# p38 mitogen-activated protein kinase dephosphorylation is regulated by protein phosphatase 2A in human platelets activated by collagen

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**Abstract Collagen and the cross-linked collagen-related peptide (CRP-XL) each induced platelet p38 mitogen-activated protein kinase (p38) phosphorylation after 2 min. Subsequent dephosphorylation occurred in platelets activated with collagen, but not with CRP-XL, demonstrating glycoprotein VI-independent regulation of p38. Okadaic acid and fostriecin, inhibitors specific for protein phosphatase 2A (PP2A), blocked p38 dephosphorylation, and PP2A co-immunoprecipitated with phospho-p38. In addition, use of phenylarsine oxide suggested that tyrosine phosphatases and PP2A may act in concert to dephosphorylate p38. Finally, regulation of p38 in collagen-stimulated Glanzmann's platelets was indistinguishable from that in normal platelets, showing that p38 regulation is independent of integrin αIIbβ3.** 

*Key words:* p38 mitogen-activated protein kinase; Protein phosphatase 2A; Collagen; Collagen-related peptide; αIIbβ3; Dephosphorylation

## 1. Introduction

The activation of platelets by collagen leads both to their aggregation and to procoagulant expression, the two branches of the hemostatic pathway. During the activation process protein kinases become activated and cytoskeletal changes occur which lead to filopodial extension and to spreading of adherent platelets. Inhibition of p38 mitogen-activated protein kinase (MAPK) has been shown to attenuate collagen-stimulated platelet aggregation [1] and to impair the blebbing of platelets, which may be an important step in their procoagulant response [2].

p38 is a member of the MAPK superfamily and four isoforms (α, β, γ and δ) of the p38 MAPK subfamily exist. The α isoform is commonly referred to as p38 [3,4]. All four species have been detected in platelets [5], where collagen is reported to activate exclusively the α and β forms. In other cells, p38 MAPK is activated by dual phosphorylation of its TGY motif [6,7], by its upstream activators, the MAPK kinases, MKK3 and MKK6 [8,9]. These proteins are yet to be detected in platelets.

The p38 MAPK family members have been categorized as stress-activated protein kinases, which respond to numerous cytokines and to environmental and chemical stress [8], leading to cellular responses such as inflammation and apoptosis. Activation of p38 leads to subsequent activation of MAPK-activated protein kinases 2 and 3 and, in turn, to the phosphorylation of small heat shock protein 27 (HSP27), involved in actin remodeling. This suggests that p38 may play an important role in cytoskeletal reorganization in platelets as well as in other cells [10–12].

We have previously shown that platelet p38 MAPK is activated by collagen, CRP-XL (cross-linked collagen-related peptide) and a thromboxane analogue [1]. Indirectly, others have shown p38 MAPK activation by α-thrombin and its involvement in the phosphorylation of cytosolic phospholipase $A_2$ ($cPLA_2$), although reports on the effect of p38 on $cPLA_2$ activity have been conflicting [13–15].

Research into platelet protein phosphorylation has mainly concentrated on the action of protein kinases. In contrast, the role of protein phosphatases, which mediate dephosphorylation, remains poorly understood. The present study examines the role of protein phosphatases as negative regulators of p38 MAPK. Protein phosphatase 2A (PP2A) is a type II serine/threonine phosphatase consisting of a 36 kDa catalytic subunit and two regulatory subunits [16]. Type II protein phosphatases are able to dephosphorylate p38 MAPK: PP2A dephosphorylates p38 in vitro [17], and PP2C has previously been described as a regulator of p38 activity in vivo [18]. Following its recruitment to the cytoskeleton after platelet aggregation, PP2A may play a role in dephosphorylating cytoskeletal proteins [19] and in microtubule rearrangement [20]. Using okadaic acid (OA), a well-established and potent inhibitor of PP2A [21,22], and other selective inhibitors, we investigated the involvement of PP2A in the regulation of p38 in platelets stimulated by collagen and CRP-XL. By immunoprecipitation of p38 we have shown a direct association between p38 and PP2A.

Platelets contain several protein tyrosine phosphatases (PTPases) and a recent report shows that glycoprotein VI (GpVI) stimulation by collagen and CRP-XL leads to the phosphorylation of PTP1C (also called SHP-1) [23]. The present study indicates a role for a PTPase, which may work together with PP2A to remove phosphate from both the tyrosine and threonine residues within the p38 activation motif, to maintain the enzyme in the predominantly non-phosphorylated form in resting platelets.

