity column also bound TRAF4 (Supplementary Figure 1).

GPIb and GPVI associate with TRAF4, p47^{phox}, Hic-5 and Pyk2 in human platelet lysates

TRAF4 does not bind to TNF receptors [31] and has not been definitively linked to any other receptor type. In endothelial cells, TRAF4 localises NADPH oxidase (Nox2)-derived ROS generation to specific subcellular regions through its independent binding partners, Hic-5, and the Nox2 subunit, p47^{phox} [32]. To ascertain whether a TRAF4/Hic-5/p47^{phox} complex is associated with GPIb-IX-V and GPVI in platelets, GPVI and GPIb were immunoprecipitated from Triton X-100-soluble platelet lysates and probed for TRAF4, Hic-5 and p47^{phox}. Both anti-GPIb and anti-GPVI antibodies, but not a control polyclonal antibody, immunoprecipitated TRAF4, p47^{phox} and Hic-5 (Figure 2A). The association of p47^{phox} with GPVI was less marked than that with GPIb, possibly reflecting an indirect association of GPVI and p47^{phox} through co-association with GPIb [14]. Hic-5 in platelets is physically and functionally associated with proline-rich tyrosine kinase, Pyk2 [33], and is constitutively bound to the Src family kinase Lyn [34], both of which also co-immunoprecipitate with GPIb and GPVI (Figure 2B and 2C, upper panels). To confirm that any observed differences in co-immunoprecipitation were not due to reduced immunoprecipitation of GPIb or GPVI, immunoblots were reprobed with anti-GPIb and anti-GPVI monoclonal antibodies (Figure 2B

and 2C, lower panels). Immunoprecipitation conditions and antibodies were consistent for all co-immunoprecipitations and the levels of GPVI and GPIb were similar for all immunoblots.

GPVI and GPIb associate with the MATH domain of TRAF4 and SH3 domains of p47^{phox}

TRAF4 (54 kDa) is comprised of a zinc finger domain, three consecutive traf-like zinc finger domains, a coiled-coil region and a meprin and traf homology (MATH) domain [31] that is required for the interaction of other TRAF-family members with receptors [35]. The core region of p47^{phox} containing tandem SH3 domains is critical for the assembly and activation of Nox2 [36]. To further characterise the association of TRAF4 and p47^{phox} with GPVI and GPIb, MBP-fusion proteins containing the MATH domain of TRAF4 (residues 307–462) or the SH3 domains of p47^{phox} were used in platelet lysate pull-down assays. Both MBP-TRAF4-MATH and MBP-p47^{phox}-SH3, but not a control fusion protein (MBP-LacZ) or amylose resin alone, pulled down GPVI and GPIb but not $\alpha_{IIb}\beta_3$ from platelet lysates (Figure 3A). The TRAF4-associated protein, Hic-5, was also pulled down by MBP-TRAF4-MATH and MBP-p47^{phox}-SH3. Pyk2 was pulled down using MBP-TRAF-MATH, as well as fusion proteins containing the cytoplasmic tail portions of GPVI and GPIbβ (Figure 3B). Together with the immunoprecipitation data, these experiments confirm the association of a TRAF4/Hic-5/p47^{phox} complex with the cytoplasmic tails of GPIb and GPVI.

Redox-sensitive phosphorylation of Hic-5 and Pyk2 following treatment of platelets with GPIb- and GPVI-dependent agonists

In endothelial cells, TRAF4, Hic-5 and Pyk2 have a strong physical and functional association in focal adhesions [32]. Localised ROS, particularly H_2O_2, modulate cell migration *via* oxidative inactivation of focal complex-targeted protein tyrosine phosphatases such as PTP-PEST [37], which binds Hic-5 [38] and is a negative regulator of Pyk2 [39] and

Hic-5 [40]. Hic-5 has previously been reported to be physically and functionally associated with Pyk2 in platelets [33], and Pyk2 is rapidly tyrosine phosphorylated following ligand binding to GPIb-IX-V [41]. To ascertain whether the platelet receptor-associated TRAF4/Hic-5/p47^{phox} complex is functional, tyrosine-phosphorylated proteins were immunoprecipitated from platelet lysates after treatment with VWF-A1/R543W COS-7 cells (GPIbα-specific agonist) or CRP (GPVI-specific agonist) and levels of Hic-5 and Pyk2 assessed by immunoblotting. A transient phosphorylation of Pyk2 (Figure 4A) and Hic-5 (Figure 4B), peaking between 1 and 5 min, was detected after treatment with either ligand. Early tyrosine phosphorylation of Hic-5 and Pyk2 was unaffected in platelets pre-treated with an RGD-containing peptide to prevent integrin engagement (data not shown), indicating that signalling through $\alpha_{IIb}\beta_3$ did not contribute to Hic-5/Pyk2 phosphorylation, consistent with other reports [41, 42]. The phosphorylation was redox-sensitive as pre-incubation of platelets with the cell-permeable Nox inhibitor apocynin significantly reduced CRP- (Figure 5) or VWF-A1/R543W-induced (data not shown) tyrosine phosphorylation of Hic-5 (Figure 5A) and Pyk2 (Figure 5B). These data demonstrate that intracellular proteins important for the generation of ROS are co-associated with platelet signalling receptors and that ligation of either GPVI or GPIb triggers phosphorylation of Pyk2 and Hic-5, components of the Nox2 signalling pathway.

