Selective dephosphorylation of the threonine^{183} residue of ERK2 upon αIIbβ3 engagement in platelets

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Abstract Thrombin-induced extracellular signal-regulated kinase 2 (ERK2) activation is negatively regulated in conditions of αIIbβ3 integrin engagement and platelet aggregation. Here we show by Western blotting with antibodies against mono- and biphosphorylated forms of ERK2 that the dephosphorylation of ERK2 by αIIbβ3 engagement affects threonine^{183} and not tyrosine^{185}. Addition of a potent serine/threonine phosphatase inhibitor, okadaic acid (OA), restored thrombin-induced threonine phosphorylation of ERK2 in conditions of platelet aggregation, whereas OA had no effect in the absence of αIIbβ3 engagement. These observations are consistent with αIIbβ3 engagement acting via at least one serine/threonine phosphatase, which dephosphorylates the phosphothreonine^{183} residue of ERK2. Moreover, a small amount (14%) of ERK2 was translocated to the αIIbβ3-dependent cytoskeleton, mostly in a monophosphorylated (i.e. inactive) form, suggesting that cytoskeleton-associated ERK2 plays only a minor role, if any. Finally, we show that negative regulation (i.e. dephosphorylation) occurs primarily or totally in the cytosol and that the αIIbβ3-dependent ERK2 Thr^{183}-specific phosphatase is different from phosphatase 1 (PP1) or PP2A. We conclude that αIIbβ3 engagement down-regulates ERK2 through selective dephosphorylation of the phosphothreonine^{183} residue by a cytosolic serine/threonine phosphatase different from known platelet phosphatases. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitogen-activated protein kinase; Dephosphorylation; Integrin; Human platelets

1. Introduction

The mitogen-activated protein kinase (MAPK) family comprises extracellular signal-regulated kinase (ERK), c-Jun-NH2-terminal kinase (JNK) and p38 MAPK. The activation of MAPKs requires phosphorylation of the threonine and tyrosine residues located within the TXY motif of kinase domain VIII [1]. The phosphorylation of both the Thr^{183} and Tyr^{185} residues of ERK2 is catalyzed by dual-specific upstream MAPK kinases, MEK1 or MEK2 [2]. The regulation of ERK2 activity involves several types of phosphatases that dephosphorylate both threonine and tyrosine activating residues. The dual-specific threonine/tyrosine phosphatase family, also called the MAPK phosphatase family, includes MKP-1, PAC-1, MKP-3 or MKP-4. The members of this family inactivate ERK, the pattern of inactivation depending on cell type and the cytosolic or nuclear location [3]. MKP-3 and MKP-4 have been shown to be more specific for ERK than JNKs and p38 MAPKs [4–5]. MKP-3 substrate selectivity appears to depend primarily on the N-terminal-specific region of the phosphatase and then on substrate recognition domains within ERK [6–7]. Serine/threonine phosphatase 1 (PP1) and PP2A have been also shown to inhibit the activity of the ERK1/2 pathway by the dephosphorylation of MEK1/2 and EKR1/2. PP2A is involved in regulation of ERK2 pathway induced by inhibitory concentrations of epidermal growth factor (EGF) in A431 cell lines [8]. PP2A has also been shown to dephosphorylate the Thr^{183} residue of ERK2 directly in PC12 cells [9]. Nevertheless, MEK1 and MEK2 activities may also be regulated by the activation of PP1 and PP2A [10]. Although the SH2 domain-containing tyrosine phosphatases (SHP1 and SHP2) have been found to regulate MAPK pathways positively or negatively [11–13] one tyrosine phosphatase family, comprising STEP, PTP-SL and He-PTP, has been shown to interact with and to dephosphorylate the Tyr^{185} residue of ERK2 [14–16].

In platelets, all three families of MAPKs are activated by physiologic agonists including thrombin [17] and collagen [18]. Thrombin-induced ERK2 activation is mediated by MEK1/2 and is dependent on both calcium and conventional protein kinase C. However, the regulation of MAPKs by phosphatases has not been investigated in these nucleus-free cells. Protein phosphatase activities in platelets seem to play an important role in regulating signaling events and consequently, platelet activation and aggregation. For example, the incubation of platelets with okadaic acid (OA), a potent inhibitor of PP1 and PP2A, results in the inhibition of activated-platelet functions [19], calcium mobilization and phosphoinositide production [20]. These serine/threonine phosphatases may also be involved in cytoskeletal reorganization, as they are translocated in this platelet fraction during thrombin-induced aggregation [21–22]. At least three non-receptor tyrosine phosphatases are expressed in platelets, and two of these phosphatases are PTP-1B and SHP2. These enzymes are activated upon thrombin-induced platelet activation and αIIbβ3 engagement [23]. The calcium-dependent enzyme calpain cleaves PTP-1B after integrin engagement, thereby activating

