**Communication**

Negative Regulation of Mitogen-activated Protein Kinase Activation by Integrin αIIbβ3 in Platelets*

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Activation of the mitogen-activated protein (MAP) kinase pathway in nucleated cells is dependent on both growth factor receptors and integrins engaged in cell adhesion. Human platelets are an interesting model for studying cell adhesion and the involvement of integrin engagement on extracellular signal-regulated kinase (ERK) activation, independently from the nuclear-DNA signal pathway. Maximal phosphorylation and activity of ERK2 occurred late during thrombin-induced platelet aggregation (90 s and later), an αIIbβ3 integrin-dependent event. Surprisingly, αIIbβ3 inhibition by the RGDS ligand peptide, or (Fab)2 fragments of the AP-2 monoclonal antibody, resulted in a 2-fold enhancement in ERK2 phosphorylation and activity. A similar 2-fold enhancement of ERK2 activation was observed in thrombasthenic platelets which are defective in αIIbβ3 and do not aggregate. This suggests that ERK2 activation in thrombin-induced platelet aggregation is dependent on thrombin rather than on αIIbβ3 and is down-regulated by αIIbβ3 engaged in ligand (fibrinogen) binding and/or aggregation. Finally, in the absence of stirring which allows fibrinogen binding to αIIbβ3 but prevents aggregation, ERK2 was again overactivated. This overactivation appears to be consecutive to inhibition of aggregation itself and to αIIbβ3 ligand binding. We conclude that in platelets, αIIbβ3 engaged in aggregation down-regulates thrombin-induced ERK2 activation. To our knowledge, this is the first report of a down-regulation of the MAP kinase pathway by integrin engagement.

Mitogen-activated protein kinases (MAPKs)† are a family of serine-threonine kinases activated by many extracellular stimuli including growth factors and hormones. They mediate intracellular phosphorylation events and regulate gene expression by transcription factors which drive cell proliferation (1). The first members of this family to be discovered in mammalian cells, the extracellular signal-regulated kinases (ERK1 and ERK2) are essential for cell proliferation (2) and differentiation (3). Recently, novel subgroups of the MAP kinase family, the c-Jun N-terminal kinase family (JNK), and p38 MAP kinase were identified (4, 5). To become active, ERK requires phosphorylation on both threonine and tyrosine residues in a Thr-X-Tyr motif (6). This dual phosphorylation is mediated by specific activators called MAP kinase kinase (MEK-1/2) (7).

Recently, it has been reported that integrin-mediated cell adhesion strongly activates ERKs (8). During adhesion, ERK activation is induced sequentially first by mitogens and then by cell matrix interaction during G1 cell cycle progression (9). This activation has been demonstrated when cells adhere to substrate coated with the adhesive proteins fibronectin, laminin, or RGD-containing peptides (8, 10). Unlike growth factors, integrin-mediated ERK activation is dependent on the integrity of the actin cytoskeleton (10, 11). Moreover, ERK activation caused by integrin ligation is dependent on activation of MEK and Raf, but the relationship between ERK activation and Ras is unclear (12).

Platelets, which are terminally differentiated and anucleated cells, are an attractive model to study coupling between cell adhesion and ERK activation, independent of nuclear DNA-dependent pathways. Recent studies have identified two forms of ERK, p42ERK2 and p44ERK1, in sheep and human platelets stimulated by thrombin (13, 14). The ERK signal pathway in platelets remains largely uncharacterized. The activation of ERKs by different agonists seems to involve MEK through protein kinase C-dependent and -independent pathways (15).