To further demonstrate a role for redox in platelet signalling/activation leading to platelet aggregation, we investigated the effect of Nox2 inhibition on platelet aggregation induced by GPVI- or GPIb-specific agonists. Treatment of platelets with increasing concentrations of apocynin inhibited the aggregation of platelets induced by CRP (Figure 6A) or VWF-A1/R543W cells (data not shown). We also examined the effect of Nox2 inhibition on GPVI dimer formation induced by CRP in the presence of the metalloproteinase inhibitor GM6001, which maintains the dimer on the platelet surface [13]. Dimer formation is

independent of Src family-dependent GPVI signalling, and indicates the generation of a rapid transient oxidative submembranous environment in activated platelets. Inhibition of Nox2 with apocynin consistently impaired CRP-induced dimer formation (optical density at 10 min: 0.39±0.15 *cf* 0.86±0.14 in the presence and absence of apocynin, respectively; $p<0.01$; n=3 independent experiments using different donors; Figure 6B). These data indicate that inhibition of Nox2 impairs GPVI dimer formation, which precedes downstream signalling. Similarly, in GPVI immunoprecipitates of CRP-treated platelets, pretreatment with apocynin or another redox inhibitor, diphenylene iodonium (DPI), prevented the CRP-induced dissociation of calmodulin from GPVI, implying that activation of the Nox2 signalling pathway precedes this early event (within 1 min; Figure 6C, upper panel) as well as the subsequent cleavage of GPVI, which occurs within 3 min (Figure 6C, lower panel). Further, while calmodulin is dissociated from GPVI following CRP treatment, TRAF4 could still be co-immunoprecipitated (Figure 6D). Taken together, these data indicate a role for intracellular platelet-derived ROS in GPVI- and GPIb-dependent platelet activation and point to a key role for Nox2 in regulating GPIb-IX-V/GPVI-dependent platelet aggregation at elevated shear rates.

DISCUSSION

Platelet adhesion at sites of atherosclerotic plaque rupture, mediated primarily though the platelet receptors, GPIb-IX-V and GPVI, is an initiating factor in cardiovascular disease. Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are also strongly linked to the pathogenesis of cardiovascular disease [43], however the precise mechanisms for how redox changes regulate, or are regulated by, platelet adhesion receptors are not well understood. Here we present evidence that key molecular players,

TRAF4, Hic-5, p47*phox* and Pyk2, central to functional redox pathways in other cell types, are physically, functionally and constitutively associated with platelet GPIb-IX-V and GPVI.

Members of the tumor necrosis factor-associated factor (TRAF) family function as adaptors in receptor-mediated signalling in several pathways and interact with TNF receptors and interleukin-1/toll-like receptor (IL-1R/TLR) family members. TRAF4 is distinctive in that it does not possess the conserved residues necessary for recognition by TNF receptors, and only weak interactions under certain conditions have been reported [44]. TRAF4 has therefore been suggested to interact with alternative transmembrane proteins [31], but the identity of these putative TRAF4-binding receptors is unknown. Several intracellular binding partners for TRAF4 have been identified, beginning with the p47*phox* subunit of the superoxide-generating enzyme complex NADPH oxidase (Nox2) [45]. Nox2 is a multi-subunit protein comprising two membrane-bound subunits: gp91*phox* (Nox2) and p22*phox*, three cytoplasmic subunits: p47*phox*, p40*phox*, p67*phox*, and a small GTP-binding protein Rac1/2 [46]. TRAF4 and p47*phox* localise subcellular production of ROS during endothelial cell migration through interaction with further TRAF4-binding partners, the focal adhesion scaffold protein Hic-5 and the redox-sensitive tyrosine kinase Pyk2 [32]. This localised activation of Nox2 is one of the key mechanisms for regulating Lyn as well as controlling other cellular functions such as lamellipodia formation and cell motility [32, 47].