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase; RGDS, Arg-Gly-Asp-Ser; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; OA, okadaic acid; CSK, cytoskeleton

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the phosphatase [24]. Moreover both SHP-1 and SHP-2 phosphatases appear to be phosphorylated in thrombin-induced activated platelets and to associate with several Src-family tyrosine kinases and the adhesion molecule PECAM-1 [25–28].

We have previously shown that, in human platelets, the thrombin-mediated activation of ERK2 and JNK1 is negatively regulated by αIIbβ3 engagement in platelet aggregation [29–30]. These were the first reports showing that an integrin could down-regulate MAPKs. In this study, we investigated the mechanism involved in ERK2 down-regulation and the possible role of phosphatases.

We found that the down-regulation of ERK2 activity upon αIIbβ3 engagement was directly correlated with dephosphorylation of the Thr^{183} residue, but not of the Tyr^{185} residue. Thrombin-induced platelet activation in the presence of the serine/threonine phosphatase inhibitor OA increased ERK2 activation in conditions of integrin engagement, providing further evidence for the involvement of a serine/threonine phosphatase. Finally, the small proportion of ERK2 translocated to the cytoskeleton (CSK) suggest that αIIbβ3-dependent negative regulation occurs in the cytosol.

2. Materials and methods

2.1. Reagents

Bovine α-thrombin, the synthetic peptide Arg-Gly-Asp-Ser (RGDS), leupeptin and aprotinin were purchased from Sigma (St. Louis, MO, USA). PD98059 was obtained from Biomol (Plymouth Meeting, PA, USA). Rabbit polyclonal antibodies directed against the C-terminal peptides of ERK1/2 (C-14), rabbit polyclonal antibody directed against the phosphorylated forms of ERKs (ERKs-P) and the phosphothreonine form of ERKs were obtained from Promega (Madison, WI, USA). Mouse monoclonal antibody directed against the phosphotyrosine of ERKs was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Donkey anti-rabbit and anti-mouse horseradish peroxidase-conjugated IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2.2. Platelet preparation and aggregation

Platelets are isolated as previously described [29]. Platelet aggregation was then initiated by adding bovine thrombin (1 NIH/ml), with constant stirring (1200 rpm) in an aggregometer cuvette (Chronolog dual-beam aggregometer). Aggregation was measured and expressed as a percent change in light transmission, with the blank sample (buffer without platelets) defined as having 100% transmission.

2.3. Immunoblotting

Samples were subjected to immunoblotting as previously described [29,30]. Briefly, platelet lysates were obtained by adding SDS denaturing buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 mM phenylarsine oxide, 5 μg/ml leupeptin, 5 μg/ml aprotinin, SDS 10%, pH 7.4) to the sample and heating at 95°C for 5 min. Nitrocellulose membranes were treated with polyclonal anti-ERKs antibody (1:20 000), anti-ERKs-P antibody (1:10 000), anti-phosphotyrosine form of ERKs antibody (1:10 000) or the anti-phosphothreonine form of ERKs antibody (1:10 000) and then with either horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20 000) or with peroxidase-conjugated rabbit