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*Abbreviations:* BSA, bovine serum albumin; CRP-XL, cross-linked collagen-related peptide, $GCO(GPO)_{10}GCOG$ (where O = hydroxyproline); GpVI, glycoprotein VI; MAPK, mitogen-activated protein kinase; OA, okadaic acid; PAO, phenylarsine oxide; PP2A, protein phosphatase 2A; PTPase, protein tyrosine phosphatase

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## 2. Materials and methods

### 2.1. Materials

Bovine tendon collagen type I fibers were a gift from Ethicon Inc. (Somerville, NJ, USA) and were dialyzed against 0.01 M acetic acid. CRP, GCO(GPO)$_{10}$GCOG was synthesized and cross-linked as described in [24]. GR144053F was a gift from Glaxo Wellcome (Stevenage, UK). Rabbit polyclonal anti-phospho-p38 (Thr180/Tyr182) antibody (Ab), rabbit polyclonal Ab which recognizes both basal and phospho-p38, and immobilized anti-phospho-p38 (Thr180/Tyr182) monoclonal Ab (mAb) were from New England BioLabs (Hitchin, UK). MAb directed against the catalytic subunit of PP2A was from TCS Biologicals (Buckingham, UK). Rabbit antiserum SAK7, which binds a p38 C-terminal peptide, i.e. both phospho- and basal p38 forms [1], was a gift from Prof. J. Saklatvala, Kennedy Institute, London, UK. Control mouse IgG was from Autogen Bioclear (Calne, UK). Phenylarsine oxide (PAO) and OA were from Calbiochem-Novabiochem (Nottingham, UK). Fostriecin was from Alexis Corp. (Nottingham, UK). Horseradish peroxidase-conjugated donkey anti-rabbit Ig and sheep anti-mouse Ig were from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents were from Sigma-Aldrich (Dorset, UK).

### 2.2. Platelet preparation

Platelets, from concentrates supplied within 48 h of donation by the National Blood Service (Cambridge, UK), were prepared by removing red blood cells after centrifugation (250×$g$, 15 min). Platelet-rich plasma, with apyrase grade VII (0.25 U/ml), was spun (700×$g$, 15 min) and washed with HEPES-buffered saline (145 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgSO_4$, 0.5 mM EGTA, 10 mM HEPES, pH 7.4) at 700×$g$ for 10 min prior to resuspension in this buffer at $1\times10^9$ platelets/ml. Glanzmann's type I platelets, from an informed donor, were from the Department of Haematology, Royal Hallamshire Hospital (Sheffield, UK), and were prepared as above from whole blood collected into acid citrate dextrose.

### 2.3. Platelet stimulation

Platelet suspensions (50 μl) were incubated at 30°C either with increasing concentrations of collagen or CRP-XL for 3 min, or with collagen (50 μg/ml), CRP-XL (25 μg/ml) or the vehicle control (0.01 M acetic acid) for up to 480 s. Phosphatase inhibitors were dissolved in dimethyl sulfoxide (DMSO) unless otherwise stated. OA (1 μM) and PAO (2 μM) were added to platelets 10 min prior to introduction of collagen. Antagonism of the integrin αIIbβ3 was achieved by preincubating platelets with GR144053F (2 μM) for 20 min before stimulation with collagen or CRP-XL. Platelet activation was terminated by addition of an equal volume of Laemmli's buffer, and then samples were boiled and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

For blockade of phosphatase activity in vitro, platelets ($5\times10^8$ in 500 μl) were stimulated for 2 min with collagen and subsequently lysed with 500 μl of 2× extraction buffer (300 mM NaCl, 20 mM Tris, 10 mM EDTA, 2% Triton X-100, 1 protease cocktail tablet/10 ml (Boehringer Mannheim), pH 7.3). A 200 μl aliquot of lysate was removed and incubated with vehicle control (DMSO), fostriecin (5 μM in $H_2O$) or OA (1 μM), at 30°C for 10 min and 30 min. A 40 μl sample was mixed with 10 μl of 5× Laemmli's buffer, boiled and subjected to SDS–PAGE.

