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Differential Involvement of ERK2 and p38 in Platelet Adhesion to Collagen*

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We investigated the role of two MAP kinases, ERK2 and p38, in platelet adhesion and spreading over collagen matrix in static and blood flow conditions. P38 was involved in collagen-induced platelet adhesion and spreading in static adhesion conditions, whereas ERK2 was not. In blood flow conditions, with shear rates of 300 or 1500 s⁻¹, ERK2 and p38 displayed differential involvement in platelet adhesion, depending on the presence or absence of the von Willebrand factor (vWF). Low collagen coverage densities (0.04 µg/cm²) did not support vWF binding. During perfusions over this surface, platelet adhesion was not affected by the inhibition of ERK2 phosphorylation by PD 98059. However, abolishing p38 activation by SB 203580 treatment reduced platelet adhesion by 67 ± 9% at 300 s⁻¹ and 56 ± 2% at 1500 s⁻¹. In these conditions, the p38 activity required for platelet adhesion depends on the α2β1 collagen receptor. At higher collagen coverage densities (0.8 µg/cm²) supporting vWF binding, the inhibition of ERK2 activity by PD 98059 decreased adhesion by 47 ± 6% at 300 s⁻¹ and 72 ± 3% at 1500 s⁻¹, whereas p38 inhibition had only a small effect. The ERK2 activity required for platelet adhesion was dependent on the interaction of vWF with GPIb. In conclusion, ERK2 and p38 have complementary effects in the control of platelet adhesion to collagen in a shear stress-dependent manner.

The adhesion and aggregation of platelets in response to vascular injury plays a key role in hemostasis and thrombosis. Fibrillar collagen is the most abundant thrombogenic molecule among the macromolecular constituents of the subendothelial layer. Following exposure to collagen, platelets rapidly adhere, spread, become active, and then aggregate (1, 2). This interaction of collagen with platelets is both direct and indirect. Under the high shear stress conditions found in small arteries, the von Willebrand factor (vWF)¹, which binds to newly exposed colla-

gen fibers, is required to capture flowing platelets (3) via the GPIb-IX-V complex on the platelet surface. This interaction (vWF-GPIb) cannot support the assembly of a stable platelet thrombus. Subsequent firm platelet adhesion and activation are mediated by two major surface receptors for collagen, the integrin α2β1 and glycoprotein VI (4–6). The relative contributions of these two receptors to collagen-mediated adhesion are unclear, and various models have been proposed (7). According to the two-site, two-step model, the major candidate for involvement in platelet deposition and stabilization on collagen is α2β1, with GPVI mediating activation and initiating the signaling pathway involved in thrombus formation (8). Platelet activation leads to an increase in the affinity of α2β1, which in turn leads to an increase in adhesion stability. Another model has been proposed in which the absence of functional GPVI impairs adhesion under static and shear conditions (9, 10). In this model, GPVI initiates signaling pathways, leading to the activation of the α2β1 integrin and of other integrins involved in firm adhesion at sites of vascular injury (11).

However, the intracellular signaling pathway occurring during platelet adhesion and spreading on collagen is poorly understood. The small GTP-binding proteins Cdc42/Rac and the effector p21-activated kinases (PAKs) play an important role in the spreading of platelet lamellipodia over the collagen matrix (12), and the Cdc42/Rac-dependent activation of PAK involves cortactin (13). A model of intracellular events during α2β1-mediated platelet spreading has been described recently (14). In this model, the α2β1 integrin mediates the activation of Src family kinases and Syk upstream from phospholipase Cγ2 and the Ca²⁺ mobilization required for platelet spreading. A second pathway may also involve phosphorylation of the tyrosine kinase focal adhesion kinase.

MAP kinases are involved in signaling for cell adhesion and spreading. They belong to a serine/threonine kinase family that includes the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and p38 MAP kinase (15). In proliferative cells, MAP kinase signaling is essential for normal cell adhesion and spreading and is regulated by growth factors and integrins (16). In platelets, ERK2, JNK1, and p38 are present and activated by various agonists (17–19), but their role in hemostatic biology and pathology remains unclear. ERK2 has been reported to be involved in the maintenance of low thrombin levels, collagen-induced platelet aggregation, and the activation of αIIbβ3 by vWF ristocetin and thrombin (20–22). Moreover, P2X1-mediated ERK2 activation amplifies col-

tetramethylrhodamine isothiocyanate; GP, glycoprotein; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAP, mitogen-activated protein.

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¹ The abbreviations used are: vWF, von Willebrand factor; PAK, p21-activated kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; Hsp, heat-shock protein; LT, lethal toxin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TRITC,

lagen-induced platelet secretion by enhancing myosin light chain kinase activation (23). In transgenic mice overexpressing the P2X1 receptor, injection of a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor protects against lethal pulmonary thromboembolism provoked by collagen and epinephrine (24). It also regulates store-mediated Ca^{2+} entry in platelets (25). In contrast to these studies implicating ERK2, Watson and co-workers (26) suggested that inhibition of the ERK pathway does not impair the primary activation of human platelets. GPIIb-dependent platelet activation has been reported to be dependent on Src kinases but not MAP kinases (27). The other MAP kinase, p38, is involved in collagen-induced platelet aggregation (28) and procoagulant activity (29). JNK1 has no known role in platelets.

We investigated the roles of p38 and ERK2 in platelet adhesion and spreading over a collagen matrix in static and blood flow conditions. We found that both p38 and ERK2 were involved in platelet adhesion. The role of p38 in platelet adhesion and spreading was independent of blood flow and involved the $\alpha 2\beta 1$ integrin. In contrast, the role of ERK2 was dependent on blood flow and vWF-GPIIb interaction. We describe here, for the first time, complementary roles for ERK2 and p38 in platelet adhesion to collagen under flow conditions.

EXPERIMENTAL PROCEDURES

Materials—Type I acid-soluble calf skin collagen, leupeptin, aprotinin, apyrase, and rabbit anti-human vWF were obtained from Sigma. The MAP kinase inhibitors, PD 98059 and SB 203580, were purchased from Calbiochem-Novabiochem. Indomethacin was purchased from Cayman Chemical Co. (Ann Arbor, MI). The anti-p38 MAP kinase, anti-ERK, and anti-PAK1/2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against the phosphorylated form of p38 MAP kinase was purchased from Promega (Madison, WI) and that against the phosphorylated form of ERK from BIOSOURCE International (Camarillo, CA). Anti-Hsp27 and anti-phospho-Hsp27 (Ser78) polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). The antibody against the phosphorylated form of PAK1/2 (threonine 423/threonine 402) was obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa fluor 488-labeled phalloidin and Alexa fluor 594-labeled DNase I were obtained from Molecular Probes (Eugene, OR). Lethal toxin (LT) purified to homogeneity from *Clostridium sordellii* (strain IP82) and toxin B from *Clostridium difficile* (strain VPI10463) were obtained from Dr M. Popoff (Institut Pasteur, Paris). The 6F1 mAb against the $\alpha 2$ integrin was generously provided by Prof. B. Coller (Mount Sinai Hospital, New York). The mAb G19H10, which blocks the binding of the vWF to GPIIb, was prepared as described previously (30). Saratin, which blocks the binding of vWF to collagen, was obtained from Merck (Darmstadt, Germany).