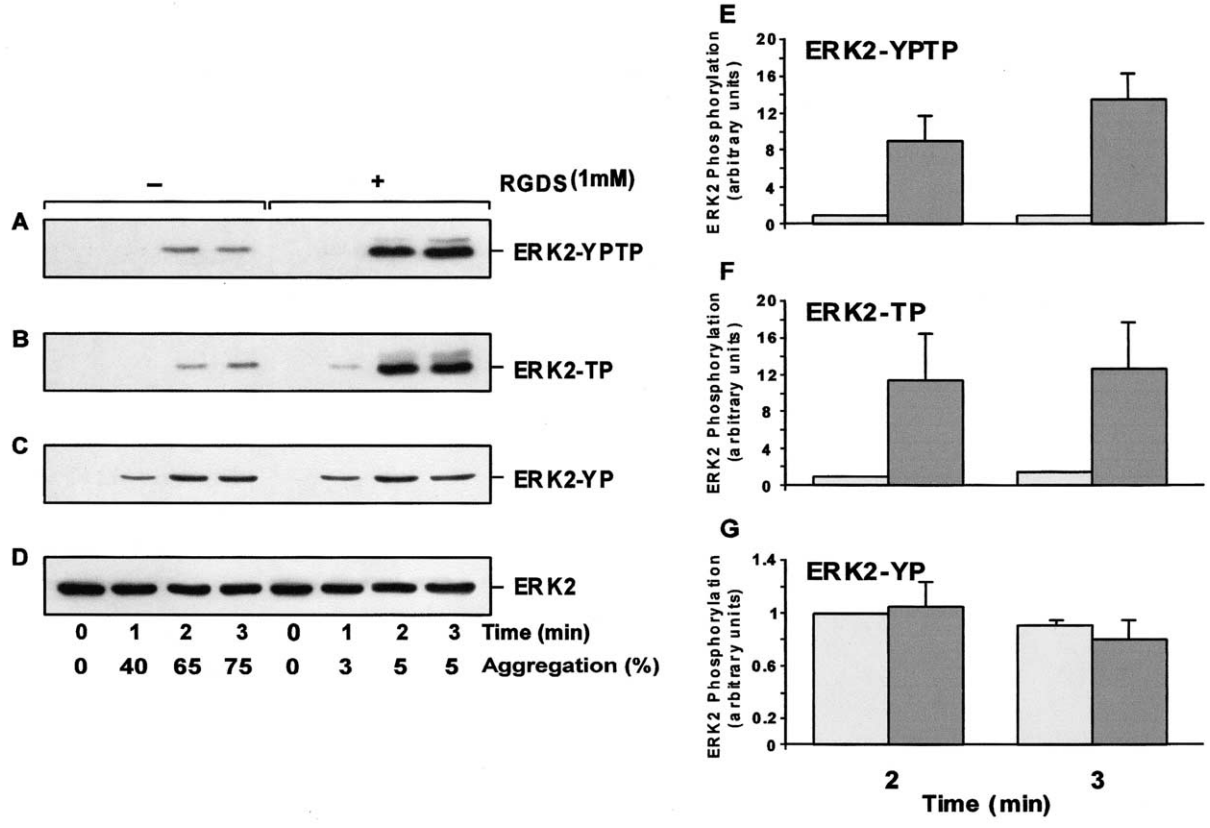

Fig. 1. Regulation of ERK2 by αIIbβ3 engagement. Washed platelets were preincubated in the presence (gray bars) or absence (white bars) of RGDS peptide (1 mM) and stimulated with thrombin (1 NIH/ml) at various times. Platelets were solubilized and analyzed by SDS–PAGE followed by Western blotting, using polyclonal antibodies recognizing the phosphotyrosine and phosphothreonine residues of ERKs (ERK-YPTP) (A, E), phosphotyrosine (ERK-YP) (B, F), or phosphothreonine (ERK-TP) (C, G) residues only. Results are representative of five experiments. ERK2 phosphorylation was measured by densitometry analysis in the presence of absence of RGDS peptide and the results were normalized with respect to platelets treated with thrombin alone (2 min), expressed as a relative intensity.