Lyn is pivotal in mediating early GPVI- and GPIb-dependent signalling events in platelets [2, 48] and thus its activation and regulation must be tightly regulated. While signalling pathways downstream of Lyn have been delineated, how receptor-associated proteins integrate their assemblage and activation of these Lyn-dependent signalling pathways is still unknown. Work presented here localises molecular components of pathways known to mediate intracellular redox, to the cytoplasmic tails of GPIb or GPVI (Figure 7). We and others have shown association of Lyn and the downstream tyrosine kinase Syk with GPIb and

GPVI [10, 48]. Further, Lyn is constitutively bound via its SH3 domain to the TRAF4-binding partner Hic-5 [34]. Our data therefore demonstrate that many of the proteins involved in the redox signalling pathway are preassembled on GPIb and GPVI enabling rapid signal transduction in response to receptor ligation. A recent study reports that GPVI-bound Lyn is maintained in an active state in platelets under resting and stimulated conditions [11], however how Lyn is initially activated or becomes activated downstream of GPIb is still unclear.

In recent years, numerous receptors on cell types involved in innate immune and inflammatory responses have been reported to signal through Nox *via* the production of ROS, and increasing evidence suggests that both platelet GPIb-IX-V and GPVI are redox-regulated. Krotz *et al* [49] reported that the GPVI agonist collagen, but not other agonists such as thrombin or ADP, increased Nox-mediated production of $O_2^{\cdot -}$, while scavenging $O_2^{\cdot -}$ with superoxide dismutase (SOD) attenuated secondary phases of aggregation, and exogenous $O_2^{\cdot -}$ increased ADP release from collagen-stimulated platelets. H_2O_2 has for many years been implicated in platelet signalling pathways initiated by collagen but not thrombin or ADP [50, 51]. Thrombus formation on collagen under high shear (1000 s^{-1}), dependent on GPIb-IX-V/GPVI binding, was blocked by the generic Nox inhibitors, diphenylene iodonium (DPI) and apocynin [52, 53]. Interestingly, platelet secretion and shape change were not affected, suggesting a distinct role for $O_2^{\cdot -}$ production in activation of $\alpha_{IIb}\beta_3$.

A potential role for TRAF4 in platelet redox signalling (Figure 7) can be inferred from endothelial cell work, where the targeting of Nox2 to focal complexes is mediated through a TRAF4/Hic-5/p47^{phox} complex. This stimulates localised ROS production that inhibits the Hic-5 bound protein tyrosine phosphatase, PTP-PEST, by oxidation of the active site cysteine [38], allowing activation of Hic-5-associated Lyn and Pyk2 [34, 47]. Activated Pyk2 leads to activation of the Nox2-associated small GTPase, Rac-1/2, by Vav and potentially other

signalling intermediates [47, 54], and activated Rac-1/2 binds and activates PAK-1, which can phosphorylate p47*phox* on multiple serine residues, leading to enhanced Nox2 activity and a positive feedback loop increasing ROS generation [32, 47]. Activated Rac-1/2 regulates lamellipodia formation [55], and further, the Rac-1/2–PAK-1 complex can activate guanylyl cyclase in a non-kinase-dependent process to increase cGMP levels (in platelets an additional potential mechanism for elevating cGMP levels) [56]. ROS can also regulate tyrosine phosphorylation-dependent signalling *via* the reversible inactivation of protein tyrosine phosphatases (PTPs) [37]. Based on previous studies [47], PTP-PEST is predicted to bind Hic-5, although it is possible in platelets that this role is served by a different protein tyrosine phosphatase such as Shp-1 or Shp-2.

Because GPVI and GPIb-IX-V are important for the initiation of platelet aggregation at high shear rates, modulation of their downstream signalling could prevent thrombosis rather than affecting normal haemostasis. Indirect evidence suggests that therapeutic targeting of the interaction between GPIb or GPVI with TRAF4 has the potential to modulate rather than abolish platelet function, thereby reducing the risk of major bleeding. Using mutant GPVI receptors expressed in a rat basophilic leukaemia (RBL) cell line, Locke *et al*. [57] demonstrated that mutation of half the basic residues in the calmodulin-binding domain of GPVI resulted in a lag in the time required for initiation of calcium signalling following convulxin stimulation of the cells. While mutating all of the basic residues in the calmodulin-binding domain disrupted the GPVI/calmodulin interaction and significantly impaired the downstream calcium signalling, mutation of only half the residues did not prevent calmodulin binding. The loss of signalling associated with these mutations was therefore likely to be due to loss of a then-unidentified protein. Of significance, the mutations were generated in the TRAF4-binding region of the GPVI cytoplasmic domain, possibly causing disruption of TRAF4/receptor interaction and providing a potential explanation for the observed reduction

in receptor signalling. TRAF4 and calmodulin can bind independently to GPVI, since under conditions where calmodulin is dissociated from GPVI in CRP-stimulated platelets in the current study, TRAF4 is still co-immunoprecipitated with GPVI, indicating that binding of TRAF4 to GPVI does not require the presence of calmodulin.