Blood Collection and Platelet Isolation—Venous blood was collected in 50 μM PPACK tubes from healthy donors free of medication for at least 2 weeks prior to blood collection. Conventional informed consent was obtained from all donors in accordance with the guidelines of the French Blood Transfusion Agency. Platelet-rich plasma was obtained by centrifuging whole blood at $120 \times g$ for 15 min at 20 °C, and platelets were isolated by differential centrifugation. The platelet pellet was resuspended in 10 mM HEPES, pH 7.4, 140 mM NaCl, 3 mM KCl, 5 mM NaHCO_3 , 0.5 mM MgCl_2 , and 10 mM glucose.

Static Platelet Deposition—Suspended platelets (50 μl at 200,000 platelets/ μl) in the presence of indomethacin (5 μM) were plated with SB 203580 (20 μM), PD 98059 (20 μM), or Me_2SO in wells previously coated with collagen (20 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$) corresponding to 0.2 $\mu\text{g/cm}^2$ or 1 $\mu\text{g/cm}^2$ for 1 h at 20 °C. Indomethacin was added to prevent nonspecific effects of SB 203580 and PD 98059 on cyclooxygenase. Plates were washed three times in phosphate-buffered saline (PBS). Nonspecific adhesion was determined in wells precoated with 0.5% bovine serum albumin in PBS (PBS-BSA). Platelet deposition was quantified by an acid phosphatase assay as described previously (31).

Flow Experiments and Image Analysis—Whole blood perfusion experiments were performed in a parallel plate perfusion chamber. Briefly, glass coverslips were coated by overnight incubation at 4 °C with 500 μl of 20 or 100 $\mu\text{g/ml}$ collagen, giving coverage densities of 0.04 and 0.8 $\mu\text{g/cm}^2$, respectively, allowing platelet adhesion but not throm-

bus formation. Various substances were added to the blood at 37 °C 15 min before perfusion: indomethacin (5 μM) in the presence of SB 203580 (20 μM), PD 98059 (20 μM), or Me_2SO . In all experiments, indomethacin was added to prevent nonspecific effects of SB 203580 and PD 98059 on cyclooxygenase. Where indicated, antibodies 6F1 (20 $\mu\text{g/ml}$) and G19H10 (30 $\mu\text{g/ml}$) and saratin (10 $\mu\text{g/ml}$) were incubated with platelet-rich plasma for 15 min at 37 °C before use in the presence or absence of a MAP kinase inhibitor. Isolated red blood cells (32) (45% of final volume) were added just before the start of the 4-min perfusion. Blood was perfused over the coverslip for 4 min with a syringe pump at shear rates of 300 or 1500 s^{-1} . Adherent platelets were washed in PBS, fixed with 4% paraformaldehyde in PBS, and permeabilized. They were then saturated by overnight incubation in PBS-BSA at 4 °C and stained for 30 min with FITC-phalloidin (1/200). They were viewed with an epifluorescence microscope (Nikon, Eclipse 600), and surfaces were analyzed. For each perfused surface, images from 30 random microscopy fields were collected. The mean area covered by adherent platelets was determined and analyzed ² using NIH Image software (rsb.info.nih.gov/nih-image/). Platelet adhesion is expressed as relative surface coverage to facilitate the comparison of different treatments.

Quantification of F-actin/G-actin—Platelet (100,000 platelets/ μl) preparations containing indomethacin were incubated with or without SB 203580 (20 μM), PD 98059 (20 μM), lethal toxin 82 (LT82) (5 $\mu\text{g/ml}$), toxin B (7.5 $\mu\text{g/ml}$), or Me_2SO as a control before plating on collagen with a surface density of 0.08 $\mu\text{g/cm}^2$. They were incubated for 1 h at 20 °C, fixed with 4% paraformaldehyde, permeabilized, and stained by incubation for 30 min with TRITC-phalloidin (2 μM) or with Alexa fluor 594-labeled DNase I (3 μM) to detect F-actin and monomeric G-actin, respectively (33). Platelets were washed three times, and TRITC-phalloidin and Alexa fluor 594 DNase I binding were measured on a plate reader (Fluoroscan Ascent FL, Labsystems) at appropriate excitation/emission wavelengths. F-actin and G-actin contents were linearly related to platelet number.

Immunoblotting—Samples were subjected to immunoblotting as described previously (22). Platelets were lysed in SDS denaturing buffer (100 mM NaCl, 50 mM Tris, 50 mM NaF, 5 mM EDTA, 40 mM β -glycerophosphate, 100 μM phenylarsine oxide, 1% SDS, 5 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin, pH 7.4) and heated at 95 °C for 5 min. Protein concentration was determined by Uptima Kit Micro BCA (Monluçon, France). Proteins were separated by SDS-PAGE according to standard protocols. The protein bands were transferred to membranes and probed with primary antibodies. Bound antibodies were visualized by chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibodies.

Statistics—Results are expressed as means \pm S.E. for at least three independent experiments. Statistical significance was assessed with Student's *t* test for paired comparisons.

RESULTS

Collagen-induced Platelet Deposition Requires p38 MAP Kinase Activation—We investigated the role of MAP kinases p38 and ERK2 in washed platelet deposition on collagen type I applied at a concentration of 20 or 100 $\mu\text{g/ml}$, corresponding to 0.2 or 1 $\mu\text{g/cm}^2$, in the presence or absence of SB 203580 (20 μM) and PD 98059 (20 μM). In all conditions, control platelets or platelets with inhibitors were pretreated with indomethacin (5 μM) to prevent nonspecific effects of the inhibitors on cyclooxygenase. At a collagen density of 0.2 $\mu\text{g/cm}^2$, the inhibitor of MEK (PD 98059) inhibited platelet deposition by $27 \pm 2\%$ (6.6×10^5 platelets versus 9.0×10^5 platelets for the indomethacin with Me_2SO control; $p < 0.001$) (Fig. 1A). SB 203580 gave even stronger inhibition, up to $67 \pm 3\%$ (3×10^5 platelets; $p < 0.001$). In contrast, at higher collagen coverage density (1 $\mu\text{g/cm}^2$), platelet deposition was not affected whatever the MAP kinase inhibitor used; deposition levels were similar to those for the indomethacin with Me_2SO control. Similar results were obtained with other MEK (U0126: 10 μM) and p38 (SB 202190: 10 μM) inhibitors and with lower concentrations of SB203580 (1–5 μM) (results not shown). In parallel, we investigated the level

² A. Mazharian, S. Roger, P. Maurice, E. Berrou, M. R. Popoff, M. F. Hoylaerts, F. Fauvel-Lafeve, A. Bonnefoy, and M. Bryckaert, personal communication.