### 2.4. Western blotting

Proteins were separated using 10% SDS gels and transferred onto nitrocellulose in a Hoefer semi-dry blotter for 2 h at 0.8 mA/cm$^2$. All blots were checked for protein loading and evenness of transfer by staining membranes with Ponceau (0.1% Ponceau, 5% acetic acid) prior to blocking. Membranes were blocked with 5% milk (milk powder in 1× Tris-buffered saline Tween (TBST), 20 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.6) for 30 min at room temperature. Anti-phospho-p38 (Thr180/Tyr182) MAPK polyclonal antibody (diluted 1/1000 in 5% bovine serum albumin (BSA)–TBST) was used to probe the membranes at 4°C overnight. Membranes were stripped as required in stripping buffer (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol, pH 6.7) for 20 min at room temperature, washed extensively with TBST, blocked with 5% milk and re-probed with total p38 MAPK antibody (1/1000 in 5% BSA–TBST) at 4°C overnight. Primary anti-phospho-p38 and anti-total p38 MAPK antibodies were detected using an anti-rabbit secondary antibody diluted 1/10 000 in 1% BSA–TBST. Washed blots were subjected to enhanced chemiluminescence (ECL). Bands representing phospho-p38 MAPK were quantified by video densitometry using a Leica Q550C image analyzer and expressed as integrated optical density (IOD) [2]. Glanzmann's platelets were used only once due to the limited access to donors, so the IOD of the band representing total p38 MAPK was used to normalize the measurement of phospho-p38.

### 2.5. Immunoprecipitation of p38 MAPK

Platelets were stimulated with collagen (50 μg/ml) for 2 min, and lysed with 2× p38 lysis buffer (40 mM Tris, 300 mM NaCl, 2 mM EDTA, 1 mM EGTA, 2% Triton X-100, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 2 mM $Na_3VO_4$, 10 μg/ml leupeptin, 2 mM PMSF, pH 7.5). The Triton-insoluble pellet was removed and the lysates were pre-cleared for 1 h at 4°C using mouse IgG. Phospho-p38 MAPK was immunoprecipitated at 4°C overnight using an agarose-immobilized mAb that binds the phosphorylated protein. Immunoprecipitates were washed three times in p38 lysis buffer (1× concentration) prior to elution of proteins with Laemmli's buffer and SDS–PAGE. After blocking the membrane with 3% milk for 30 min, PP2A was detected using a mAb (1 μg/ml in milk) at 4°C overnight, followed by anti-mouse secondary antibody (1/2000 in milk). As a control, agarose-immobilized anti-phospho-p38 Ab was subjected to the immunoprecipitation (IP) protocol in the absence of platelet proteins to allow Ab-derived bands to be detected. Mouse IgG was also subjected to the IP protocol and captured using protein A/G agarose beads. Proteins were also eluted from 30 μl of beads alone and run alongside IP samples. Phospho- and non-phosphorylated p38 MAPK were also immunoprecipitated using SAK7 (5 μl) from platelets stimulated for 0, 2 or 8 min with collagen (50 μg/ml) and then lysed. Lysates were pre-cleared for 1 h using rabbit IgG. Protein A/G agarose beads were coated with antiserum for 2 h at 4°C and then mixed with platelet lysates for 5 h at 4°C. The beads were washed and proteins eluted as above.

### 2.6. Statistical analysis

Results shown are representatives of at least three experiments. Means and S.E.M. are shown on graphs and the number of experimental repeats indicated in the figure legends. Linear regression analysis of the time course of collagen-induced and CRP-XL-induced p38 phosphorylation between 90 and 480 s was done using the InStat® program.

## 3. Results

### 3.1. Collagen causes p38 dephosphorylation whereas CRP-XL does not

Phosphorylation of p38 MAPK was examined using platelets stimulated with either native type I collagen fibrils or the GpVI-specific ligand, CRP-XL. Dose–response curves for p38 MAPK phosphorylation (Fig. 1A) indicated that maximal p38 phosphorylation was achieved using collagen at a dose of 25–100 μg/ml or CRP-XL at 25–50 μg/ml, thus allowing saturating concentrations of ligands to be defined. After a 30 s lag, collagen (50 μg/ml) caused an increase in p38 phosphorylation, which reached a peak between 90 and 120 s after activation and then continuously declined by 480 s as shown in Fig. 1D. CRP-XL-induced (25 μg/ml) p38 MAPK phosphorylation also showed a 30 s delay, peaking between 90 and 240 s and was maintained without decline for up to 480 s. Representative Western blots for activation by collagen and CRP-XL, respectively, are shown in Fig. 1B and C. The lower panels in Fig. 1B and C indicate total p38 levels in each lane and demonstrate that the decrease in p38 phosphorylation in the presence of collagen is not a result of protein degradation. Regression analysis of p38 phosphorylation in CRP-XL-stimulated platelets showed that peak activity did not decline between 90 and 480 s ($P = 0.98$). In separate ex-

