Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin αIIbβ3

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Integrin activation (inside-out signaling) in platelets can be initiated by agonists such as von Willebrand factor (VWF) and thrombin. Here we show that a mitogen-activated protein kinase (MAPK), p38, plays an important role in the activation of integrin αIIbβ3 induced by VWF and thrombin. A dominant-negative mutant of p38, p38AF, inhibits αIIbβ3 activation induced by VWF binding to its receptor, the platelet glycoprotein Ib-IX (GPIb-IX), and p38 inhibitors diminish platelet aggregation induced by VWF or low-dose thrombin. The inhibitory effect of p38 inhibitor is unlikely to be caused by the previous suggested effect on cyclo-oxygenase, as inhibition also was observed in the presence of high concentrations of cyclo-oxygenase inhibitor, aspirin. VWF or thrombin induces p38 activation, which is inhibited in cGMP-dependent protein kinase (PKG)–knockout mouse platelets and PKG inhibitor–treated human platelets, indicating that activation of p38 is downstream from PKG in the signaling pathway. p38AF or p38 inhibitors diminish PKG-induced phosphorylation of extracellular stimuli-responsive kinase (ERK), which also is important in integrin activation. Thus, p38 plays an important role in mediating PKG-dependent activation of ERK. These data delineate a novel signaling pathway in which platelet agonists sequentially activate PKG, p38, and ERK pathways leading to integrin activation.

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Materials and methods
Preparation of platelets
For studies using human blood, approval was obtained from the University of Illinois at Chicago institutional review board. Informed consent was provided according to the Declaration of Helsinki. Fresh blood was drawn by venipuncture from healthy volunteers, with one-tenth volume of 3.8% trisodium citrate used as anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation at 300g for 20 minutes at room temperature. To prepare washed platelets, blood was anticoagulated with ACD (2.5% trisodium citrate, 2% dextrose, and 1.5% citric acid) (1.4 mL ACD/10 mL blood). PRP was further centrifuged at 1200g for 15 minutes. Platelets were then washed twice with CGS (sodium chloride 0.12 M, trisodium citrate 0.0129 M, and glucose 0.03 M, pH 6.5) containing 0.1% bovine serum albumin (BSA) and resuspended in the HEPES-buffered Tyrode solution. They were then allowed to recover to resting state at 25°C for 1 to 2 hours as previously described. In experiments for the detection of p38 phosphorylation, platelets were resuspended in Tyrode buffer to give a concentration of 5 × 10^9/mL.

Platelet aggregation
Platelet aggregation was measured using a turbidometric platelet aggregometer at 37°C with a stirring speed of 1000 rpm. VWF-dependent platelet aggregation was induced by addition of VWF modulators, ristocetin or botrocetin, to PRP. Washed platelets were used in platelet aggregation induced by α-thrombin (Enzyme Research Laboratories, South Bend, IN) or thrombin receptor activating peptides, SFLLRN or AYPGKF. In some experiments, aggregation was induced by addition of VWF modulators, ristocetin or with thrombin (0.05 U/mL) in an aggregometer. To detect ERK phosphorylation, cells were solubilized by adding an equal volume of SDS-PAGE sample buffer containing 0.2 mM E64, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 M NaCl, pH 7.5 (Tris-buffered saline [TBS]), PVDF membranes were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in 5% dried skim milk in 0.01 M Tris, pH 7.5 (Tris-buffered saline [TBS]), PVDF membranes were probed with a monoclonal anti–flag M2 antibody specifically recognizing the phosphorylation-dependent epitope in the Thr180/Tyr182 site of p38 (New England Biolabs, Ipswich, MA; 1:1000). After washing, the membranes were probed with horseradish peroxidase–conjugated goat anti–rabbit IgG for 45 minutes. After further washing, blots were developed using the Enhanced Chemiluminescence kit from Pharmacia-Amersham Biotech (Uppsala, Sweden).