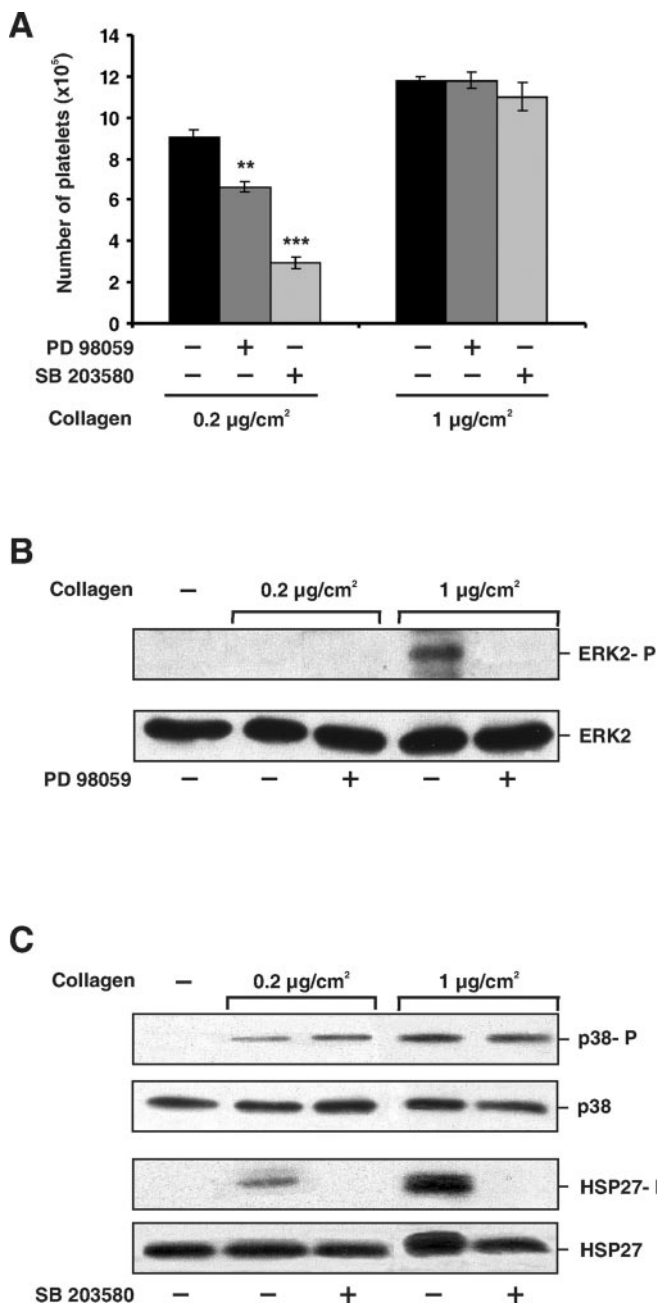


FIG. 1. Role of ERK2 and p38 in adhesion of washed platelets to type I collagen. Platelet suspensions were first incubated with indomethacin (5 µM) for 15 min at 37 °C in the presence of the MAP kinase inhibitors PD 98059 (20 µM), SB 203580 (20 µM), or Me₂SO for the controls. They were then added to wells coated with collagen (0.2 or 1 µg/cm²) for 1 h at 20 °C. A, platelet deposition was assessed by acid phosphatase assay. Western blotting was carried out in the presence or absence of MAP kinase inhibitors with suspended platelets in the *first lane* and adherent platelets in the *lanes* with collagen. B and C, we probed for phosphorylated and nonphosphorylated forms of ERK2 (B) and p38 and its indirect substrate Hsp27 (C). Data are expressed as the mean ± S.E. of at least three independent experiments. Data are statistically significant according to Student's *t*-test (**, *p* < 0.01; ***, *p* < 0.001).

of phosphorylation of ERK2 (ERK2-P) and p38 (p38-P) required for activity in the presence and absence of inhibitors (Fig. 1B and C). ERK2-P was detected only in platelets deposited on the higher density of collagen (1 µg/cm²) and was inhibited by PD 98059. p38-P was detected whatever the density of collagen used (Fig. 1C). As SB 203580 inhibits p38 activity but not p38 phosphorylation, we assessed the level of

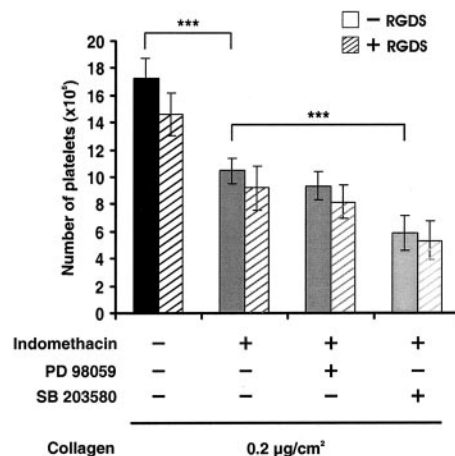


FIG. 2. Role of αIIbβ3 integrin in adhesion of washed platelets to type I collagen. Platelet suspensions were first incubated for 15 min at 37 °C with MAP kinase inhibitors PD 98059 (20 µM), SB 203580 (20 µM), or Me₂SO with or without indomethacin (5 µM) in the presence (hatched bars) or absence of RGDS peptide (1 mM). The samples were then added to wells coated with collagen (0.2 µg/cm²) and incubated for 2 h at 20 °C. Platelet deposition was assessed by acid phosphatase assay. Data are expressed as the mean ± S.E. of at least six independent experiments. Data are statistically significant according to Student's *t*-test (***, *p* < 0.001).

phosphorylation of an indirect substrate of p38, the heat-shock protein Hsp27 (Hsp27-P). As expected, Hsp27-P but not p38-P was inhibited by SB 203580.

In static adhesion conditions, much of the adhesion observed was secondary to platelet-platelet interaction through the αIIbβ3 integrin. We investigated the role of p38 in adhesion and/or aggregation using the RGDS peptide (1 mM) and inhibitors of MAP kinases. Adhesion in the presence or absence of the RGDS peptide indicated that αIIbβ3 interaction was not involved at this density of collagen (0.2 µg/cm²) and that the inhibition of adhesion observed with indomethacin (30%) was because of thromboxane A₂, which is involved in platelet adhesion only (Fig. 2). The addition of SB 203580 in the presence and absence of the RGDS peptide confirmed that p38 affected platelet adhesion but not platelet aggregation. Our results indicate that p38 plays a major role in collagen-induced platelet adhesion, whereas ERK2 has only a very small effect.

Collagen-induced Platelet Spreading Requires p38 MAP Kinase Activation but Not ERK2 Activation—We investigated the roles of p38 and ERK2 in platelet spreading over a collagen matrix (0.2 µg/cm²) under conditions in which MAP kinases have been shown to be involved in platelet adhesion. Polymerized actin was stained with FITC-phalloidin. As expected, polymerized actin formed characteristic filipodia and lamellipodia (Fig. 3A). In the presence of PD 98059 (20 µM), the formation of filipodia and the spreading of lamellae were systematically observed. In contrast, platelet spreading was inhibited by SB 203580 (20 µM); platelets were round without lamellipodial extensions. We quantified the surface area of each platelet labeled with FITC-phalloidin in an attempt to confirm these results (Fig. 3B). SB 203580 inhibited platelet spreading by 35 ± 3%, whereas PD 98059 had no inhibitory effect (4 ± 2%). Similar platelet spreading results were obtained after 1 h in the presence of SB203580 (results not shown). Thus, p38 is involved in platelet spreading, whereas ERK2 is not.

The Involvement of p38 Activation in Platelet Spreading Does Not Depend on Actin Polymerization—Actin-rich filipodial and lamellipodial protrusions and activation of the small guanosine triphosphate GTPase Rac1 are required for platelet spreading (13).