Platelets possess a number of adhesive receptors (16), including integrin αIIbβ3 (GPIIb-IIIa), the receptor of fibrinogen, involved in platelet to platelet adhesion or aggregation. Engagement of αIIbβ3 in aggregation following fibrinogen binding and clustering triggers cytoskeletal rearrangement and activation of tyrosine kinases (17, 18). As early events in integrin-mediated signaling involve the ERK cascade in proliferative cells, we examined the contribution of αIIbβ3 integrin to the activation of ERK2 during platelet aggregation. Interestingly, αIIbβ3 down-regulated ERK2 activation when engaged in fibrinogen-mediated platelet to platelet adhesion or aggregation. This observation contrasts with studies in other cell systems which describe integrin-mediated adhesion as being linked to activation of the MAPK pathway.

**EXPERIMENTAL PROCEDURES**

Reagents—Bovine thrombin, synthetic peptides Arg-Gly-Asp-Ser (RGDS), Arg-Gly-Glu-Ser (RGES), leupeptin, aprotinin, and myelin basic protein were from Sigma. Protein G-Plus-agarose and rabbit polyclonal antibody raised against a C-terminal peptide of ERK2 (C-14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal antibody raised only against the phosphorylated form of ERK2 (ERK2-P) was from New England Biolabs (Beverly, MA). Donkey anti-rabbit horseradish peroxidase-conjugated IgG was obtained from Jackson ImmunoResearch (West Grove, PA). [γ-32P]ATP (167 TBq/mmol) was from ICN (Irvine, CA). (Fab)2 fragments of the mouse monoclonal IgG AP-2 specific for αIIbβ3 were an extremely generous gift from Dr. T. J. Kunicki (19).

Patients and Normal Donors—The two patients with Glanzmann’s thrombasthenia studied herein have been described previously (20, 21). They are characterized by an absence of platelet aggregation and total absence of fibrinogen binding. Normal donors and thrombasthenic pa...
tients had not received any drug during the 2 weeks before blood donation.

Platelet Preparation and Aggregation—Venous blood was obtained from both healthy donors and patients with Glinzamann’s thrombathemia with their informed consent. The platelets were isolated and washed by differential centrifugation in citrate buffer, pH 6, containing 10^{-4} mM prostaglandin E1, 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, and 12.5 mM sucrose and then in the same buffer without prostaglandin E1. The platelet pellet was resuspended in 10 mM HEPE, pH 7.4, 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl2, 5 mM NaHCO3, and 10 mM glucose. The cell concentration was adjusted to 5 x 10^8/ml. Platelet aggregation was initiated by the addition of bovine thrombin with constant stirring (1,200 rpm) in an aggregometer cuvette (Chronolog dual beam aggregometer). Aggregation was measured and is expressed as a percent change in the transmission of light, with the blank sample (buffer without platelets) defined as 100%.

Immunoblotting—Samples were subjected to immunoblotting as described previously (22). Proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose filters, and then filters were incubated with the polyclonal primary antibody anti-ERK2 (1:10,000) or a monoclonal antibody anti-ERK2-P (1:1,000). After 5 washings, nitrocellulose membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) or anti-mouse IgG (1:10,000). Immunoreactive bands were visualized by chemiluminescence using the Amersham ECL enhanced chemiluminescence system.

Immunoprecipitation—Platelet lysates were incubated for 4 h at 4 °C with anti-ERK2 antibody (0.2 μg/sample), then further incubated with Protein G-Plus-agarose (30 μl, v/v) for 1 h at 4 °C for kinase activity (23). Samples were subjected to SDS-polyacrylamide gel electrophoresis, and the gel was stained with Coomassie Blue, dried, and autoradiographed.