TRAF4 knockout mice display different phenotypes with variable penetrance, dependent on their genetic background [58, 59]. Mice generated in a mixed 129/SvJ x C57BL/6 background exhibit a developmental defect of the upper respiratory tract resulting in respiratory abnormalities and pulmonary inflammation [58]. In a pure 129/SvJ genetic background, TRAF4 deficiency not only results in a tracheal defect but also in embryonic lethality and severe changes in the development of the axial skeleton (a high incidence of spina bifida) and the nervous system [59]. The question of platelet function in TRAF4 mice has not been addressed, and is likely to be compromised by proinflammatory abnormalities in these animals, however no overt bleeding has been reported. Interestingly, although unlike other TRAF family members the role of TRAF4 in the immune system is unknown, immune function in TRAF4-deficient animals is not impaired. Immune cell development, ROS production and phagocytic capacity of neutrophils in these mice was unaffected by the absence of TRAF4, whereas migration of dendritic cells was reduced, indicating that the role of TRAF4 in ROS generation is more likely to be in a signalling capacity rather than a high output, antimicrobial defence mechanism.

In conclusion, the present study reveals that TRAF4, a binding partner of the regulatory subunit of Nox, p47^{phox}, is physically coupled to platelet receptors, GPIb-IX-V and GPVI. This novel interaction is potentially responsible for linking these receptors to redox signalling pathways. Further studies will need to be undertaken to determine the functional consequences of disrupting the TRAF4 association and whether this interaction can be targeted therapeutically.

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Disclosures

None.

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FIGURE LEGENDS

Figure 1. Interaction partners of the MBP-GPIbβ affinity column. 2 μl of each flow-through or guanidine-HCl eluted fraction were blotted onto a nitrocellulose membrane and probed for the following proteins: MBP, GPIbβ, TRAF4, Hic-5, p47*phox* and Fyb.

Figure 2. Co-immunoprecipitation of GPVI and GPIb with TRAF4, p47*phox* and Hic-5. Human washed platelets were immunoprecipitated with polyclonal antibodies directed against GPVI (anti-GPVI) or GPIb (anti-glyc.) or with a control polyclonal antibody (Cont.). **(A)** Immunoblots were probed with antibodies against TRAF4, p47*phox* and Hic-5 and visualized using an HRP-conjugated secondary antibody and enhanced chemiluminescence. **(B)** Immunoblots were probed with anti-Pyk2 polyclonal antibody (upper panel) and reprobed with anti-GPVI and anti-GPIb monoclonal antibodies (lower panel). **(C)** Immunoblots were probed with anti-Lyn monoclonal antibody (upper panel), then reprobed simultaneously with anti-GPVI and anti-GPIb monoclonal antibodies (lower panel). Data are representative of at least three independent experiments. Vertical line indicates a repositioned gel lane. In the case of **(C)**, 100 μl platelet lysate load was substituted for the 20 μl load seen in the Lyn blot above, to allow visualisation of GPVI in the lysate. P.L., platelet lysate; WB, western blot.

Figure 3. Pull-down of GPVI and GPIb by MBP-p47*phox*-SH3 and MBP-TRAF4-MATH. **(A)** MBP-TRAF4-MATH and MBP-p47*phox*-SH3 specifically pull down GPIb, GPVI and Hic-5 from human platelet lysates MBP-LacZ and amylose-resin are negative controls; platelet integrin α_{IIb} was not pulled down as a further control. **(B)** MBP-GPIbβ cytoplasmic tail, MBP-GPVI cytoplasmic tail, or MBP-TRAF4-MATH were used to pull down Pyk2 from platelet lysates. P.L., platelet lysate; WB, western blot.

Figure 4. Phosphorylation of Hic-5 and Pyk2 following platelet activation. Washed platelets were treated with the GPVI agonist CRP (upper panel) or the GPIb agonist VWF-A1/R543W (lower panel) in the aggregometer for up to 5 min, then lysed and immunoprecipitated with a combination of anti-phosphotyrosine antibodies, 4G10 and PY20. Immunoblots were sequentially probed for **(A)** Hic-5 and **(B)** Pyk2. Data are representative of three independent experiments using different donors. NT, no treatment; P.L., platelet lysate; PTyr, anti-phosphotyrosine antibodies.