We investigated the role of p38 and ERK2 in actin cytoskeletal remodeling by determining the F-actin/G-actin ratio, which reflects F-actin polymerization. The F-actin/G-actin ratio of platelets adhering to collagen had increased by $60 \pm 10\%$ after 5 min (Fig. 4A). In these conditions, SB 203580 and PD 98059 did not affect the F-actin/G-actin ratio in response to collagen. Thus, p38 is involved in platelet spreading but not in actin polymerization.

We then used toxin B from *C. difficile* and LT82 from *C. sor-dellii* to determine whether the small G protein Rac required for platelet spreading was involved in p38 activation. Toxin B inactivates Rho, Rac, and Cdc42, whereas LT82 inactivates p21^{ras}, Ra1, Rap, and Rac proteins (34, 35). LT82 (5 $\mu\text{g/ml}$) and toxin B (7.5 $\mu\text{g/ml}$) decreased the collagen-induced F-actin/G-actin ratio by 79 and 84%, respectively (Fig. 4B). It also inhibited collagen-induced PAK2 phosphorylation, one of the most precisely characterized effectors of Rac/Cdc42 involved in lamellipodia formation (Fig. 4C). In these conditions, p38 phosphorylation was inhibited by 57 and 69%, respectively (Fig. 4D). Our findings suggest that p38 activation is largely dependent on Rac activation. This Rac activation, and possibly the activation of other small G proteins, induces p38 phosphorylation and actin polymerization by two different pathways, both of which are required for spreading.

Involvement of p38 and ERK2 in Platelet Adhesion to Collagen under Blood Flow Conditions—We investigated the roles of p38 and ERK2 in blood perfusion in conditions of low collagen coverage density (0.08 and 0.4 $\mu\text{g/cm}^2$), allowing platelet adhesion but not thrombus formation. Blood was perfused over immobilized type I collagen at a low (300 s^{-1}) or high (1500 s^{-1}) shear rate to ensure GPIb-dependence. Blood was first treated with indomethacin to prevent PD 98059 and SB 203580 from having nonspecific effects on cyclooxygenase and consequently on thromboxane A₂ formation. We compared adhesion at 1500 s^{-1} in the presence and absence of indomethacin. We found that thromboxane A₂ played only a minor role (7%) in blood flow (results not shown). After 4 min of blood perfusion over collagen at low coverage density (0.08 $\mu\text{g/cm}^2$), the p38 inhibitor (SB 203580) decreased collagen-induced platelet adhesion by 67 ± 9 and $56 \pm 2\%$ at 300 and 1500 s^{-1} , respectively (Fig. 5). Similar results were obtained with the other p38 inhibitor, SB 202190 (10 μM) (results not shown). In contrast, the MEK inhibitor had little ($8 \pm 2\%$) or no effect ($15 \pm 8\%$), regardless of shear rate (300 and 500 s^{-1}). Thus, p38 is also involved in collagen-induced platelet adhesion under shear conditions. The inhibition by SB 203580 of platelet deposition in both static and shear conditions strongly suggests that p38 acts independently of shear conditions and therefore, probably also independently of vWF-GPIb interaction.

We investigated the roles of p38 and ERK2 at a higher density of collagen (0.4 $\mu\text{g/cm}^2$) at a shear stress of 300 or 1500 s^{-1} . SB 203580 weakly but significantly inhibited ($28 \pm 9\%$) platelet adhesion (Fig. 6) at 1500 s^{-1} . Surprisingly, PD 98059 reduced platelet adhesion by $47 \pm 6\%$ at 300 s^{-1} and by $72 \pm 3\%$ at 1500 s^{-1} . Similar inhibition was obtained with lower concentrations (2–10 μM) of two inhibitors of MEK (U0126 and PD98059) at 1500 s^{-1} (results not shown). Thus, ERK2 is involved in platelet adhesion at higher collagen density, and the involvement of ERK2 seems to depend on shear conditions.

Role of Platelet Receptors $\alpha 2\beta 1$ and GPIb in MAP Kinase Activation and Platelet Adhesion—Our results suggest that activated ERK2 and p38 are involved in platelet adhesion via different pathways. We therefore investigated the roles of the collagen receptor, $\alpha 2\beta 1$ integrin, and GPIb-vWF interaction in platelet adhesion and MAP kinase activation in high-shear stress conditions (1500 s^{-1}).

We first determined whether vWF was deposited on collagen

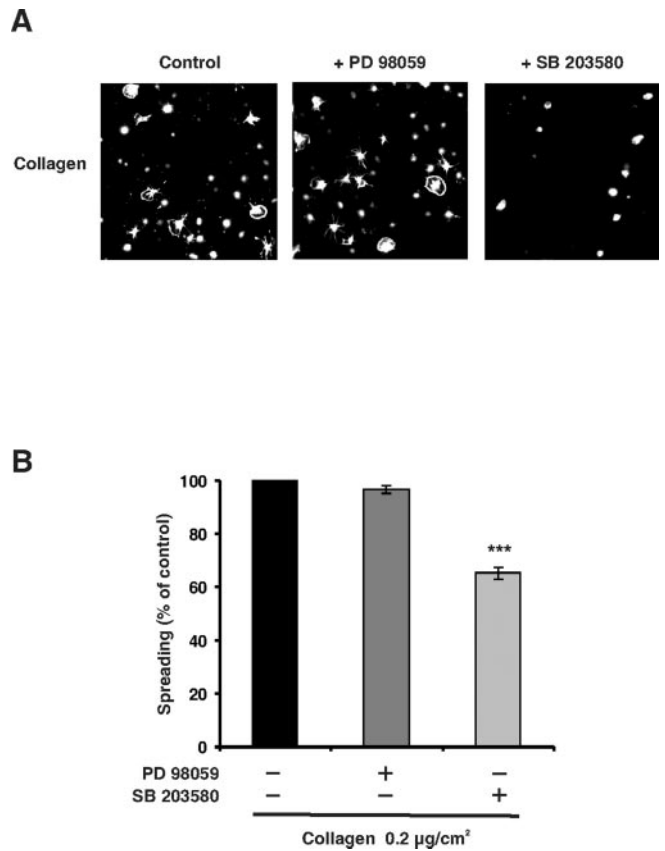


FIG. 3. Role of ERK2 and p38 platelet spreading on type I collagen. Platelet suspensions were incubated with indomethacin (5 μM) in the presence of the MAP kinase inhibitors PD 98059 (20 μM), SB 203580 (20 μM), or Me_2SO for 15 min at 37 °C. They were then added to wells coated with collagen (0.2 $\mu\text{g/cm}^2$) for 2 h at 20 °C (A) and stained with FITC-phalloidin. B, the surface area was quantified as described under "Experimental Procedures." Data are expressed as the mean \pm S.E. of four independent experiments. Data are statistically significant according to Student's *t*-test (***, $p < 0.001$).

at low and higher coverage densities (0.08 or 0.4 $\mu\text{g/cm}^2$) after blood perfusion (4 min). At low collagen density (0.08 $\mu\text{g/cm}^2$), only small amounts of vWF were deposited. Significantly larger amounts of vWF were deposited at higher density (0.4 $\mu\text{g/cm}^2$) (Fig. 7A). This strongly suggests that the involvement of p38 in adhesion depends on $\alpha 2\beta 1$, whereas that of ERK2 depends on GPIb-vWF interaction and/or $\alpha 2\beta 1$.