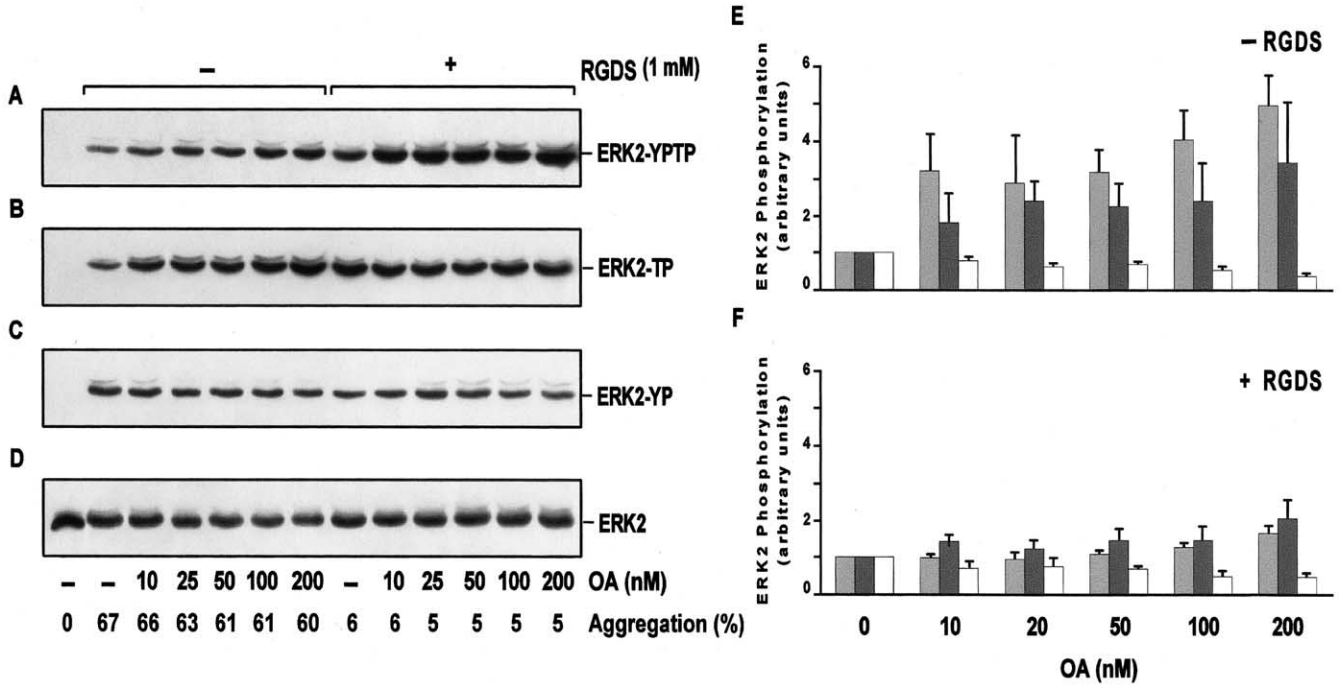

Fig. 2. Effect of a serine/threonine phosphatase inhibitor (OA) on ERK2 activation. Washed platelets were stimulated with thrombin (2 min) and various concentrations of OA (0–200 nM) in the presence or absence of RGDS peptide. Platelet lysates were analyzed by Western blotting, using polyclonal antibodies recognizing the phosphotyrosine and phosphothreonine residues of ERKs (ERK-YPTP) (A, E, F) (gray bars), phosphotyrosine (ERK-YP) (C, E, F) (white bars) and phosphothreonine residues (ERK-TP) (B, E, F) (black bars). Results are representative of four experiments. ERK2 phosphorylation was measured by densitometry analysis in the presence (F) or absence (E) of RGDS peptide and the results were normalized with respect to platelets treated with thrombin alone (2 min), expressed as a relative intensity.

anti-mouse IgG (1:20 000). Immunoreactive bands were visualized with enhanced chemiluminescence detection reagents (Pierce).

2.4. CSK extraction

Briefly, we isolated the CSK of control or activated platelet suspensions by adding 0.4 ml of ice-cold CSK buffer (100 mM Tris–HCl, 2% Triton, 50 mM NaF, 150 mM β-glycerophosphate, 5 mM EDTA, 2 mM orthovanadate, 5 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.5). After 30 min of incubation at 4°C, Triton X-100-soluble and -insoluble fractions were separated by centrifugation at 12 000 × *g* for 15 min at 4°C. Total CSK, corresponding to the Triton X-100-insoluble fraction, and equal amounts of samples from total lysates and the Triton X-100-soluble fraction were subjected to 12% SDS–polyacrylamide gel electrophoresis (PAGE). Western blotting was performed with an antibody recognizing total ERK2 (ERK2-P, ERK2) or various forms of phosphorylated ERK2: T^{183}-phosphorylated ERK2 (ERK2-TP, and ERK2-TPYP) or Y^{185}-phosphorylated ERK2 (ERK2-YP and ERK2-TPYP).