Figure 5. Inhibition of GPVI-dependent Hic-5 and Pyk2 phosphorylation by Nox inhibitor apocynin. Human washed platelets were treated with CRP for 5 min, in the presence or absence of 100 μM apocynin, then lysed and immunoprecipitated with a combination of anti-phosphotyrosine antibodies, 4G10 and PY20. Immunoblots were sequentially probed for **(A)** Hic-5 and **(B)** Pyk2. Data are representative of 3 independent experiments using different donors. NT, no treatment; P.L., platelet lysate; Cont., irrelevant monoclonal antibody; PTyr, anti-phosphotyrosine antibodies.

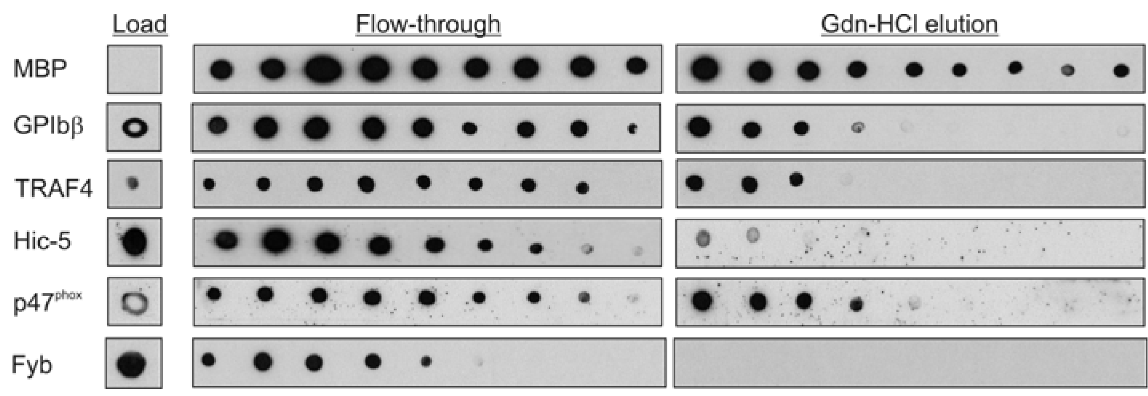
Figure 6. Inhibition of GPVI-dependent platelet aggregation, GPVI homodimer formation and calmodulin dissociation by NADPH oxidase inhibition. **(A)** Representative aggregation traces from human washed platelets treated with CRP in the presence or absence of increasing concentrations of the Nox inhibitor, apocynin. Traces are representative of four independent experiments using different donors. **(B)** Immunoblot of washed platelets treated with 5 μg/ml CRP in the presence or absence of Nox inhibition (apocynin) following preincubation with the metalloproteinase inhibitior, GM6001, to maintain the dimer on the platelet surface. Dimer induced by 0.5 μg/ml convulxin (Cvx) is included for comparison. Immunoblot was probed with the GPVI monoclonal antibody 4B8. Optical density measurements are shown below the corresponding lanes. Data are representative of 3

independent experiments. **(C)** Washed platelets were treated in the aggregometer with 5 μg/ml CRP in the presence or absence of redox inhibitors (300 μM apocynin or 10 μM DPI), then lysed and immunoprecipitated with an anti-GPVI polyclonal antibody. Immunoblots were probed for calmodulin (upper panel) and GPVI (lower panel). Vertical lines represent repositioned lanes. **(D)** Washed platelets were treated in the aggregometer with 5 μg/ml CRP for 1 to 3 min, then lysed and immunoprecipitated with an anti-GPVI polyclonal antibody. Data are representative of 2 independent experiments. Immunoblots were probed for TRAF4. NT, no treatment; PL, platelet lysate; Apo, apocynin; DPI, diphenylene iodonium; Cont, irrelevant control antibody.

Figure 7. Potential role for TRAF4 in the redox-dependence of GPIb and GPVI signalling. TRAF4 directly associated with the cytoplasmic tails of GPIbβ/GPVI could provide a novel pathway for ligand-induced activation of both Lyn (*via* Hic-5) and Nox2 (*via* p47^{phox}). Downstream of TRAF4, Lyn-dependent activation of Syk, or p47^{phox}-dependent activation of Rac1/2 and Rac/PAK1 together stimulate separate pathways leading to platelet integrin activation. Key proteins thus far demonstrated by pull-down and IP/WB to be part of the complex with GPIb-IX-V and GPVI are shaded (this study). The association of Syk with GPIb and GPVI is published elsewhere [10, 48].