We further investigated the effect of p38 inhibition on platelet adhesion to collagen (0.08 $\mu\text{g/cm}^2$) in the presence of mAb 6F1 (20 $\mu\text{g/ml}$) to block the integrin $\alpha 2\beta 1$, mAb G19H10 (30 $\mu\text{g/ml}$) to block the vWF-GPIb interaction, or saratin (10 $\mu\text{g/ml}$) to block the binding site for the vWF on collagen. We found that 6F1 inhibited platelet adhesion by $87 \pm 1\%$, whereas no significant inhibition (9 ± 3 and $12 \pm 5\%$) was observed with G19H10 or saratin (Fig. 7B), as expected given the small amount of vWF present. In the presence of the p38 inhibitor alone, platelet adhesion was inhibited by $78 \pm 1\%$, strongly suggesting that p38 is required for the effect of $\alpha 2\beta 1$. A similar level of inhibition was observed in the presence of 6F1 ($88 \pm 0.5\%$). In contrast and as expected, the weak effect on platelet adhesion of mAb G19H10 (9%) alone was strongly increased by adding p38 inhibitor ($85 \pm 0.6\%$). Similar results were obtained with saratin in the presence or absence of the p38 inhibitor. The pathways involving p38 and vWF-GPIb were therefore different. Thus, the role of p38 in platelet adhesion is dependent on the collagen receptor ($\alpha 2\beta 1$). We investigated the roles of GPIb and the collagen receptor ($\alpha 2\beta 1$) in the ERK2 acti-

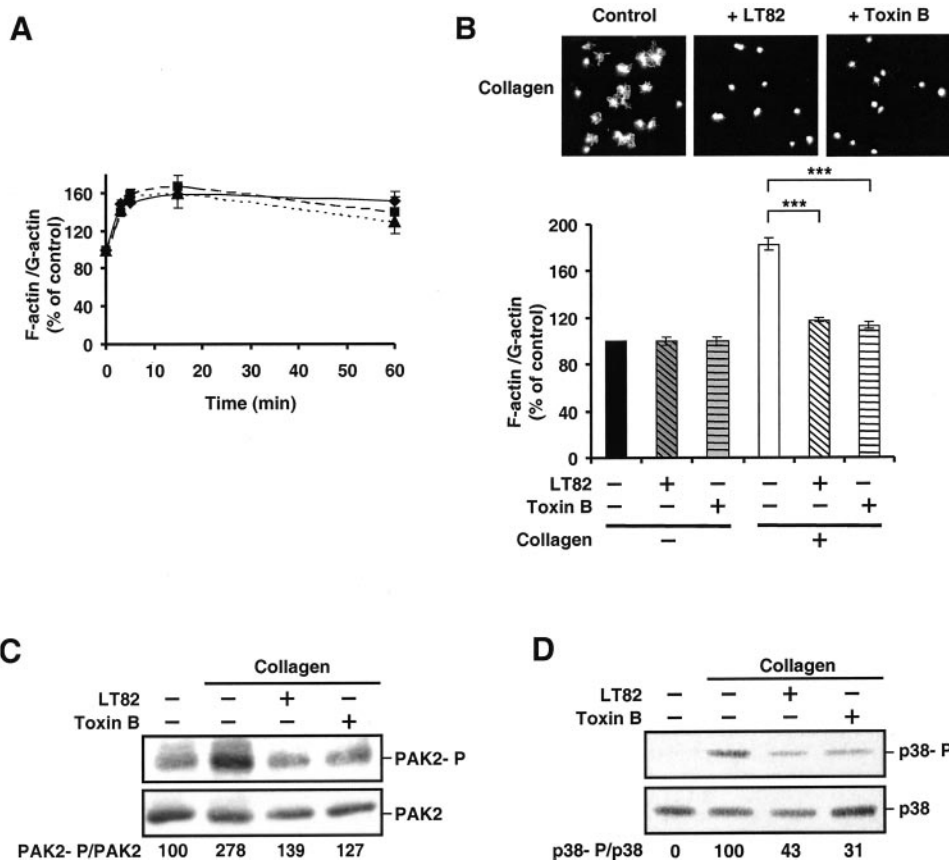


FIG. 4. **Activation and roles of ERK2 and p38 in the actin polymerization required for spreading.** Platelet suspensions were incubated with indomethacin ($5 \mu\text{M}$) in the presence of the MAP kinase inhibitors PD 98059 ($20 \mu\text{M}$), SB 203580 ($20 \mu\text{M}$), or Me_2SO for 15 min at 37°C (A) or with LT 82 ($5 \mu\text{g/ml}$) (hatched) or toxin B ($7.5 \mu\text{g/ml}$) (horizontal stripes) for 2 h at 37°C (B–D). The platelets were then added to wells coated with collagen ($0.2 \mu\text{g/cm}^2$) for 1 h at 20°C and were stained with TRITC-phalloidin (F-actin) and DNase I (G-actin). The F-actin/G-actin ratio was determined, and the data are expressed as the mean \pm S.E. of four independent experiments. In parallel, we immunoblotted the phosphorylated forms of PAK1/2 (C) and p38 (D) in the presence or absence of LT 82 and toxin B. Data are expressed as the mean \pm S.E. of at least three different experiments. Data are statistically significant according to Student's *t*-test (***, $p < 0.001$).

vation required for platelet adhesion in conditions of higher collagen density ($0.4 \mu\text{g/cm}^2$) at a shear stress of 1500 s^{-1} . G19H10 and saratin inhibited platelet adhesion by 83 ± 0.8 and $83 \pm 1\%$, respectively, whereas 6F1 gave only weak inhibition ($22 \pm 3\%$) (Fig. 7C). In the presence of the MEK inhibitor alone, platelet adhesion was inhibited by $71 \pm 2\%$, suggesting that the effect of ERK2 is largely, if not entirely, dependent on GPIb-vWF interaction. Moreover, similar levels of inhibition were observed with G19H10 and saratin in the presence (83 ± 0.8 ; $87 \pm 1\%$) and absence (85 ± 0.7 ; $87 \pm 0.8\%$) of the MEK inhibitor, confirming that the involvement of ERK2 was totally dependent on vWF-GPIb interaction.