3. Results

3.1. Negative ERK2 regulation by αIIbβ3 integrin involves dephosphorylation of the phosphothreonine^{183} residue

As αIIbβ3 engagement down-regulates ERK2 activity [29–30] it must decrease the phosphorylation of either T^{183} and Y^{185}, or both. This raised the question as to which step in the ERK2 activation pathway was the target of αIIbβ3. We therefore compared the phosphorylation states of the tyrosine^{185} and threonine^{183} residues of ERK2 after thrombin induction, in the presence or absence of RGDS peptide (i.e. with and without aggregation), by Western blotting using antibodies specific for ERK2 with phosphorylated threonine residues, tyrosine residues, or both. As previously reported, ERK2 phosphorylation levels were 10 times higher in the presence of RGDS peptide than in its absence after 2 min of thrombin induction, as shown by Western blotting with the dual phosphorylation antibody (ERK2-TPYP) (Fig. 1A,E). As various phosphatases present in platelets have been reported to be activated upon thrombin-induced platelet activation and αIIbβ3 engagement [23], we suggested that RGDS peptide blocked the αIIbβ3-dependent dephosphorylation of ERK2 by phosphatase. A similar up-regulation of phosphorylated ERK2 (increase by a factor of 11) was observed in the presence of RGDS peptide with the T^{183}P-specific antibody (Fig. 1B,F). In contrast, no significant change in the tyrosine phosphorylation of ERK2 was observed, in the presence or absence of RGDS peptide, in Western blots with the Y^{185}P-specific antibody (Fig. 1C,G). A non-specific band detected throughout corresponded to a cross-reaction with JNK1. In conclusion, our data show that only the phosphothreonine residue was negatively regulated by αIIbβ3 engagement.

3.2. A threonine phosphatase dependent on αIIbβ3 engagement decreases ERK2 activation

Specific dephosphorylation of T^{183} but not of Y^{185} strongly suggests the involvement of a serine/threonine phosphatase. We thus investigated the effect of OA, an inhibitor of serine/threonine phosphatases PP1 and PP2A, on ERK2 phosphorylation. In conditions of platelet aggregation, a dose-dependent increase in ERK2 activity was observed in the presence of various concentrations of OA (0–200 nM), with activity reaching 534% ± 144% ($P < 0.05$) at a concentration of 200 nM (Fig. 2A,E). Moreover, using an anti-T^{183}P antibody, we showed that OA increased ERK2 phosphorylation by 400 ± 133% ($P < 0.05$) at OA concentrations of 200 nM

(Fig. 2B,E). In contrast, in the presence of RGDS peptide, in conditions in which αIIbβ3 was not engaged, no statistically significant variation in ERK2 activity or in levels of the phosphothreonine form of ERK2 (even at 200 nM OA) was observed (Fig. 2A,B,F). Thus, the threonine phosphorylation of ERK2 was negatively regulated by αIIbβ3 engagement and a serine/threonine phosphatase was involved in this negative regulation. The incomplete restoration of T^{183} phosphorylation in the presence of OA (increase in phosphorylation by a factor of five) whereas RGDS peptide increased the level of phosphorylation by a factor of 10, may be accounted for by the use of only a low concentration of OA (200 nM), to prevent the inhibition of platelet aggregation. Surprisingly, increasing the concentration of OA decreased the level of phosphorylation of the Y^{185} residue of ERK2 (halved at 200 nM OA) in the presence or absence of RGDS peptide (Fig. 2C,E,F). Our data suggest that a serine/threonine phosphatase is also involved in thrombin-induced ERK2 phosphorylation, independent of αIIbβ3 engagement and increasing the amount of the phosphotyrosine form of ERK2. Nevertheless, ERK2 activity is negatively regulated by serine/threonine phosphatases in conditions of platelet aggregation.

3.3. Subcellular localization of activated ERK2 and regulation

3.3.1. Some ERK2 is translocated to the CSK after thrombin induction. As αIIbβ3 engagement induces the reorganization of the actin CSK and the translocation of a number of signaling molecules, we assessed the extent to which the phosphorylated and unphosphorylated forms of ERK2 were associated with the CSK. We fractionated the total lysate into Triton X-100-soluble and -insoluble (corresponding to CSK) fractions. To optimize sensitivity, total CSK from 800 μl of platelet suspension was subjected to Western blotting whereas only a fraction (50 μl of 800 μl) of total lysate and the Triton X-100-soluble fraction was analyzed. Comparison of total ERK2 (ERK2 and ERK2-P) levels in whole lysate, detergent-soluble fraction and CSK showed that ERK2 and ERK2-P (ERK2-YPTP, ERK2-YP, ERK2-TP) were present in the detergent-soluble fraction and the CSK (Fig. 3A–D). Quantitative analysis showed that in resting platelets, 6.2 ± 1.1% of total ERK2 was detectable in the CSK (Fig. 4A,B). This level increased to 14.0 ± 3.5% after 3 min of thrombin induction, showing that only a small amount of ERK2 was translocated. Moreover, CSK-associated ERK2 was mostly un- or dephosphorylated (Fig. 3A,D). These results are consistent with two hypotheses: (1) translocation of ERK2 into the CSK occurs independently from the state of ERK2 phosphorylation; (2) ERK2-P is the species of ERK2 translocated, but is rapidly dephosphorylated in the CSK. As the amount of ERK2 in the CSK is not affected by PD98059, a MEK1/2 inhibitor (i.e. in conditions of ERK2 inactivation; Fig. 5), we conclude that ERK2 translocation to the CSK is independent of activation state, and therefore also of phosphorylation state. Finally, comparison of ERK2-YPTP, ERK2-YP, and ERK2-TP levels (Fig. 3B–D), suggests that ERK2 is rapidly activated (30 s after the addition of thrombin) and that a serine/threonine phosphatase acts as soon as 30 s to 1 min after thrombin induction.