Supplementary data

Supplementary Figure 1. Interaction partners of the MBP-GPVI affinity column. 2 μl of each flow-through or guanidine-HCl eluted fraction were blotted onto a nitrocellulose membrane and probed for the following proteins: MBP, GPVI, TRAF4, Hic-5, p47^{phox} and Fyn.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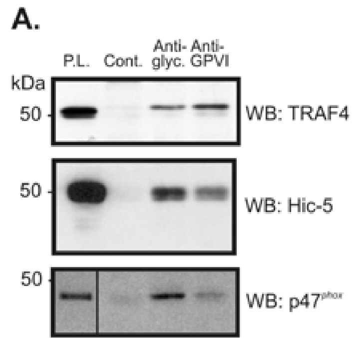
GPIbβ
TRAF4
Hic-5
p47phox
Fyb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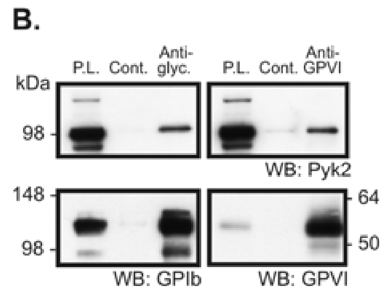
WB: p47phox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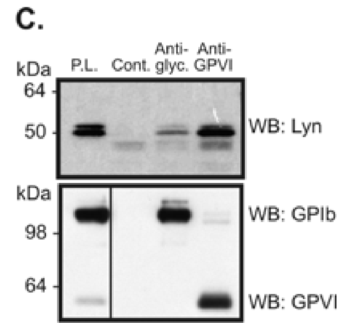
50
WB: GPVI


C.
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64
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WB: Lyn
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98
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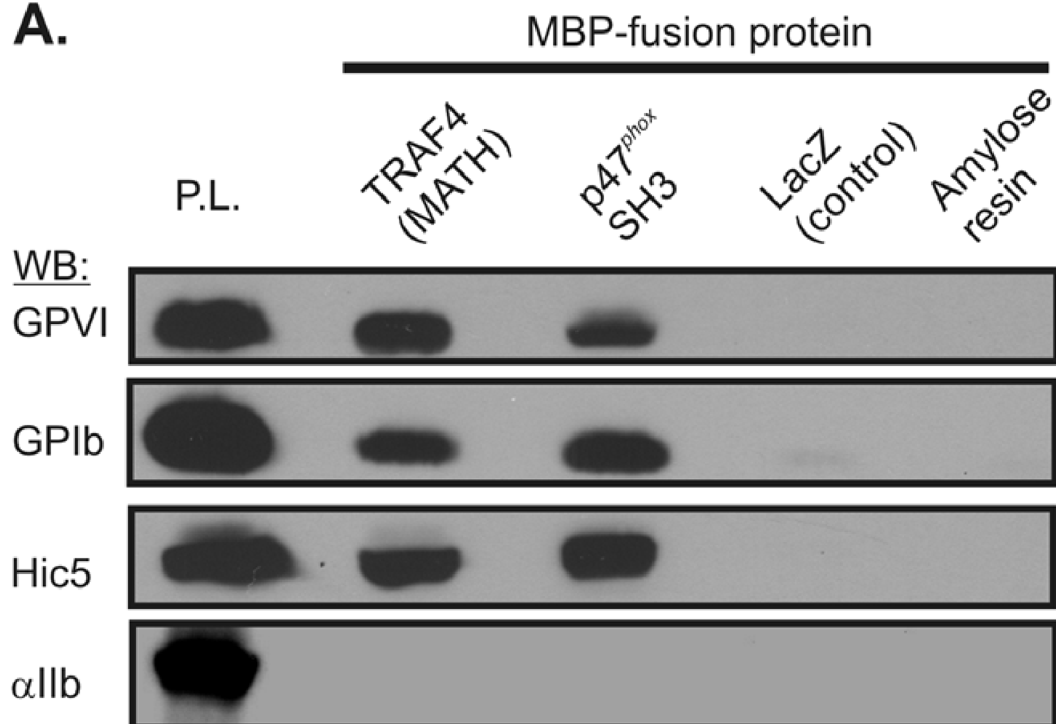
WB: GPIb
WB: GPVI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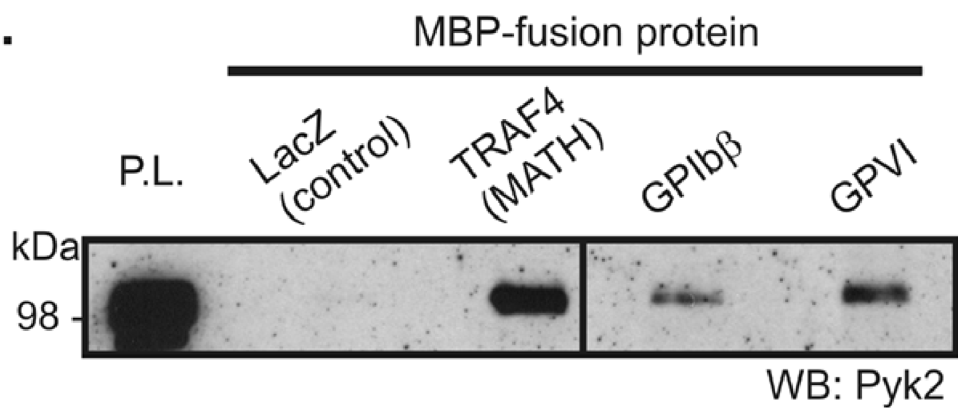
P.L.
TRAF4 (MATH)
p47phox SH3
LacZ (control)
Amylose resin
WB:
GPVI
GPIb
Hic5
αIIb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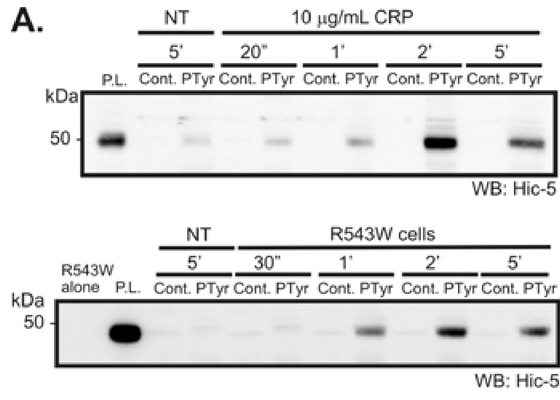
P.L.
LacZ (control)
TRAF4 (MATH)
GPIbβ
GPVI
kDa
98
WB: Pyk2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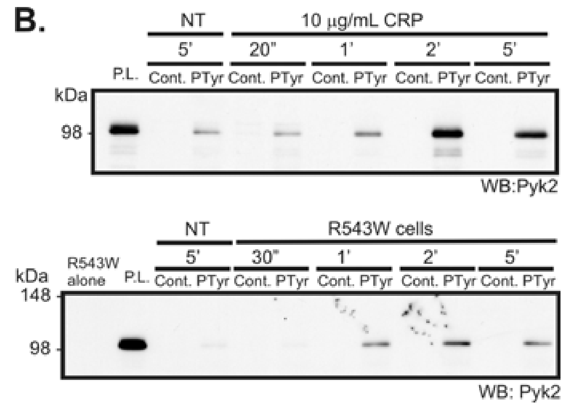
kDa
50
WB: Hic-5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WB:Pyk2
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kDa
148
98
WB: Pyk2