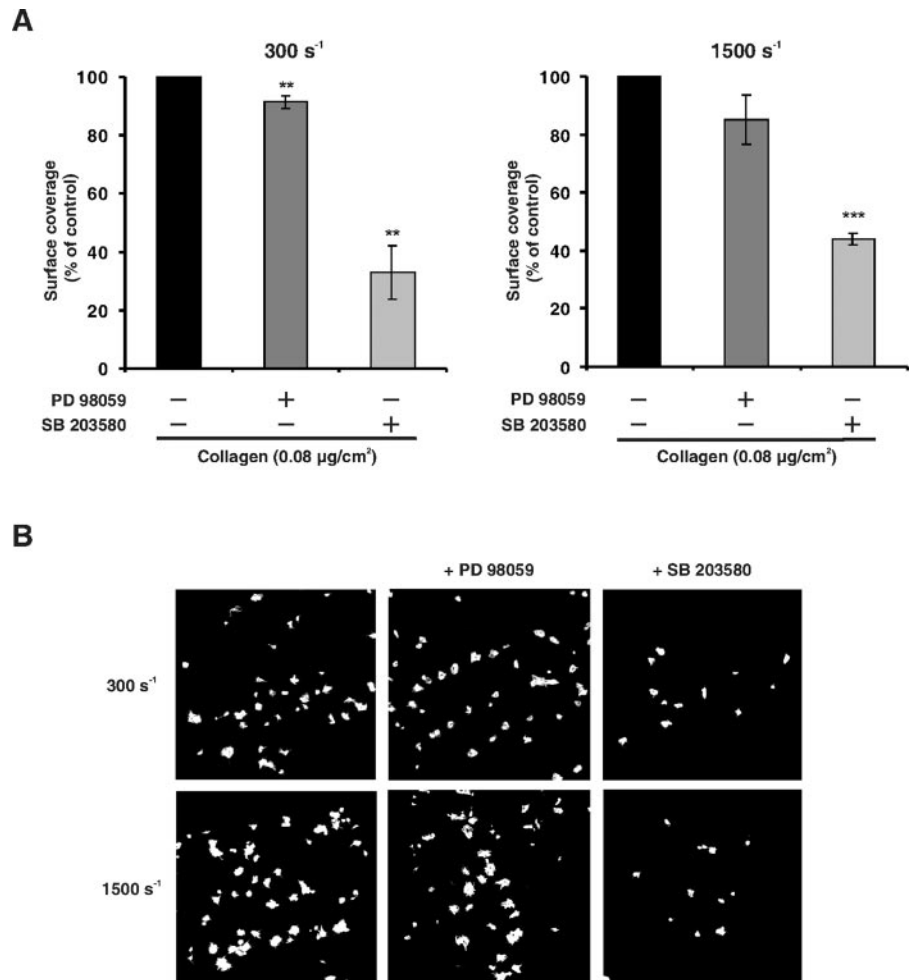
DISCUSSION

This study shows that ERK2 and p38 play different roles in platelet adhesion over collagen matrix under static and shear conditions. The involvement of p38 in platelet adhesion and spreading is independent of shear stress, requires the $\alpha 2\beta 1$ integrin, and is probably initiated by the GPVI signaling pathway. Conversely, the involvement of ERK2 in platelet adhesion is dependent on shear stress and requires vWF-GPIb interaction. We also provide evidence that a small G protein, probably activated Rac (results not shown), induces p38 phosphorylation and actin polymerization by two different pathways, both of which are required for platelet spreading.

In recent years, much progress has been made toward understanding collagen interactions with specific platelet receptors, but the intracellular signaling involved in platelet adhesion and spreading on collagen-coated surfaces remains poorly

characterized. MAP kinase-signaling molecules activated during adhesion and spreading in proliferative cells have been described extensively (36). In platelets activated by a thromboxane A_2 analog, p38 activation has been reported to be involved in adhesion to immobilized fibrinogen (37). In this study, we showed that two MAP kinases, ERK2 and p38, were activated during adhesion to immobilized collagen in static conditions and that only p38 was required for adhesion and spreading. Moreover, p38 acted on platelet adhesion but not on platelet aggregation. $\alpha 2\beta 1$ has been reported to be involved in platelet deposition on collagen-coated surfaces. Only 10% of GPIa-deficient platelets adhered to type I collagen versus 80% of control platelets (38). Anti- $\alpha 2\beta 1$ antibody also inhibits platelet adhesion or deposition on collagen, whereas anti-GPVI antibody has no effect (39). The lack of involvement of the vWF in the static adhesion of washed platelets at collagen densities of 0.2 and $1 \mu\text{g/cm}^2$ (results not shown) and the involvement of p38 only at a collagen density of $0.2 \mu\text{g/cm}^2$ strongly suggest that different signaling pathways via the collagen receptors ($\alpha 2\beta 1$ and GPVI) are engaged during platelet deposition at low and high collagen densities. Platelet spreading on the $\alpha 2\beta 1$ -specific GFOGER peptide was recently reported to require the activation of Src kinases and phospholipase $\text{C}\gamma 2$ (14). We show here that the role of p38 in adhesion depends largely on $\alpha 2\beta 1$, suggesting that platelet spreading via $\alpha 2\beta 1$ involves p38 activity. Spreading requires actin polymerization. Several lines of evidence suggest that the indirect substrate of p38, Hsp27, controls cytoskeletal reorganization and actin polymerization.

FIG. 5. Roles of ERK2 and p38 in platelet adhesion to collagen (0.08 $\mu\text{g}/\text{cm}^2$) under flow conditions. The blood cell suspension was incubated with indomethacin (5 μM) in the presence or absence of PD 98059 (20 μM), SB 203580 (20 μM), or Me_2SO for 15 min at 37 $^\circ\text{C}$ (A and B). Blood was perfused for 4 min at a shear rate of 300 or 1500 s^{-1} over immobilized collagen (0.08 $\mu\text{g}/\text{cm}^2$). Adherent platelets were labeled with FITC-phalloidin. Data are expressed as the mean \pm S.E. of four independent experiments. Data are statistically significant according to Student's *t*-test (**, $p < 0.01$; ***, $p < 0.001$).