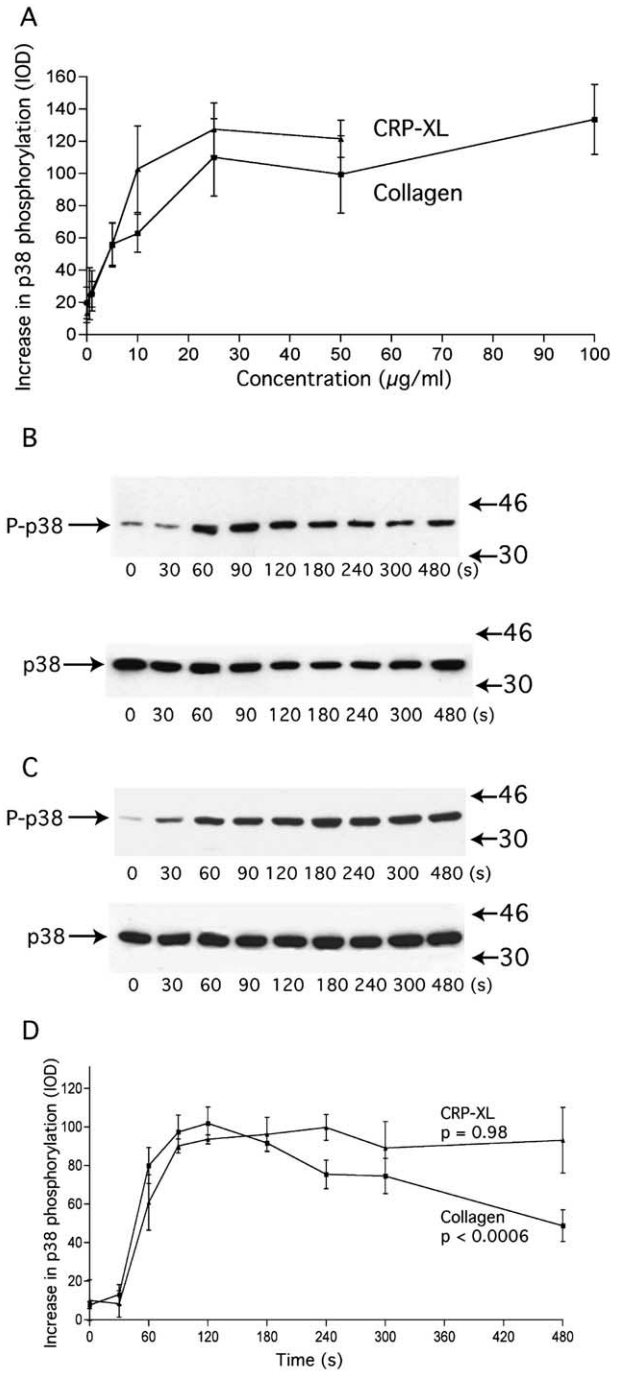


Fig. 1. Collagen and CRP-XL cause an equivalent increase in p38 phosphorylation, but subsequent p38 dephosphorylation is mediated by collagen only. A: Integrated optical density (IOD) plot for p38 phosphorylation in response to increasing doses of collagen (■) or CRP-XL (▲). Data from four similar experiments represent means and S.E.M. Platelets in suspension were stimulated with 50 μg/ml collagen fibers (B) or 25 μg/ml CRP-XL (C) for the indicated times. Lower panels in B and C show total p38 levels in the same blots after stripping and re-probing for p38 irrespective of its phosphorylation status. Phospho-p38 and total p38 were detected as detailed in Section 2.4. D: IOD data for p38 phosphorylation with time from five similar blots for collagen (■) and four similar blots for CRP-XL (▲). Data represent means and S.E.M.

periments (not shown), there was no decrease in p38 phosphorylation levels even after 20 min exposure to CRP-XL. In marked contrast, in collagen-treated platelets there was a significant, linear decline to about half peak levels of phosphorylation by 480 s ($P < 0.0006$). These activation profiles suggest that collagen regulates p38 activity in a different manner to CRP-XL and that signals from collagen result in a more rapid down-regulation of the phosphorylated state.