Immunoblot detection of p38 phosphorylation in human platelets
Washed platelets were incubated with VWF in the presence of botrocetin or ristocetin, or with thrombin (0.05 μg/mL), in a platelet aggregometer at 37°C for various lengths of time. Platelets also were incubated with 8-bromo-cGMP (100 μM), 8-bromo-cAMP (100 μM), 8-pCPT-cGMP (100 μM), or SNAP1 (100 μM). In some experiments, platelets were preincubated with KT5823 (5 μM), Rp-cPT-cGMPS (0.2 mM), Rp-Bi-PET-cGMPs (0.1 mM), KT 5720 (5 μM), or H89 (50 μM) for 5 minutes prior to adding agonists or PKG activators. Platelets were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 0.2 mM E64 and 2 mM phenylmethylsulfonyl fluoride (PMSF). Proteins in lysates were separated by SDS-PAGE on a 4% to 15% polyacrylamide gel and then electrotransferred to polyvinylidene fluoride (PVDF) membranes. After blocking in 5% dried skim milk in 0.01 M Tris, 0.15 M NaCl, pH 7.5 (Tris-buffered saline [TBS]), PVDF membranes were probed with the polyclonal anti-p38 antibody or an anti-p38 antibody specifically recognizing the phosphorylation-dependent epitope in the Thr180/Tyr182 site of p38 (New England Biolabs, Ipswich, MA; 1:1000). After 3 washes, the membranes were probed with horseradish peroxidase–conjugated goat anti–rabbit IgG for 45 minutes. After further washing, blots were developed using the Enhanced Chemiluminescence kit from Pharmacia-Amersham Biotech (Uppsala, Sweden).

Immunoblot detection of p38 phosphorylation in mouse platelets
Generation of a PKG I null (−/−) (PKG I−/−) allele by homologous recombination has been described previously. The PKG I−/− mice are on a mixed 129sv/C57BL background. Both the wild-type controls and PKG I−/− mice were age-matched littermates produced by heterozygous breeding. Washed mouse platelets were prepared as described previously. Briefly, mice (6-8 weeks old) were anesthetized by intraperitoneal injection of pentobarbital. Whole blood from homozygous PKG knockout mice or wild-type mice was collected from the inferior vena cava using one-seventh volume of ACD as anticoagulant. The platelets were then washed twice with CGS and resuspended in Tyrode buffer at 3 × 10^9/mL and incubated at 25°C for 1 hour before use. Washed platelets were then incubated with VWF (20 μg/mL) and botrocetin (5 μg/mL) in a platelet aggregometer at 37°C for 30 seconds. Platelets were solubilized in SDS-PAGE sample buffer containing 0.2 mM E64 and 2 mM PMSF. Phosphorylation of p38 was analyzed by Western blot.

Expression of p38 dominant-negative mutant
Wild-type p38 and a dominant-negative mutant of p38 (TGY-AGF) (p38 AF) were generated as described previously. Wild-type p38 and p38AF mutants were cloned into the pCDNA3 expression vector with the tagged Flag epitope and were transfected into 123 cells using Lipofectamine plus (Invitrogen, Carlsbad, CA). Expression of p38 and p38AF mutant was assessed by Western blotting with a monoclonal anti–flag M2 antibody (Sigma, Saint Louis, MO). Expression levels of GPIb-IX and αIIBβ3 were examined by flow cytometry using a monoclonal antibody against GPIbα, SZ2 (kindly provided by Dr Changgeng Ruan, Soochow University, Suzhou, China) and a monoclonal antibody against αIIBβ3 complex, D57 (kindly provided by Dr Mark Ginsberg, Scripps Research Institute, La Jolla, CA).

GPIb-IX–mediated integrin activation in reconstituted CHO model
VWF-induced GPIb-IX–mediated activation of integrin αIIBβ3 was examined by flow cytometry analysis of Oregon green 488–labeled fibrinogen (Molecular Probes, Eugene, OR) binding to integrin αIIBβ3, as described previously. Briefly, Chinese hamster ovary (CHO) cells coexpressing integrin αIIBβ3 and GPIb-IX (123 cells) were detached from the tissue culture plates by 0.53 mM EDTA in phosphate-buffered saline solution (PBS). After washing, the cells were resuspended in modified Tyrode buffer. The cells were incubated with Oregon green 488–labeled fibrinogen and ristocetin in the presence or absence of purified human VWF (20 μg/mL). Nonspecific binding of fibrinogen was estimated by measuring fibrinogen binding in the presence of a specific integrin inhibitor RGDS (4 μg/mL). We have previously shown that RGDS but not RGES peptides specifically inhibit fibrinogen binding to integrin αIIBβ3.