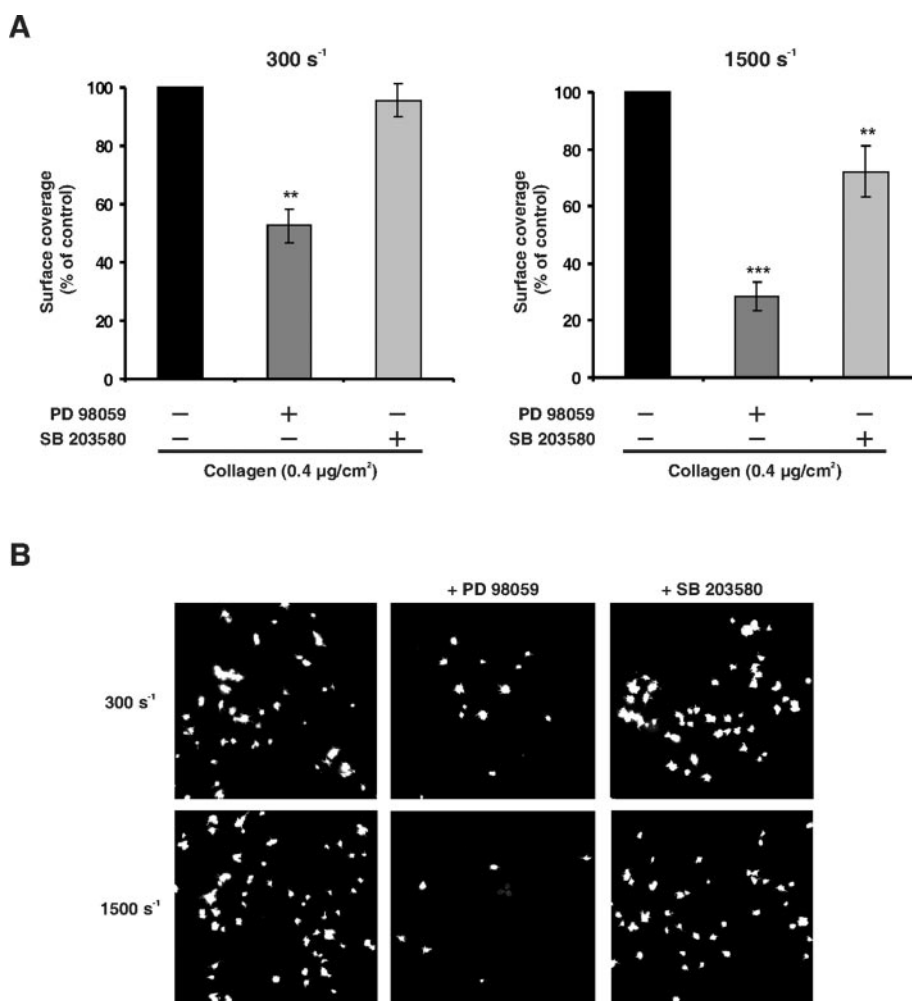


In smooth muscle cells, the p38 cascade induced by platelet-derived growth factor controls the actin polymerization required for lamellipodium formation via the indirect substrate Hsp27 (33). In platelets, thrombin-induced aggregation leads to the activation of p38 and MAP kinase-activated protein kinase and the phosphorylation of Hsp-27, resulting in actin polymerization (40). In our model, the p38 cascade was not involved in actin polymerization on collagen, suggesting the existence of two different pathways between actin polymerization and p38 activation, both of which are involved in platelet spreading. The activation of Rac, a small guanosine triphosphate-binding protein, and of PAK is required for actin polymerization and spreading on collagen-coated surfaces (12). Src family kinases and the phosphatidylinositol 3-kinase are involved in this process (12). In proliferative cells, the link between Rac/PAK activation and p38 activation is unclear. The activation of p38 has been reported to be dependent on Ras but independent of Rac (41) and to involve the serine/threonine kinase PAK (42). Rac activation was observed in this study (results not shown), and we investigated the relationship between Rac activation and p38 activation. Clostridial toxin B and lethal toxin 82 inhibited actin polymerization as reported previously (13). Spreading on a collagen-coated surface was inhibited in the presence of these toxins. In these conditions, phosphorylated p38 levels were very low, demonstrating that p38 phosphorylation is largely dependent on a small G protein, probably Rac. In platelets, p38 activation and actin polymerization, both of which are required for spreading, are probably dependent on Rac activation.

We show here, for the first time, that in blood flow conditions and at low collagen coverage density (0.08 $\mu\text{g}/\text{cm}^2$), p38 is

involved in adhesion, whereas ERK2 plays only a minor role. The effect of p38 seemed to be independent of shear stress. Collagen and the vWF have been reported to activate p38 in platelet aggregation (19). In our low collagen density conditions, even at shear rates as high as typically encountered in arterioles (1500 s^{-1}), only small amounts of plasma vWF bound to collagen, strongly suggesting that p38 exerted its effects on adhesion via the collagen receptors ($\alpha 2\beta 1$ and GPVI). These results were confirmed by the results for platelet adhesion to collagen-coated surfaces (0.08 $\mu\text{g}/\text{cm}^2$ and 1500 s^{-1}), which was fully dependent on the collagen receptor $\alpha 2\beta 1$ (87%). This apparent discrepancy with published results showing that both GPIb and $\alpha 2\beta 1$ contribute to primary adhesion under flow in these shear stress conditions (43) may be due to the low density of collagen (0.08 $\mu\text{g}/\text{cm}^2$), precluding plasma vWF binding, and/or the low levels of fibrillar collagen at this density of collagen (0.08 $\mu\text{g}/\text{cm}^2$). In either case, platelet $\alpha 2\beta 1$ function is essential for irreversible platelet attachment, even at high shear stress (45). Savage *et al.* (44) reported that $\alpha 2\beta 1$ inhibition completely prevented platelet adhesion to acid-soluble collagen but had no effect on binding to native insoluble fibrillar collagen. We cannot rule out the possible gradual production of fibrillar collagen from acid-soluble collagen, leading to the activation of GPVI, enhancing adhesion by inside-out signaling to $\alpha 2\beta 1$. Such differences in collagen structure have been reported to be directly related to differences in vWF content (45). In pathological conditions such as atherogenesis, *in vivo* studies have shown that collagen fibers may be degraded because of an increase in the matrix metalloproteinase activity of infiltrating macrophages and activated smooth muscle cells (46).

FIG. 6. Roles of ERK2 and p38 in platelet adhesion to collagen ($0.4 \mu\text{g}/\text{cm}^2$) under flow conditions. The blood cell suspension was first incubated with indomethacin ($5 \mu\text{M}$) in the presence or absence of PD 98059 ($20 \mu\text{M}$), SB 203580 ($20 \mu\text{M}$), or Me₂SO for 15 min at 37°C (A and B). Blood was perfused for 4 min at a shear rate of 300 or 1500 s^{-1} over immobilized collagen ($0.4 \mu\text{g}/\text{cm}^2$). Adherent platelets were labeled with TRITC-phalloidin. Data are expressed as the mean \pm S.E. of four independent experiments. Data are statistically significant according to Student's *t*-test (**, $p < 0.01$; ***, $p < 0.001$).



These findings suggest that $\alpha 2\beta 1$ plays a major role in these conditions.

At a collagen density ($0.4 \mu\text{g}/\text{cm}^2$) allowing adhesion but not thrombus formation (44), ERK2 was involved in platelet adhesion, and its role seemed to depend on shear stress. In static conditions, ERK2 had no effect on platelet adhesion, whereas at 1500 s^{-1} , adhesion was inhibited by 75% in the presence of the MEK inhibitor. This apparent discrepancy between static adhesion and blood flow could be explained by the presence of the vWF, involved in ERK2 activation, in blood flow. At higher collagen densities, the vWF was readily detected bound to the collagen matrix, and adhesion depended largely (83%) on vWF-GPIb interaction and to a lesser extent (22%) on collagen receptors (such as $\alpha 2\beta 1$), strongly suggesting that much of the effect of ERK2 (71%) on adhesion was dependent on the vWF. This hypothesis was confirmed by the inhibition of adhesion (22%) observed in the presence of 6F1 and its increase to 79% after the addition of the MEK inhibitor. There are two possible reasons why the MEK inhibitor alone inhibited platelet adhesion (71%) to a lesser extent than mAb G19H10 (85%) or sartin (87%). The vWF-GPIb interaction involved in platelet adhesion may depend on at least two different pathways, with the major pathway dependent on ERK2 activation and the second independent of ERK2 activation. Alternatively, ERK2 activity may not be totally inhibited by PD 98059. Moreover, whereas adhesion at 300 s^{-1} was largely dependent on $\alpha 2\beta 1$ (62%) and to a lesser extent on GPIb-vWF interaction (39%; results not shown), the inhibition of adhesion by PD 98059 was weaker and depended on GPIb-vWF interaction. This strongly suggests that ERK2 plays a major role in platelet adhesion to the arte-

rial subendothelium. Our results show, for the first time, that the role of ERK2 in human platelet adhesion under shear conditions is largely dependent on the vWF. In previous studies, the vWF has been reported to activate $\alpha \text{IIb}\beta 3$ and consequently to induce platelet aggregation via ERK2 activation (20). In our collagen model, using two inhibitors of MEK (PD98059 and U0126), vWF/GPIb interaction induced platelet adhesion via ERK2, showing that ERK2 plays a major role in adhesion and thrombus formation. However, in mouse platelets, Watson and co-workers (27) showed that ERK2 was not involved in adhesion to collagen in blood flow conditions. Differences between these models (mouse *versus* human platelets) may account for the divergent findings concerning the role of ERK2 at high shear rates. Our data are consistent with the involvement of the ERK2 pathway in platelet aggregation on collagen under flow conditions in transgenic mice via the platelet P2X₁ ion channel (24) and suggest that, in human platelets, the P2X₁ pathway and the vWF bound to collagen are involved in platelet activation and the development of thrombosis via the MAP kinase ERK2. Finally, the role of ERK2 at high shear rate (1500 s^{-1}) dependent on vWF-GPIb interaction and the role of ERK2 dependent on the P2X₁ ion channel in thromboembolism (24) are two different and complementary pathways, both of which contribute to platelet thrombus formation.