### 3.2. PP2A is responsible for the decline in p38 phosphorylation

Pretreatment of platelets for 20 min with 1 μM OA increased the phosphorylation of p38 MAPK by at least 50 IOD units. Both the 30 s lag in p38 phosphorylation after the addition of collagen and the decline subsequent to peak p38 phosphorylation levels were abolished in the presence of OA (Fig. 2A and B). OA is a more potent inhibitor of PP2A ($IC_{50} \cong 0.1$ nM) than PP1 ($IC_{50} \cong 10$ nM) in vitro, but concentrations required for inhibition in vivo can be much higher, requiring up to 1 μM for complete blockade of PP2A [25]. We found that lower concentrations (10 nM and 100 nM) were without effect.

The inhibitor fostriecin has a high affinity and specificity for PP2A ($IC_{50} \cong 3.2$ nM) compared to PP1 ($IC_{50} \cong 131$ μM) [26]. It therefore allowed better discrimination of the serine/threonine phosphatase causing the decline in p38 MAPK phosphorylation. Treatment of intact platelets with up to 1 μM fostriecin had no effect on basal or collagen-stimulated p38 phosphorylation and suggested that the compound was membrane impermeant at the concentrations tested (data not shown). To allow access of fostriecin to PP2A and to ensure complete inhibition of the phosphatase, phospho-p38 levels were measured in lysates from collagen-stimulated platelets in the presence and absence of fostriecin (5 μM) or OA

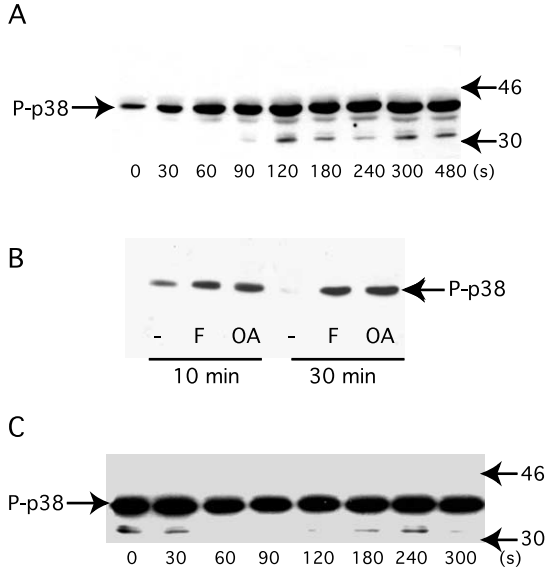


Fig. 2. PP2A and a PTPase mediate platelet p38 dephosphorylation. A: Platelets were preincubated with 1 μM OA for 10 min before activation by collagen (50 μg/ml) for the indicated times. B: Platelets were activated with collagen (50 μg/ml) for 2 min, lysed and incubated with vehicle control (DMSO), fostriecin (5 μM) or OA (1 μM) for 10 min and 30 min. C: PTPase involvement is shown by preincubation with 2 μM PAO for 10 min prior to activation with collagen (50 μg/ml) for the indicated times. These experiments were conducted at least three times. Phospho-p38 levels were detected using the immunodetection methods described in Section 2.4.

(1 μM). Both inhibitors caused an accumulation of phospho-p38, which was clearly evident after a 10 min or 30 min incubation as seen in Fig. 2B. Finally, we used tautomycin, an inhibitor shown to be more selective towards PP1 than PP2A [27] and found that there was no effect on p38 phosphorylation when it was employed at 1 μM (data not shown).

### 3.3. A PTPase dephosphorylates p38 MAPK

PAO has been shown to inhibit PTPase activity in platelets [28] and was used to investigate the involvement of the latter in p38 dephosphorylation. When PAO was employed in our system at 2 μM, a substantial increase in p38 phosphorylation was apparent (Fig. 2C). The high level of phospho-p38 seen under basal conditions was retained throughout the time course of activation by collagen.