Detection of ERK phosphorylation
Washed platelets were incubated with or without VWF and ristocetin in a platelet aggregometer at 37°C for various lengths of time. Platelets were solubilized in SDS-PAGE sample buffer containing 0.2 mM E64 and 2 mM PMSF. Proteins in lysates were separated by SDS-PAGE on a 4% to 15% polyacrylamide gel and then electrotransferred to PVDF membranes. To determine the role of p38 on agonist-induced ERK phosphorylation, washed platelets were preincubated with SB203580, SB202190, or PD98059 (20 μM) for 5 minutes, then incubated with VWF in the presence of ristocetin or with thrombin (0.05 U/mL) in an aggregometer. To detect ERK phosphorylation in transfected CHO cells, cells (5 × 10^6 cells/mL) resuspended in modified Tyrode buffer were stimulated with VWF (20 μg/mL) and 1 mg/mL ristocetin at 22°C for 5 minutes. In some experiments, the cells also were stimulated with PKG activators 8-bromo-cGMP (0.1 mM), 8-pCPT-cGMP (0.1 mM), or glyco-SNAP1 (0.1 mM). When examining the effect of p38 inhibitors, SB203580 and SB202190 (20 μM) were preincubated with cells at 22°C for 5 minutes before addition of agonists. Washed platelets were solubilized in SDS-PAGE sample buffer containing 0.2 mM E64 and 2 mM PMSF. Proteins in lysates were separated by SDS-PAGE on a 4% to 15% polyacrylamide gel and then electrotransferred to PVDF membranes. To determine the role of p38 on agonist-induced ERK phosphorylation, washed platelets were preincubated with SB203580, SB202190, or PD98059 (20 μM) for 5 minutes, then incubated with VWF in the presence of ristocetin or with thrombin (0.05 U/mL) in an aggregometer. To detect ERK phosphorylation in transfected CHO cells, cells (5 × 10^6 cells/mL) resuspended in modified Tyrode buffer were stimulated with VWF (20 μg/mL) and 1 mg/mL ristocetin at 22°C for 5 minutes. In some experiments, the cells also were stimulated with PKG activators 8-bromo-cGMP (0.1 mM), 8-pCPT-cGMP (0.1 mM), or glyco-SNAP1 (0.1 mM). When examining the effect of p38 inhibitors, SB203580 and SB202190 (20 μM) were preincubated with cells at 22°C for 5 minutes before addition of 8-bromo-cGMP. Cells were solubilized by adding an equal volume of SDS-PAGE sample buffer containing 0.2 mM E64, 20 μg/mL aprotinin, and 2 mM PMSF, and nuclear DNA was removed by centrifugation. Phosphorylation of ERK was detected by Western blot as described previously. The membranes were immunoblotted with an anti-ERK2 antibody (Santa Cruz Biotechnology,
Results
GPIb-IX–mediated activation of αIBβ3 is inhibited by a dominant-negative mutant of p38

We have previously shown that activation of the platelet integrin αIBβ3 can be reconstituted in CHO cells expressing both recombinant human GPIb-IX and integrin αIBβ3 (123 cells).25 In this reconstituted integrin activation model, binding of VWF to GPIb-IX triggers activation of integrin αIBβ3, allowing specific binding of an integrin ligand, fibrinogen. Reconstitution of integrin activation in this CHO cell line thus allows analysis of integrin activation pathways using molecular biology techniques. To identify the role of the p38 pathway in GPIb-IX–mediated integrin activation, 123 cells were transfected with cDNA constructs encoding a dominant-negative mutant of p38, which was created by replacing 2 residues in the phosphorylation sites (Thr180 and Tyr182) with alanine and phenylalanine, respectively (p38AF) (Figure 1).33 Three different levels of expression of this dominant-negative mutant resulted in inhibition of GPIb-IX–mediated integrin activation.

The role of p38 in integrin activation in human platelets

To investigate the role of p38 in VWF-induced integrin activation in platelets, we examined the effects of p38 inhibitors SB203580 and SB202190 on VWF-induced integrin-dependent platelet aggregation. As expected, addition of ristocetin (or botrocetin [not shown]), which allowed VWF to bind to GPIb-IX, induced αIBβ3 activation and the first wave of partially integrin-dependent aggregation/agglutination of platelets, leading to the totally integrin-dependent second wave of platelet aggregation (Figure 2). Either SB203580 or SB202190 inhibited integrin-dependent platelet aggregation induced by ristocetin (Figure 2A,B) or botrocetin (not shown). It was previously suggested that SB203580 might affect cyclo-oxygenase activity in platelets.29 To exclude the possibility that the inhibitory effect of SB203580 is dependent on a possible effect on cyclo-oxygenases, ristocetin-induced platelet aggregation/agglutination was examined in the presence of a high concentration of aspirin, a cyclo-oxygenase inhibitor. To verify that aspirin abolished cyclo-oxygenase function, we showed that aspirin at 0.5 mM or higher concentrations completely inhibited arachidonic acid–induced platelet aggregation that is mediated via the cyclo-oxygenase pathway (Figure 2C). As expected, TXA2-dependent second wave of platelet aggregation induced by ristocetin was completely abolished in the presence of aspirin (Figure 2D). However, the ristocetin-induced first wave of platelet aggregation/agglutination was not affected by aspirin but was significantly (but partially) inhibited by RGDS peptide, even in the presence of aspirin (Figure 2D), suggesting that VWF-induced integrin activation precedes the second wave of platelet aggregation and is independent of TXA2 pathway. Addition of SB203580 or SB202190 to the aspirin-treated platelets inhibited ristocetin-induced platelet aggregation in a manner similar to RGDS peptide (Figure 2D). These results exclude the possibility that the inhibitory effects of SB203580 or SB202190 on GPIb-mediated integrin activation are caused by their effects on cyclo-oxygenases. Thus, we conclude that the p38 pathway is important in VWF-induced integrin activation in platelets.