In conclusion, we report here the differential involvement of ERK2 and p38 in adhesion during the perfusion of whole blood over a collagen matrix (Fig. 8). In models of vessel wall damage resembling thrombosis, small numbers of collagen fibers are exposed and vWF binding cannot take place. In these conditions, platelets adhere directly via collagen receptors ($\alpha 2\beta 1/$

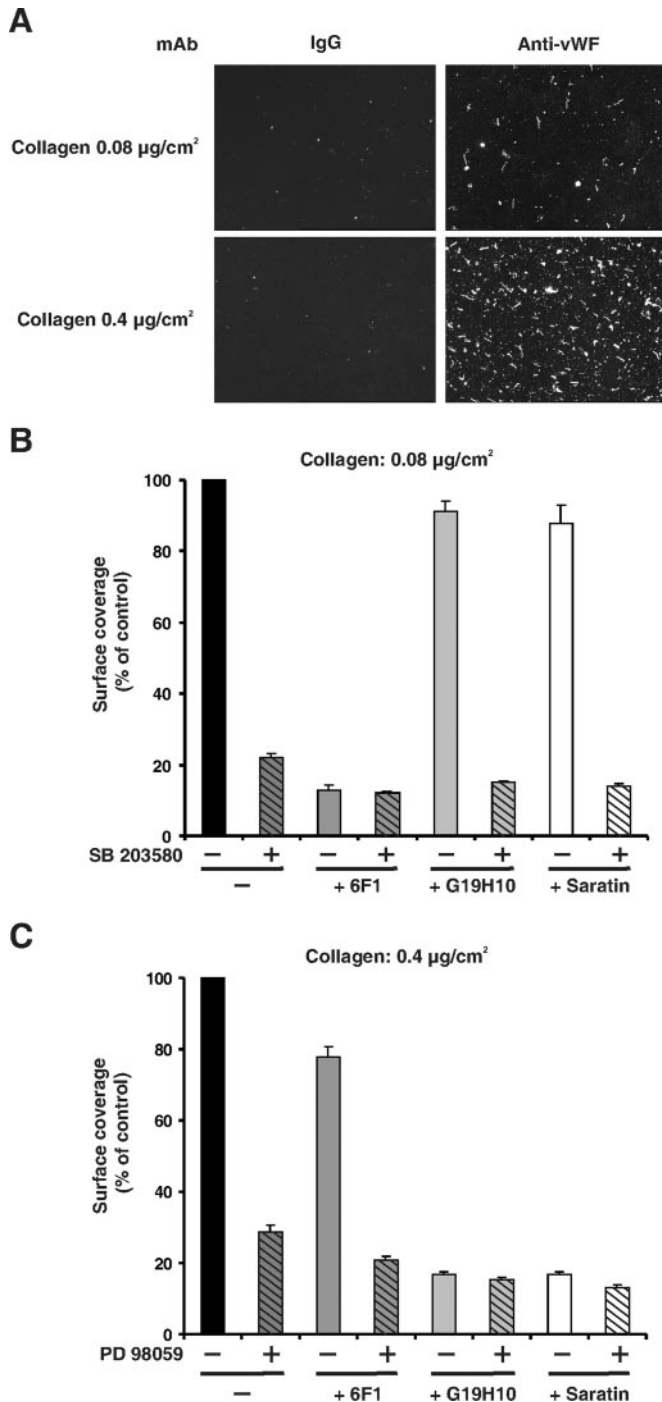


FIG. 7. Role of collagen receptor $\alpha 2\beta 1$ and GPIb-vWF interaction in platelet adhesion and MAP kinase activation. A, platelet-rich plasma with red cells was perfused at a shear rate of 1500 s^{-1} over immobilized collagen (0.08 and $0.4 \mu\text{g}/\text{cm}^2$). The vWF was visualized by immunofluorescence microscopy. B and C, platelet-rich plasma was incubated with mAbs 6F1 ($20 \mu\text{g}/\text{ml}$) or G19H10 ($30 \mu\text{g}/\text{ml}$) or saratin ($10 \mu\text{g}/\text{ml}$) and indomethacin ($5 \mu\text{M}$) in the presence or absence of SB 203580 ($20 \mu\text{M}$) and PD 98059 ($20 \mu\text{M}$) or Me_2SO for 15 min at 37°C before the addition of red cells. The blood was then perfused at a shear rate of 1500 s^{-1} for 4 min over immobilized collagen ($0.08 \mu\text{g}/\text{cm}^2$ (B) and $0.4 \mu\text{g}/\text{cm}^2$ (C)). Adherent platelets were labeled with FITC-phalloidin. Data are expressed as the mean \pm S.E. of four independent experiments.

GPVI), and this adhesion is dependent on p38. In another type of vessel wall damage, at high shear rates (as in arteries) collagen fibers are exposed and vWF binds. In this model, platelets initially interact via glycoprotein Ib-V-IX and vWF bound to collagen. This process is dependent on ERK2 activa-

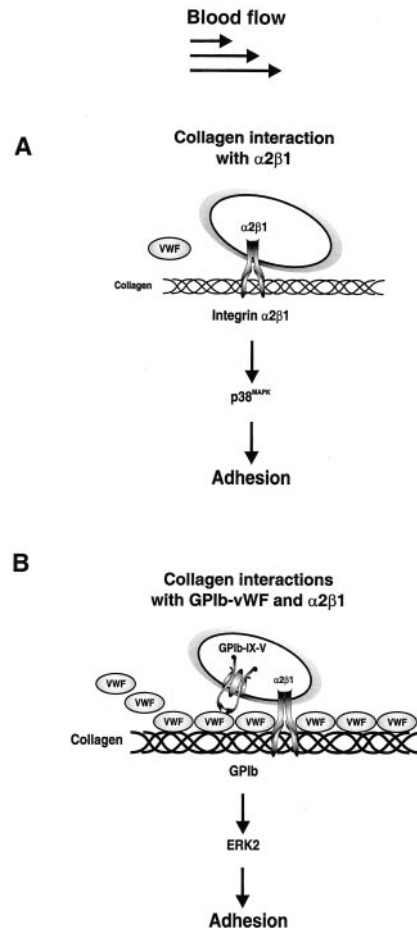


FIG. 8. Complementary roles of ERK2 and p38 in platelet adhesion. First model (A), in small vessel wall damage, a low level of collagen fibers does not support vWF binding. Platelets adhere directly via collagen receptor $\alpha 2\beta 1$, and this adhesion is dependent on p38. Second model (B), in vessel injury (high shear rates, as in arteries), which allow the binding of the vWF to collagen, platelets initially interact via glycoprotein Ib-V-IX with the von Willebrand factor bound to collagen; this process is dependent on ERK2 activation, after which irreversible binding to collagen receptors occurs.

tion followed by irreversible adhesion to the collagen receptor.

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