### 3.4. PP2A directly interacts with p38 MAPK

The use of inhibitors suggested a functional role for PP2A as a regulator of p38 MAPK. We therefore sought to demonstrate a physical interaction between PP2A and p38 by examining proteins, which co-precipitate with phospho-p38. Platelets were stimulated with collagen for 2 min, at the peak of p38 phosphorylation, and the MAPK was immunoprecipitated with a phospho-p38-specific mAb (Fig. 3A). The isolated protein complexes were subjected to SDS–PAGE and Western blotting as described in Section 2. Blots were probed with a mAb raised against the 36 kDa catalytic subunit of PP2A. Fig. 3A (lane 2) shows that phospho-p38 is pulled down by the immunoprecipitating Ab. The presence of phospho-p38 in the whole cell lysate from collagen-activated platelets (lane 1) is also indicated and can be used to identify phospho-p38 in the IP sample. The blot from the same experiment was stripped and re-probed for PP2A. As seen in Fig. 3B, PP2A associated with p38 MAPK (lane 2), and the antibody control (lane 3) showed that the band that corresponded to PP2A was not contamination from the immunoprecipitating antibody. The interaction of PP2A with p38 was specific, as mouse IgG did not precipitate the phosphatase (Fig. 3B, lane 5). Present in all three panels of this figure are a series of dense bands, which migrate between approximately 27 and 34 kDa (Fig. 3A and B, lanes 4–5 and C, lanes 3–5). These bands were proteins eluted from the protein A/G agarose beads themselves, as deduced from lane 4 where beads not subjected to the immunoprecipitation protocol were treated with Laemmli's buffer and boiled (Fig. 3A and B). These proteins cross-reacted with the secondary antibodies to produce the dark bands. Further, Fig. 3C shows that when p38 was immunoprecipitated using SAK7, PP2A was much more prominently detected in lysates of platelets stimulated for 2 min with collagen compared with those from platelets stimulated for 0 or 8 min. This is consistent with an obligatory role for PP2A in the dephosphorylation of p38. In contrast, immunoprecipitates of PP2A (not shown) contained no detectable p38. It is likely that this reflects the small proportion of PP2A that binds p38, given that a wide range of phosphoproteins will compete with p38 for PP2A activity.

### 3.5. αIIbβ3 activation is not required for p38 phosphorylation or its subsequent dephosphorylation in platelets

An essential role for platelet aggregation and the fibrinogen receptor, αIIbβ3, has been described in the recruitment of PP2A to the cytoskeleton, allowing PP2A to dephosphorylate