To examine whether p38 was required for platelet activation induced by other platelet agonists, washed platelets were preincubated with SB203580 or SB202190 and stimulated with a low dose of thrombin (0.05 U/mL). Thrombin-induced platelet activation was inhibited by the p38 inhibitors SB203580 and SB202190 (Figure 2E). To exclude the possibility that inhibitory effects of SB203580 or SB202190 resulted from a possible effect on cyclo-oxygenase,29 platelets were preincubated with 1 mM aspirin and stimulated with the same concentration of thrombin. We found...
that aspirin had no significant inhibitory effect on 0.05 U/mL thrombin-induced platelet aggregation (Figure 2E). Thus, the inhibitory effect of SB203580 or SB202190 is unlikely to result from their effects on cyclo-oxygenase. It is known that low-dose thrombin-induced platelet activation requires both GPIb-IX and protease-activated receptors (PARs). To investigate whether p38 also plays a role in GPIb-IX–independent platelet activation pathways, we examined the effects of SB203580 on platelet aggregation induced by thrombin receptor activating peptides (TRAPs), SFLLRN (tethered ligand for PAR1), or AYPGKF (ligand for PAR4). SB203580 significantly inhibited platelet aggregation induced by both PAR1 and PAR4 ligands (Figure 3A, B). These data indicate that the p38 pathway not only plays an important role in GPIb-IX–mediated platelet activation but also in platelet activation-induced by G-protein–coupled receptors.

**Activation of p38 in platelets**

If p38 mediates integrin activation signals induced by VWF and thrombin, these agonists would be expected to stimulate p38 activation. It is known that p38 activation is a consequence of p38 phosphorylation at Thr\(^{180}\) and Tyr\(^{182}\) by the upstream MAPK kinases (MKK), MKK3/MKK6. Thus, to determine whether p38 is activated following ligand binding to GPIb-IX, we examined phosphorylation of p38 using an anti–p38 antibody recognizing the phosphorylated Thr\(^{180}/\)Tyr\(^{182}\) site. Figures 4A and 4C show that p38 was rapidly activated in platelets following stimulation by VWF in the presence of ristocetin. VWF-induced p38 phosphorylation peaked after only 1 to 2 minutes (variable between different donors) but became significantly reduced within 5 minutes (Figure 4A, C, D), suggesting that p38 phosphorylation is an early transient event. The time course of p38 phosphorylation induced by VWF coincided with the time course of phosphorylation of ERK2 (Figure 4B) that we previously showed to be transiently activated by VWF. Activation of p38 also was confirmed by an immunoprecipitation-kinase assay, using myelin basic protein (MBP) as a p38 substrate (data not shown). To determine whether phosphorylation of p38 is GPIb-IX dependent, platelets were preincubated with the anti–GPIbα monoclonal antibody, SZ2, before addition of VWF and ristocetin. VWF-induced p38 phosphorylation was inhibited by SZ2 but not by control mouse IgG (Figure 4E). Similarly, low-dose thrombin also induced p38 phosphorylation (Figure 4F, G). Furthermore, the integrin inhibitor, RGDS peptide, or the integrin α\(^{IIb}\)β\(^{3}\) blocking antibodies, 2G12 and SZ21, had no inhibitory effect on the phosphorylation of p38 (Figure 4G–E), and RGDS by itself did not induce p38 phosphorylation. Therefore, p38 phosphorylation is unlikely to be caused by integrin outside-in signaling. These data indicate that VWF and thrombin activate p38 in platelets.