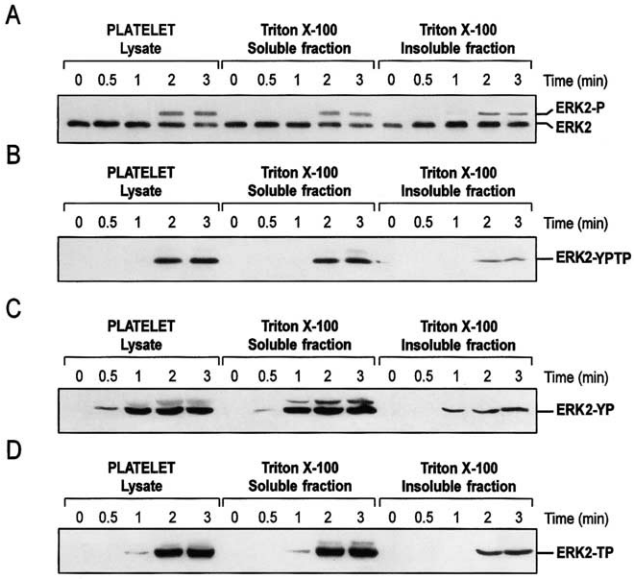

Fig. 3. Distribution of ERK2 after thrombin activation. Washed platelets were stimulated by thrombin (1 NIH/ml) for various lengths of time with stirring. Platelets were solubilized in Triton X-100 as described in Section 2. A sample (50 μl) of whole lysate and of Triton X-100-soluble fraction (in contrasts to the total Triton X-100 insoluble fraction), corresponding to the CSK, were separated by SDS–PAGE followed by Western blotting using (A) an anti-total ERK2 antibody (ERK2-P, ERK2), (B) an anti-ERK2-YPTP, (C) (ERK2-YP) or (D) (ERK2-TP). Results are representative of four experiments.

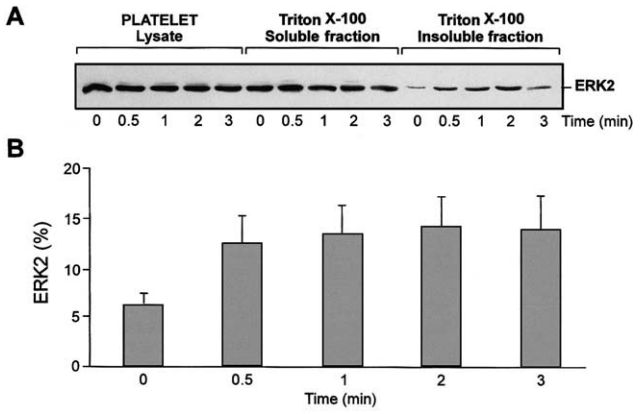

Fig. 4. Translocation of ERK2 in the CSK. Washed platelets were stimulated by thrombin (1 NIH/ml) in the presence or absence of PD 98059 (20 μM) for various lengths of time, with stirring. Platelets were solubilized in Triton X-100 as described in Section 2. The Triton X-100-soluble fraction, corresponding to the CSK, was resolved as previously described and Western blotted, using a polyclonal antibody recognizing both phosphorylated and non-phosphorylated ERK2 (A–D). Results are representative of three experiments.

Thus, our results show that only small amounts of ERK2 are translocated to the CSK, in an unphosphorylated and monophosphorylated inactive form, and that the active substrates of ERK2 are in the cytosol.