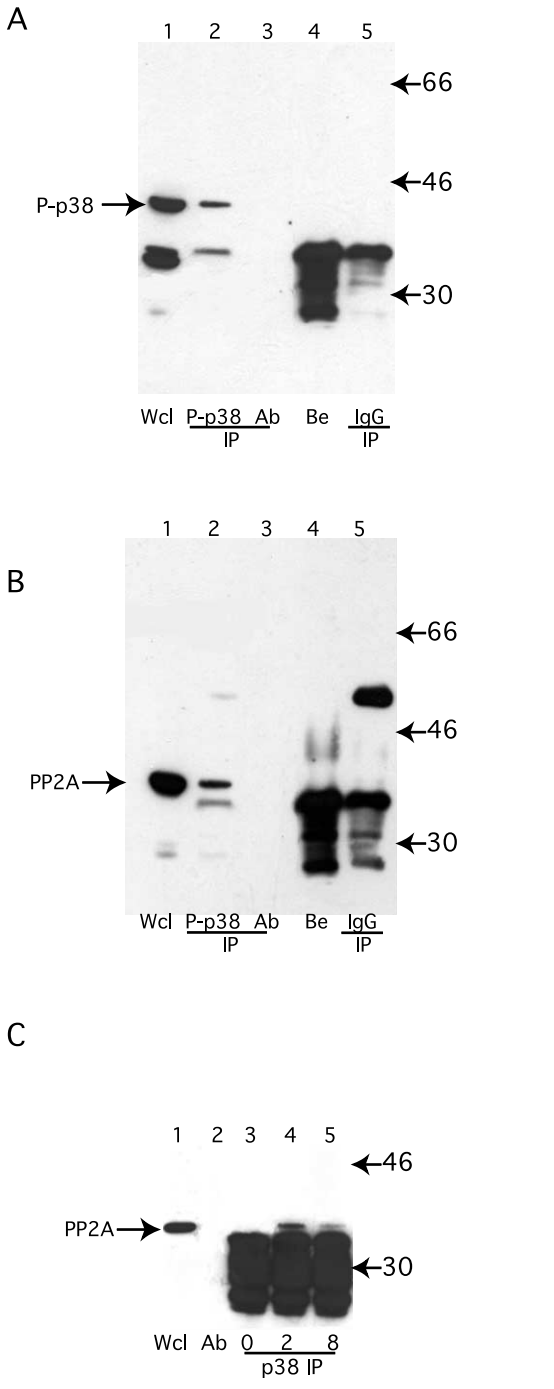


Fig. 3. PP2A directly associates with phospho-p38. A: Immunoprecipitation of phospho-p38 (P-p38) from collagen-stimulated platelets using an agarose-immobilized monoclonal Ab followed by immunodetection of phosphorylated protein using a polyclonal Ab. Lane 1 shows whole cell lysate from platelets stimulated with collagen for 2 min (Wcl). Lane 2 depicts the IP sample (P-p38) and lane 3 shows the phospho-p38 mAb control (Ab). Lane 4 demonstrates proteins that are eluted from the protein A/G agarose beads alone (Be), and lane 5 shows a control IP with mouse IgG (IgG). B: The blot from A was stripped and re-probed for the presence of PP2A with an antibody directed against the catalytic subunit of PP2A. C: Immunoprecipitation of total p38 using SAK7 followed by immunodetection of PP2A. Lane 1 shows proteins present in the whole cell lysate (Wcl) following immune capture of p38. Lane 2 shows the SAK7 antibody control (Ab) and lanes 3–5 show immunoprecipitates of p38 under resting (0 min) and collagen-stimulated conditions (2 min and 8 min). Blots shown are representative of at least four similar experiments.

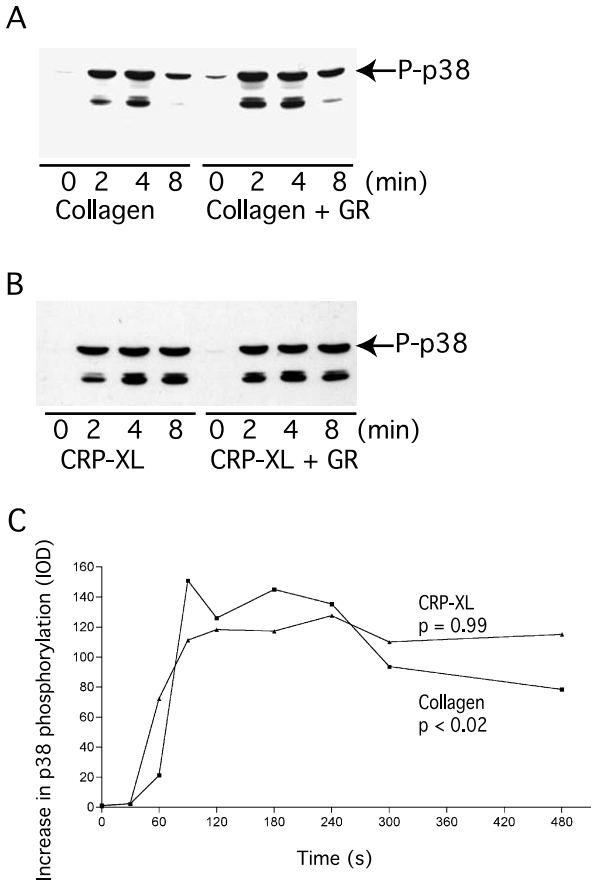


Fig. 4. p38 phosphorylation and subsequent dephosphorylation occurs independently of integrin αIIbβ3. Normal platelets preincubated with GR144053F (2 μM) for 20 min were activated by collagen 50 μg/ml (A) and CRP-XL 25 μg/ml (B) for the indicated times. C: Glanzmann's platelets were stimulated with collagen (■) or CRP-XL (▲) and phospho-p38 levels as well as whole p38 levels were measured densitometrically and IOD values were normalized to account for total p38 levels.

cytoskeletal proteins [19]. Similar considerations may apply to PTP1B and PTP1C [29]. To investigate whether occupancy of αIIbβ3 played a role in p38 phosphorylation and its subsequent dephosphorylation in collagen-stimulated platelets, we employed the αIIbβ3 antagonist GR144053F with normal platelets, and also used αIIbβ3-deficient platelets from a Glanzmann's thrombasthenic patient. Fig. 4A and B show immunoblots of normal platelets treated with collagen and CRP-XL respectively. Preincubation with GR144053F had no effect on the rise in p38 phosphorylation stimulated by either ligand, measured densitometrically. Decline in phosphorylation was still obvious in platelets stimulated with collagen for 8 min. Regardless of the presence of GR144053F, p38 phosphorylation showed a 35% fall in IOD between 2 and 8 min in the presence of GR144053F, compared with a 37% fall in control levels. Fibrinogen binding to αIIbβ3 was not necessary for the onset of p38 phosphorylation, its increase with time, nor its subsequent dephosphorylation, when treated with collagen or CRP-XL. The use of Glanzmann's thrombasthenic platelets supported this conclusion. The curve in Fig. 4C is similar to that in Fig. 1D. Once again, p38 phosphorylation in collagen-stimulated platelets peaked by 90 s and significantly declined by 480 s ($P < 0.02$), whereas with CRP-XL-stimulated platelets, p38 phosphorylation remained at a high level ($P = 0.99$).