RESULTS AND DISCUSSION

ERK2 Is Activated during Thrombin-induced Platelet Aggregation—To examine the phosphorylation and activity of ERK2 during platelet activation, we determined the conditions of thrombin-induced ERK2 activation by incubating platelets with increasing concentrations of thrombin (0–2 NIH units/ml) for 2 min under stirring. The state of phosphorylation, which correlates with a reduction in the electrophoretic mobility of ERK2, was investigated by Western blotting using one antibody specific for ERK2, whether phosphorylated or not, and another antibody which recognized only phosphorylated ERK2 (ERK2-P). In resting platelets and after induction with thrombin (0.02–0.1 NIH units/ml), only nonphosphorylated ERK2 was detectable (Fig. 1A). A retardation in ERK2 mobility, characteristic of ERK2-P, was detected after treatment with 0.2 NIH unit/ml thrombin and at higher concentrations. The identity of the low mobility ERK2 band was confirmed by reprobing the Western blot with an anti-ERK2-P antibody. No detectable band was visible in resting platelets or when platelets were stimulated with thrombin between 0 and 0.1 NIH units/ml. ERK2-P was detected at 0.2 NIH unit/ml of thrombin at 60% platelet aggregation and reached maximum intensity at 1 NIH unit/ml. Scanning densitometry indicated that 30% of total ERK2 was ERK2-P following thrombin treatment. We tested ERK2 kinase activity under the same thrombin conditions after ERK2 immunoprecipitation, by in vitro phosphorylation of MBP as substrate (see “Experimental Procedures”). No MBP phosphorylation was observed in resting platelets or when platelets were stimulated with between 0.02 and 0.1 NIH units/ml of thrombin. In contrast, a significant increase in ERK2 activity was detectable with 0.2 NIH unit/ml of thrombin and reached a maximum at 1 NIH unit/ml of thrombin. Therefore, there was a direct correlation between electrophoretic mobility, phosphorylation, and ERK2 activity. A thrombin concentration as low as 0.02 NIH unit/ml resulted in detectable platelet aggregation but not ERK2 activation suggesting that ERK2 activation is not required to initiate platelet aggregation.

We next used gel mobility shift assay and kinase assay to investigate the time course of ERK2 activation upon treatment with 1 NIH unit/ml of thrombin (Fig. 1B). In resting platelets and platelets stimulated with thrombin for 30 s, only the nonphosphorylated form of ERK2 was detected, and there was no ERK2 activity. In contrast, ERK2 phosphorylation and activity were detected after 60 s of induction, when platelet aggregation was about 34%, reached maximum between 150 and 180 s, and then decreased. Thus ERK2 activation is a late event in thrombin-induced platelet aggregation. One interpretation consistent with these results is that ERK2 activation is dependent on α1β3 integrin-dependent platelet to platelet adhesion or aggregation. Alternatively, ERK2 activation may be dependent on thrombin platelet stimulation. These two interpretations are not mutually exclusive.

ERK2 Activation Is Dependent on Thrombin and Is Up-regulated by Inhibition of α3β1α3β1—To distinguish between these hypotheses, we investigated thrombin-induced ERK2 activation conditions of inhibition of fibrinogen binding to integrin α1β3 and inhibition of aggregation. The involvement of α1β3 in ERK2 activation was assessed first by use of the RGDS peptide, a competitive inhibitor of fibrinogen for binding to α1β3 integrin-dependent platelet to platelet adhesion or aggregation. Alternatively, ERK2 activation may be dependent on thrombin platelet stimulation. These two interpretations are not mutually exclusive.

**Fig. 1. Effect of thrombin-induced ERK2 activation in platelets.** Washed platelets were stimulated under stirring for 2 min with various concentrations of thrombin (A) or with 1 NIH unit/ml at different times (B). ERK2 phosphorylation was analyzed by SDS-PAGE followed by Western blotting using a polyclonal antibody recognizing ERK2 (phosphorylated and nonphosphorylated forms) and a monoclonal antibody specific for phosphorylated ERK2-P. ERK2 activity was measured by detection of 32P-MBP as described under “Experimental Procedures.” Corresponding aggregation rates are indicated (Aggregation %). These autoradiograms and autoradiographs are representative of at least three separate experiments.

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phosphorylation and activity were increased dose dependently by AP-2 (Fab')2, reaching a plateau at 10 μg/ml (Fig. 2B). At this concentration (10 μg/ml), the values obtained were comparable with those obtained with RGDS: 240.0% ± 63.4% (p = 0.02) ERK2 phosphorylation and 245.0 ± 75.6% (p = 0.02) ERK2 activity. Thus both experiments (RGDS and (Fab')2 fragments) suggest that activation of the ERK2 pathway is not dependent on aggregation and is up-regulated when fibrinogen binding to αIIbβ3 is inhibited.