**Activation of p38 is downstream from PKG in the platelet activation pathway**

We recently have reported that PKG I is an important early stimulatory mediator in the GPIb-IX– and PAR-mediated platelet activation pathway. To determine whether PKG is involved in VWF-induced p38 activation, we examined the effect of PKG inhibitors (Rp-pCPT-cGMPS or Rp-Br-PET-cGMPS) on GPIb-IX–dependent phosphorylation of p38. PKG inhibitors significantly inhibited VWF-induced p38 phosphorylation, indicating that PKG is important in GPIb-IX–mediated p38 activation (Figure 5A). Similarly, low-dose thrombin-induced p38 phosphorylation also was inhibited by Rp-Br-PET-cGMPS (Figure 5B). To exclude the possibility of nonspecific effects of PKG inhibitors, we examined VWF-induced phosphorylation of p38 in PKG I-knockout mouse platelets. VWF significantly enhanced phosphorylation levels of p38 in platelets from wild-type mice but failed to enhance p38 phosphorylation in PKG I knockout mouse platelets (Figure 5C). Thus, agonist-induced phosphorylation of p38 is downstream from PKG signaling.

To investigate whether activation of PKG is sufficient to stimulate activation of p38, platelets were treated with cGMPS analogs 8-bromo-cGMPS or 8-pCPT-cGMPS, or the nitric oxide donor glyco-SNAP1, and then examined for p38 phosphorylation. All these PKG activators induced phosphorylation of p38 (Figure 6A). cGMPS analog–induced phosphorylation of p38 was inhibited by PKG inhibitors KT5823 (Figure 6B) or Rp-pCPT-cGMPS (data
Figure 4. VWF-induced phosphorylation of p38 in human platelets. (A,B) Washed platelets (5 × 10^7/mL, 200 μL) were incubated for 0.5, 1, 2, or 5 minutes in a platelet aggregometer stirring at 1000 rpm with buffer, ristocetin alone (0.6 mg/mL), or with ristocetin and VWF (20 μg/mL). (A) The platelets were then solubilized and immunoblotted with a rabbit antibody specifically recognizing phosphorylated form of p38 (P-p38) or with a rabbit anti-p38 antibody to indicate comparable loading levels (p38). Note that VWF or thrombin-induced p38 phosphorylation is inhibited by p38 inhibitors and in PKG knockout platelets. (B) Solubilized platelets also were immunoblotted with a rabbit antibody specifically recognizing phosphorylated form of ERK (P-ERK) or with a rabbit anti-ERK2 antibody to indicate comparable loading levels (ERK). (C) Washed platelets (5 × 10^7/mL, 200 μL) were incubated for 0.5 or 5 minutes in a platelet aggregometer (stirring at 1000 rpm) with buffer (ctrl), 1 mg/mL ristocetin (risto), and VWF (20 μg/mL). The platelets were then solubilized and immunoblotted with a rabbit antibody specifically recognizing phosphorylated forms of p38 (P-p38) or immunoblotted with a rabbit anti-p38 antibody to indicate comparable loading levels (p38). Immunoblotting results as in experiments described in panel C were scanned and quantitated using NIH Image software. Results shown are from 4 experiments (mean ± SD). (D) Platelets were preincubated with a mouse IgG (40 μg/mL), an anti-GPIbα monoclonal antibody, SZ2 (40 μg/mL), or RGDS (1 mM) at 22°C for 5 minutes, and then stimulated with WVF and ristocetin (WVF). (E) Platelets were preincubated with a mouse IgG (20 μg/mL) or anti-human integrin αIIbβ3 monoclonal antibodies SZ21 or 2G12 (20 μg/mL), or RGDS (1 mM) at 22°C for 5 minutes, and then incubated without or with thrombin (0.05 μM/mL). (F) Platelets were preincubated with buffer (control) or RGDS (1 mM) at 22°C for 5 minutes and then incubated with or without thrombin (0.05 μM/mL). Immunoblotting results were scanned and quantitated using NIH Image software. Results shown are from 3 experiments (mean ± SD).

Discussion

Two major findings presented in this study are significant to the signaling mechanisms of platelet integrin activation. First, we have not shown). In contrast, PKA inhibitors KT5720 and H89 had no inhibitory effect on cGMP-induced p38 phosphorylation (Figure 6B). Thus, cGMP induces p38 activation by activating PKG. Like VWF and thrombin, cGMP-induced p38 phosphorylation was significantly increased after only 30 seconds but returned to near background level within 5 minutes (Figure 6C,D). These data indicate that activation of p38 is downstream from the cGMP-PKG pathway during integrin activation in platelets.

The p38 pathway is important in PKG-mediated activation of ERK pathway

We recently have shown that VWF and thrombin induce activation of the ERK pathway, which plays an important role in integrin activation.21 We also have shown that activation of ERK is downstream from PKG in the GPIb-IX signaling pathway.21 To investigate the relationship between ERK and p38 pathways during integrin activation, we examined the effects of dominant-negative mutants of p38 on VWF-induced phosphorylation of ERK in 123 cells. VWF-mediated phosphorylation of ERK was abolished by expression of p38AF (Figure 7A), indicating that p38 is required for VWF-induced activation of the ERK pathway.