A.

NT CRP
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36
WB: Hic-5

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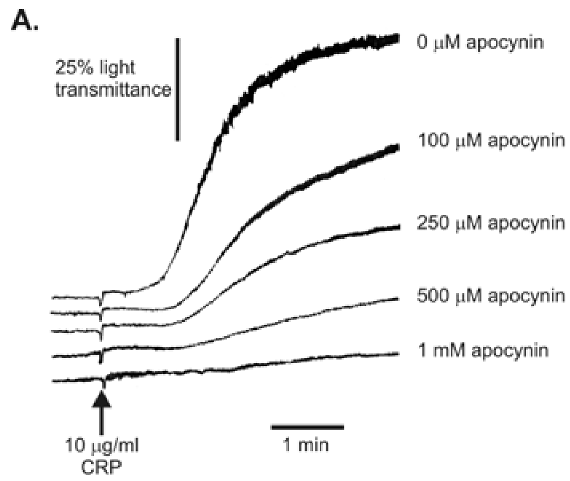
WB: Pyk2

A.



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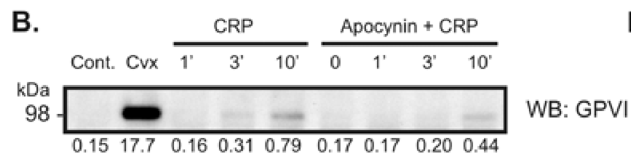