We next investigated ERK2 activation in platelets from two Glanzmann thrombasthenia patients which are characterized by absence of aggregation due to quantitative or qualitative defects in αIIbβ3. Platelets from patients I and II had 2-fold higher ERK2 activities than control platelets (Fig. 3). The enhancement in ERK activation (patient I, 190%; patient II, 240%) was thus similar whether αIIbβ3 was absent (patient I) or nonfunctional (patient II) or fibrinogen binding was competitively inhibited (by RGDS peptide or AP-2 (Fab')2). This confirms that the absence of αIIbβ3 integrin engagement up-regulates ERK2 activation and that ERK2 activation is independent of platelet aggregation.

αIIbβ3 Integrin Engagement Negatively Regulates Thrombin-induced ERK2 Activation—All conditions used so far prevented or led to the absence of fibrinogen binding to αIIbβ3 and consequently to the absence of aggregation. To distinguish between fibrinogen binding and aggregation itself, we compared ERK2 overactivation in unstirred (fibrinogen-bound, no aggregation) and stirred (fibrinogen-bound and aggregation) thrombin-activated platelets. The RGDS peptide was used as a negative control for fibrinogen binding. As illustrated in Fig. 4, in unstirred platelets which prevent aggregation but allow fibrinogen binding, ERK2 activation was higher than in stirred thrombin-activated platelets. In control platelets, absence of stirring induced ERK2 overactivation, 181% and 182% at 2 and 3 min of thrombin stimulation. Since platelets did not aggregate in these conditions, this suggests that events precluding aggregation in that include fibrinogen binding to αIIbβ3 and subsequent clustering may participate in ERK2 overactivation. However maximal ERK2 overactivation reached 254% and 202% at 2 and 3 min, respectively, in the presence of RGDS in both stirred and unstirred platelets. Thus both platelet aggregation and fibrinogen binding to αIIbβ3 (and/or clustering) appear to negatively control thrombin-induced ERK2 activation.

Aggregation is a complex phenomenon consecutive to fibrinogen binding to αIIbβ3 integrin and cytoskeletal rearrangements (24), in the course of platelet to platelet adhesion. It is possible that the partial negative control of thrombin-activated ERK2 is due to inhibition of upstream effectors of ERK or activation of phosphatases. Several kinases that act upstream from ERK have been identified in platelets. MEK can directly phospho-
The relationship between αIbβ3 and integrin engagement and the MAP kinase activation in cells of the megakaryocytic lineage. A third possible explanation is that integrin signaling differs according to the physical state of the ligand, for example adhesion to a solid support-associated ligand leads to activation of tyrosine kinases different from those activated by interaction with the soluble form of the ligand (8, 10). A similar situation may exist for coupling integrins and the MAP kinase pathway; this may explain why integrin-mediated cell adhesion to an immobilized ligand activates the MAP kinase pathway, whereas platelet aggregation, a cell-to-cell adhesion process mediated by interaction of αIbβ3 integrin with soluble fibrinogen triggers down-regulation of the MAP kinase pathway. In conclusion, this is the first report showing that integrin engagement down-regulates ERK activation, suggesting that integrin may regulate the MAP kinase pathway in various ways depending on the cell and function.

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FIG. 4. Kinetics of thrombin-induced ERK2 activation of stirred and unstirred platelets and in the presence and absence of RGDS peptide. Washed stirred and unstirred platelets were preincubated in the presence or absence of RGDS peptide (1 mM) and then stimulated with thrombin (1 NIH unit/ml). The platelets were then solubilized in buffer containing SDS and analyzed for ERK2 phosphorylation and activity. The experiment shown is representative of four separate experiments.