Previously, we showed that overexpression of PKG in 123 cells (123/PKG cells) significantly enhanced VWF-induced ERK2 phosphorylation.21 To further determine the role of p38 on PKG-dependent activation of the ERK pathway, we also examined the effects of p38AF on ERK activation in 123/PKG cells. Figure 7B shows that expression of p38AF abolished VWF-induced ERK2 phosphorylation in 123/PKG cells. Furthermore, addition of cGMP analogs (8-Br-cGMP, 8-pCPT-cGMP, or 8-dibutyl-cGMP) to 123/PKG cells was sufficient to induce ERK phosphorylation, which was abolished by expression of p38AF (Figure 7C) or by specific p38 inhibitors, SB203580 and SB202190 (Figure 7D). Similarly, ERK2 phosphorylation induced by VWF or low-dose thrombin in human platelets was inhibited by p38 inhibitors (Figure 7E,F). However, VWF-induced p38 phosphorylation was not significantly affected by PD98059 (Figure 7E). Thus, we conclude that platelet agonists induce a sequential activation of cGMP-PKG, p38, and ERK pathways, leading to integrin activation.

Figure 5. VWF- or low-dose thrombin-induced p38 phosphorylation is PKG dependent. (A) Human platelets were preincubated without or with a PKG inhibitor, Rp-pCPT-cGMPS (200 μM), at 22°C for 5 minutes, and then stimulated with botrocetin in the presence or absence of VWF at 37°C for 1 minute. (B) Human platelets were treated with or without the PKG inhibitor Rp-Br-PET-cGMPS (100 μM) and then stimulated with or without a low-dose thrombin (0.05 μM/mL) at 37°C for 2 minutes (the order of lanes from the same blot was rearranged for better presentation). (C) Platelets from wild-type mice (WT) or PKG I knockout mice (KO) were incubated with buffer or with botrocetin (5 μg/mL) in the presence or absence of VWF (20 μg/mL) at 37°C for 0.5 minute. Platelets from panels A, B, and C were then solubilized and immunoblotted with an antibody specific for phosphorylated forms of p38 (P-p38) or with an antibody against phosphorylation-independent epitopes of p38 (p38). Note that VWF or thrombin-induced p38 phosphorylation is inhibited by PKG inhibitors and in PKG knockout platelets.
found an important role for p38 MAPK in VWF- and thrombin-induced activation of integrin αIIbβ3. Second, we have delineated a novel signaling pathway in which platelet agonists induce sequential activation of cGMP-PKG, p38, and ERK pathways, leading to the activation of integrin αIIbβ3. These findings also are of general significance to the understanding of the interaction between intracellular signaling pathways, as we have identified a mechanism by which both p38 and ERK MAPK pathways are activated and a downstream signaling pathway of the important intracellular secondary messenger cGMP.

Previous studies on the role of p38 in platelet activation have been based mainly on the p38 inhibitor SB203580. This inhibitor inhibited platelet aggregation induced by low concentrations of several platelet agonists, including collagen and thromboxane A2 (TXA2).28 However, these results have been interpreted as a consequence of the direct inhibitory effect of SB203580 on TXA2 production by cyclo-oxygenase.29 In this study, we show that p38 plays an important role in platelet activation induced by human VWF and low-dose thrombin by using a dominant-negative mutant of p38 and by using SB203580 under conditions where the reported effect of SB203580 on cyclo-oxygenase is excluded. Thus, the data presented in this study allow us to conclude that p38 indeed plays an important role in platelet activation. Our data also demonstrate that the effect of SB203580 on platelet activation is unlikely to result from its effect on cyclo-oxygenase. It is important to note here that cyclo-oxygenase does not play a significant role in platelet aggregation induced by thrombin at the concentrations used in this study, which is not significantly affected by high concentrations of aspirin (Figure 2). Therefore, it is reasonable to use SB203580 as a relatively specific inhibitor to examine the role of p38 in platelet activation induced by agonists such as thrombin.

The effects of SB203580 on VWF-induced platelet activation also have been controversial, with studies showing inhibitory effects or showing no effect of this inhibitor on VWF-dependent platelet aggregation.35,36 Using dominant-negative mutants of p38 as well as p38 inhibitors under conditions that exclude the role of cyclo-oxygenase, our study provides clear evidence that p38 plays a critical role in the regulation of platelet activation induced by VWF.