3.3.2. ERK2 is negatively regulated in the Triton X-100-soluble fraction after αIIbβ3 engagement. We compared the subcellular location and activation of ERK2 in the presence and absence of RGDS peptide. ERK2 and ERK2-P were detected in both compartments in the absence of RGDS peptide (Fig. 6A,B). Similar increases in ERK2-P levels were observed in the presence of RGDS peptide in the soluble fraction and in total lysate after thrombin induction, suggesting that T^{183} dephosphorylation occurred in the cytosol (results

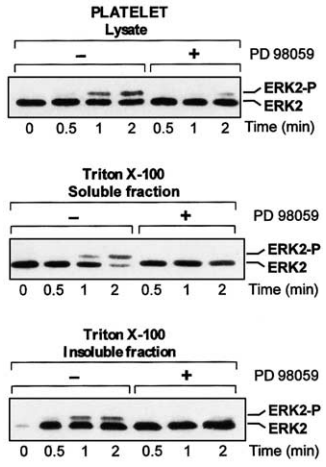

Fig. 5. Quantification of ERK2 in the CSK. Washed platelets were stimulated by thrombin (1 NIH/ml) in the presence or absence of PD 98059 (20 μM) for various lengths of time, with stirring. Platelets were solubilized in Triton X-100 as described in Section 2. The Triton X-100-soluble fraction, corresponding to the CSK, was resolved as previously described and subjected to Western blotting, using a polyclonal antibody recognizing both phosphorylated and non-phosphorylated ERK2. ERK2 was measured by densitometry analysis and the results are representative of three independent experiments.

not shown). In contrast, RGDS peptide decreased the amount of ERK2 and ERK2-P in the CSK by 73%, confirming that the translocation of ERK2 and ERK2-P depended principally on the αIIbβ3-dependent CSK. Finally, our results strongly suggest that negative regulation by αIIbβ3 engagement occurs mainly in the cytosol. The fact that only 14% of ERK2 was translocated to the CSK suggests that even if negative regulation occurred in the CSK, it played only a minor role in overall regulation.

In conclusion, our results show that the translocation of ERK2 to the CSK depends principally on αIIbβ3 engagement and that negative regulation occurs mostly or entirely in the cytosol.

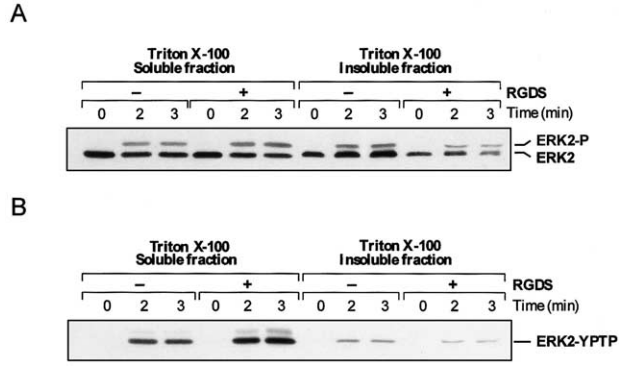

Fig. 6. Subcellular localization of the negative regulation of ERK2. Washed platelets were stimulated with thrombin (1 NIH/ml) for various lengths of time, with stirring, in the presence or absence of RGDS peptide (1 mM). Samples were solubilized in Triton X-100 as described in Section 2. A sample (50 μl) of total lysate and of the Triton X-100-soluble fraction, corresponding to the CSK, was separated by SDS–PAGE and Western blotted, using (A) an anti-ERK2 or (B) an anti-ERK2-YPTP. Results are representative of three experiments.