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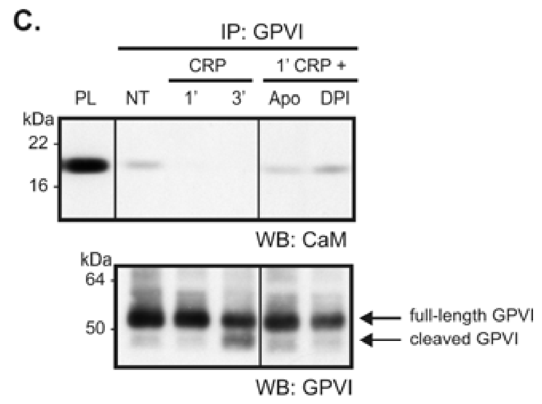
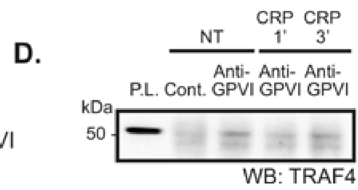
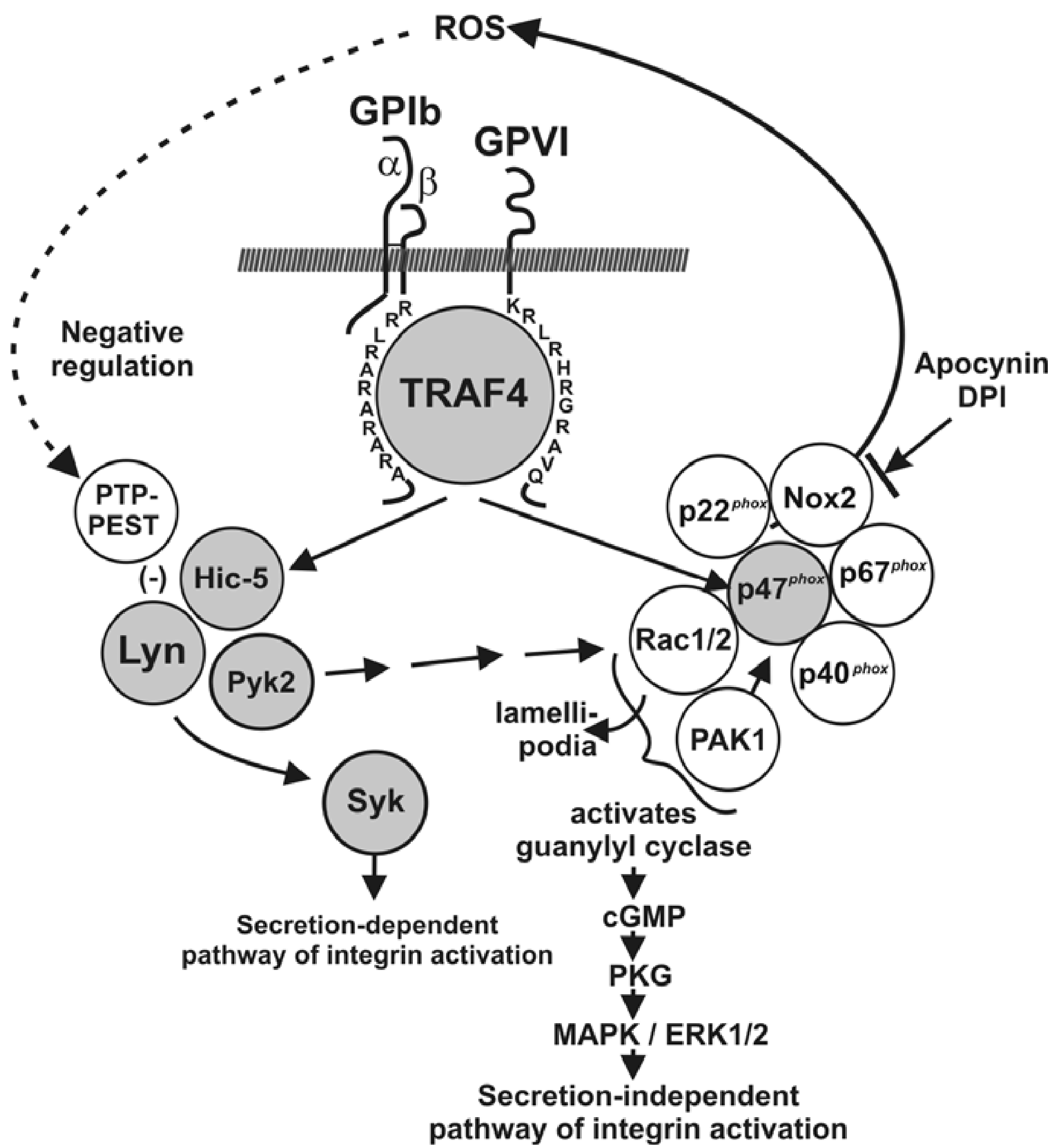


D.




ROS
GPIb
α
β
GPVI
Negative
regulation
TRAF4
Apocynin
DPI
PTP-
PEST
Nox2
p22phox
Hic-5
(-)
p47phox
p67phox
Lyn
Rac1/2
Pyk2
p40phox
lamelli-
podia
PAK1
Syk
activates
guanylyl cyclase
cGMP
PKG
MAPK / ERK1/2
Secretion-dependent
pathway of integrin activation
Secretion-independent
pathway of integrin activation