Figure 6. cGMP analogs or a nitric oxide donor–induced phosphorylation of p38 in platelets. (A) Washed human platelets (200 μL) were incubated under stirring in the platelet aggregometer with 2 μL water (control) or PKG activators 8-bromo-cGMP (100 μM), 8-pCPT-cGMP (100 μM), or glyco-SNAP1 (100 μM) at 37°C for 0.5 minute. Platelets were then solubilized and analyzed by immunoblotting with a rabbit antibody recognizing phosphorylated form of p38 to detect p38 phosphorylation (P-p38) and with a rabbit anti-p38 antibody to indicate comparable loading levels (p38). (B) Platelets were preincubated without (control) or with DMSO, a PKG inhibitor, KT5823 (5 μM), or PKA inhibitors, KT5720 (5 μM) or H89 (50 μM), at 22°C for 5 minutes and then stimulated with 8-pCPT-cGMP (100 μM) for 0.5 minute. These platelets were solubilized and analyzed by immunoblotting with a rabbit antibody recognizing phosphorylated form of p38 (P-p38) and with a rabbit anti-p38 antibody to indicate comparable loading levels (p38). (C,D) Washed human platelets were treated with 8-bromo-cGMP for 0.5 minute or 5 minutes and then immunoblotted with P-p38 antibody and with p38 antibody as described in panel A. Immunoblotting results were scanned and quantitated using NIH Image software. Quantitative results from 3 experiments (mean ± SD) are shown in panel C, and a representative experiment is shown in panel D.

Figure 7. The role of p38 in agonist- and cGMP-induced phosphorylation of ERK2. (A) 123 cells were transfected with vector (123/vector) or the dominant-negative mutant p38AF cDNA (123/p38AF), 123/vector cells, or 123/p38AF cells were incubated with buffer, ristocetin (1 mg/mL), or ristocetin and VWF (20 μg/mL) at 25°C for 5 minutes. Cells were solubilized by adding equal volume of 2 × SDS sample buffer and immunoblotted with an antibody specific for phosphorylated Thr202/Tyr204 of ERK (New England Biolabs) (P-ERK2) to detect ERK phosphorylation, and with an anti-ERK2 antibody to monitor loading. (B) 123/PKG cells were transfected with vector (PKG/vector) or p38AF cDNA (PKG/p38AF). PKG/vector cells or PKG/p38AF cells were incubated with buffer, ristocetin (1 mg/mL), or ristocetin and VWF (20 μg/mL) at 25°C for 5 minutes. Phosphorylation of ERK was analyzed as described in panel A. (C) PKG/vector cells or PKG/p38AF cells were incubated with 8-bromo-cGMP (100 μM), 8-pCPT-cGMP (100 μM), or 8-dibutyryl-cGMP (100 μM) at 25°C for 5 minutes. Phosphorylation of ERK was analyzed as described in panel A. (D) PKG/vector cells were preincubated with DMSO, SB203580 (20 μM), or SB203580 (20 μM) for 5 minutes and then stimulated with 8-bromo-cGMP (100 μM) at 25°C for 5 minutes. Phosphorylation of ERK was analyzed by Western blot. (E) Washed human platelets were incubated with p38 inhibitor SB203580 or SB202190, or MEK inhibitor, PD98059, or with DMSO as a control. Platelets were stimulated with botrocetin or botrocetin plus VWF at 37°C for 1 minute, solubilized, and then analyzed for ERK2 phosphorylation as in panel A. (F) Washed human platelets were incubated with p38 inhibitor SB203580 or SB202190, or MEK inhibitor PD98059, or with DMSO as a control. Platelets were stimulated with 0.05 μM thrombin at 37°C for 2 minutes, solubilized, and then immunoblotted to detect ERK phosphorylation by Western blot.
an important role in VWF-induced platelet integrin activation. While the reasons for the difference between different studies are not clear, we noted that if the platelets become partially activated during preparation, VWF stimulation would have minimal stimulatory effects on p38 activation and the effects of SB203580 on platelet activation would be diminished (not shown). Since we show here that p38 activation is an early and transient event during platelet activation, it is possible that inadvertent activation of p38 during platelet preparation may render further p38 activation unnecessary during subsequent agonist stimulation.