## 4. Discussion

We have previously demonstrated that collagen and CRP-XL can activate p38 MAPK in human platelets [1]. Here, as others have suggested, we show that the effect of collagen differs from that of the GpVI-specific ligand, CRP-XL [30]. Both ligands caused rapid onset of p38 phosphorylation, which peaked between 90 and 120 s. However, in the case of collagen, but not CRP-XL, p38 phosphorylation levels fell by almost 50% after 480 s (Fig. 1). These data suggest that collagen can regulate p38 MAPK dephosphorylation through receptors other than GpVI, although these remain to be identified.

We report here both a functional and a physical association between p38 and PP2A. The use of the selective PP2A inhibitors, fostriecin and OA, reveals that PP2A is responsible for p38 dephosphorylation in platelets (Fig. 2A and B). Further, the presence of PP2A in immunoprecipitates of phospho-p38 (Fig. 3B) together with the greater selectivity of fostriecin, provides strong support for PP2A directly regulating platelet p38 MAPK activity. To the best of our knowledge, this is the first report to demonstrate a physical association between PP2A and p38. The greater amount of PP2A detected at 2 min compared with either 0 or 8 min of stimulation indicates that PP2A interacts more avidly with the phosphorylated form of the kinase, as hardly any PP2A was detected under resting conditions (Fig. 3C). The same phenomenon was found with PP2C, which exclusively bound to the phosphorylated form of p38 and implies a common mode of binding to p38, by type II protein phosphatases [18].

PP2A is known to inhibit p38 activity in vitro [17]. Similar experiments support such a role for PP2A in the rat tumor cell line 9L RBT and in both human skin fibroblasts and NIH 3T3 cells [31,32], where the use of OA similarly led to an increase of p38 activity. Together with these reports, our data suggest that there may be a widespread role for PP2A in terminating signaling through p38 MAPK. It has recently been reported that PTPase HePTP in T cells [33] can dephosphorylate p38 MAPK, which provides a precedent for a PTPase acting in concert with PP2A in this process. Our own results with platelets treated with the general PTPase inhibitor PAO support this concept, as p38 phosphorylation was also markedly increased under these conditions (Fig. 2C). Thus, tyrosine and serine/threonine phosphatases act together to maintain p38 MAPK predominantly in the non-phosphorylated form seen in resting platelets, and later dephosphorylate p38 subsequent to signaling through collagen.

Both kinase and phosphatase activity appear to be constitutive in the resting platelet, with the phosphorylated and basal forms of p38 existing in dynamic equilibrium, since preincubation with OA in the absence of ligand shifted the balance towards increased p38 phosphorylation. Basal phosphatase activity may also contribute to the kinetic profile of p38 activation by collagen, since the 30 s lag preceding the rise in p38 phosphorylation (Fig. 1D) was abolished in the presence of OA (Fig. 2A). The decline in control phospho-p38 levels between 90 and 480 s (Fig. 1D) suggests a relatively slow increase in PP2A activity after platelet activation by collagen fibers.

Experiments using αIIbβ3-deficient platelets demonstrate that regulation of p38 MAPK phosphorylation and its subsequent dephosphorylation are independent of the platelet fibrinogen receptor and platelet aggregation (Fig. 4C). These data were confirmed by applying an αIIbβ3-specific antagonist, GR144053F, to normal platelets, which was without effect on p38 phosphorylation (Fig. 4A and B. These lines of argument suggest that, in this respect, p38 appears to differ from the related MAPKs ERK2 and JNK1 whose dephosphorylation has been reported to involve αIIbβ3 occupancy in thrombin-stimulated platelets [34].

p38 is considered to be the protein involved with actin remodeling via its downstream target HSP27 [10], which may account for its involvement in the platelet aggregation process [1]. A possible role for platelet p38 may be to mediate reorganization of the cytoskeleton and formation of signaling complexes, required for the morphological changes during aggregation. Cytoskeletal changes which lead to blebbing [35] and platelet microparticle generation also require the activation of p38 MAPK [2]. Protein tyrosine dephosphorylation is reported to play an integral role in microvesiculation [28], and perhaps p38 dephosphorylation represents one target for such activity. Further work will be needed to identify the PTPase, which jointly acts with PP2A to achieve complete dephosphorylation of p38 MAPK.

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