Our data not only provide evidence that p38 plays an important role in VWF and thrombin-induced platelet activation but, more importantly, also demonstrate that p38 serves as a downstream mediator of PKG in the platelet activation pathway. We have reported that PKG plays an important stimulatory role in platelet activation induced by VWF and low doses of other platelet agonists such as thrombin, thromboxane A2, and collagen.20,22 Here, we further show that VWF- and thrombin-induced phosphorylation of p38 is inhibited in PKG knockout mouse platelets and by PKG inhibitors and that cGMP analogs or NO donors are sufficient to induce p38 phosphorylation in platelets. The results obtained with PKG inhibitors and those obtained with PKG I knockout mice are very similar and are consistent with the data obtained with PKG activators and recombinant protein expression in cultured cell lines, excluding the possible nonspecific effects of pharmacologic inhibitors. Thus, the data presented in this study provide a novel downstream effector mechanism of PKG. In addition, these data further support our previous findings and numerous previous publications that PKG inhibitors such as Rp-pCPT-cGMPs and KT5823 are effective and relatively specific in vitro, in platelets and in other cells.20,22,37-42 It remains unclear how PKG activates p38. Our data indicate that PKG induces activation of the upstream kinase of p38, MEK3 (data not shown), suggesting that PKG mediates activation of p38 indirectly via activation of upstream kinases in the p38 pathway.

While we show that GPIb-IX-induced activation of the p38 pathway is mediated by PKG, we also have previously shown that the cGMP-PKG pathway induces activation of a different MAPK pathway in platelets: the ERK pathway,21,37 which has been shown by us as well as other investigators to play an important role in platelet activation.21,43 Early studies on the ERK and p38 pathways suggest that these 2 pathways are activated by distinct stimuli and often play opposing roles in cell survival and death. Thus, there have been reports of negative regulation of the ERK pathway by the p38 pathway and vice versa.44 However, there also have been data showing that certain stimuli induce activation of both the p38 and ERK pathways. For example, oncogenic ras mutants induce activation of both ERK and p38, leading to senescence.45,46 The mechanisms that induce activation of both MAPK pathways remain unclear. One report suggests that oncogenic ras mutants may activate the ERK pathway, which then activates p38 in the senescence pathway of human fibroblast.45 Our data indicate that the cGMP-PKG pathway is a mechanism for the simultaneous activation of both the p38 and ERK MAPK pathways in platelets. This conclusion is consistent with previous data that individual MAPKs can be activated via cGMP pathway in other cell types.40,41,47 More importantly, we show that PKG-mediated activation of the ERK pathway requires activation of p38, indicating a novel signaling cascade of sequential activation of cGMP-PKG, p38, and ERK pathways, leading to integrin activation. Since PKG is expressed in many cell types, such as leukocytes, vascular endothelial cells, and smooth muscle cells, identification of the sequential activation of PKG, p38, and ERK is significant not only because this finding delineates a novel integrin inside-out signaling pathway in platelets, but also because it suggests a signaling pathway that may be of general importance in cGMP-dependent regulation of MAPK activity during cell activation, adhesion, proliferation, and apoptosis.

Although we have identified that the cGMP-PKG-p38-ERK pathway mediates integrin activation, the downstream mechanism leading to integrin activation remains to be elucidated. One possible mechanism is the activation of platelet secretion by this signaling pathway, as we have shown that PKG induces platelet secretion of ADP and other granular contents that activate integrins.22 However, the PKG, p38, and ERK-dependent integrin activation also occurs in the reconstituted CHO cell model, which is not known to have platelet granules. Therefore, secretion may not be the only mechanism responsible for integrin activation induced by this pathway. Thus, it would be interesting to further investigate how the PKG-p38-ERK pathway is involved in integrin activation. It is important to note here that activation of the PKG-MAPK pathway by adding cGMP analogs in the absence of agonist stimulation is not sufficient to activate integrin αIIbβ3.20 Thus, it appears that additional parallel signaling pathways induced by VWF or thrombin are required for integrin activation in addition to the cGMP-PKG-MAPKs pathway. In the case of GPIb-IX pathway, it has been reported that VWF-induced platelet activation involves Src kinase pathway,10,16,48-50 phosphotyrosinylsitol 3-kinase (PI3K) pathway,5,9,10,48,51 and Fc receptor γII (or Fc receptor γ-chain)-protein-tyrosine kinase Syk-signaling pathway.16-19 In addition, GPIb-IX is associated with several intracellular signaling proteins including 14-3-3,52 phosphotyrosinylsitol 3-kinase (via 14-3-3),51 Src family of kinases,1,6,50 and calmodulin.53 Thus, it is possible that integrin activation requires coordination of 2 or more signaling pathways, one of which is the cGMP-PKG-MAPK signaling pathway. It is also possible that the PKG-p38-ERK pathway is important in the amplification and stabilization of integrin activation.

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References

Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin \( \alpha_{IIb}\beta_3 \)

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