4. Discussion

In this report, we investigated the mechanism leading to ERK2 down-regulation following αIIbβ3 engagement [29,30]. We demonstrated that the phosphothreonine^{183} and phosphotyrosine^{185} residues necessary for ERK2 activity were differentially regulated in conditions of platelet aggregation. As previously reported [30], ERK2 activity was up-regulated (by a factor of 10) in the presence of RGDS peptide, conditions corresponding to thrombin-induced platelet activation without aggregation. Similar levels of RGDS-induced up-regulation (increase by a factor of 11) were observed for the phosphothreonine^{183} residue, with an antibody directed against this residue, whereas no significant change was observed in levels of the phosphotyrosine^{185} residue of ERK2. To our knowledge, this is the first report showing the differential regulation by integrin engagement of phosphorylation of the T^{183} and Y^{185} residues of ERK2. Moreover, both experiments showed that this down-regulation involved activation of serine/threonine phosphatases dependent on αIIbβ3 integrin engagement that act directly on ERK2. We evaluated the role of phosphatases in this regulation by investigating the effect of OA, a potent inhibitor of the serine/threonine phosphatases PP1 and PP2A. It has been reported that PP2A regulates ERK2 under conditions of growth inhibition by EGF in A431 cells [8]. In these conditions, a correlation between PP2A activity induced by EGF and inhibition of ERK2 activity was observed [8]. PP2A has also be shown to be involved in the negative regulation of MAPK activity [9]. In our model, the addition of OA in conditions of platelet aggregation up-regulated ERK2 activity and phosphothreonine^{183} residue levels whereas no significant variation was observed in the absence of fibrinogen binding, suggesting that a serine/threonine phosphatase dependent on αIIbβ3 engagement in platelets was involved. In the presence of OA, the level of ERK2 activity was not completely restored in the presence of RGDS peptide. This may be due to the effects of different serine/threonine phosphatases acting on ERK2, one of which was unaffected by OA. Alternatively, given the low concentrations used (200 nM), OA may have not completely inhibited serine/threonine phosphatase activity. The serine/threonine phosphatases PP2A and PP1, which are inhibited by low concentrations of OA (IC_{50} PP2A: 0.2 nM; IC_{50} PP1: 20 nM) may be involved in this T^{183} dephosphorylation. Surprisingly, in conditions of thrombin-induced platelet aggregation, no association of ERK2 with PP2A or PP1 was detected in coimmunoprecipitation experiments, in the presence or absence of RGDS peptide (results not shown). This suggests that PP2A and PP1 may not be the phosphatases involved in dephosphorylation of the threonine^{183} residue of ERK2. Alternatively, PP2A and/or PP1 may actually involved, but the association–dissociation of ERK2 and phosphatase may not result in the formation of a complex stable enough to be detected in our conditions. A direct association between ERK2 and PP2A was observed only in in vitro experiments based on purified enzymes (ERK2 and PP2A). It is possible that our conditions (total cell lysate and IgG-bound enzymes) hindered the formation of such an association. A third possibility is that other phosphatases such as PP4 and PP5, which have been reported to be sensitive to OA [31], may be involved. Finally, we cannot exclude the possibility of an unknown serine/threonine phosphatase acting on ERK2.

We also observed that OA decreased the level of phosphorylation of Y^{185}. However, this phosphorylation was independent of αIIbβ3 engagement. As changes in pY^{185} mediated by OA occurred after thrombin activation, the simplest interpretation is that OA inhibited a serine/threonine phosphatase involved in the negative regulation of a tyrosine phosphatase. PTP-1B, which is present in platelets, has been reported to be phosphorylated on the serine residue responsible for its enzymatic activation [32]. Moreover, the treatment of cells with OA resulted in the serine phosphorylation of PTP-1B and stimulation of PTP-1B phosphatase activity [33].

ERK2 is known to associate with the CSK [34] and, because αIIbβ3 controls the actin CSK, we investigated whether αIIbβ3-dependent down-regulation was associated with CSK. As previously reported [35], we found that ERK2 was present in both the detergent-soluble fraction and the CSK. Comparison of ERK2-P, in the shift assay, and ERK2-YP and ERK2-TP in the detergent-soluble fraction and CSK, suggested that monophosphorylated forms (ERK2-YP and ERK2-TP) of ERK2 were present in both fractions, whereas only a low level of biphosphorylated forms (ERK2-YPTP) was present in CSK. At early time points (30 s and 1 min), only the tyrosine form of ERK2 was detected, suggesting that ERK2 is activated after 30 s of thrombin induction and that a serine/threonine phosphatase masks ERK2 activity up to that time point. Moreover, the small proportion of ERK2 (6%) in resting platelets increased to 14% after thrombin induction. Maximal ERK2 translocation was observed at 30 s when ERK2 is still mostly inactive, which suggests that ERK2 activation is not required for translocation. This hypothesis was confirmed in conditions in which ERK2 was inhibited by PD 98059, a MEK1/2 inhibitor, that did not affect the translocation of unphosphorylated ERK2 to the CSK. Finally, we observed that cytochalasin D did not affect ERK2-YPTP activity after thrombin activation, strongly suggesting that actin CSK is not involved in T^{183} down-regulation (results not shown).

The fact that 90% of ERK2 translocation was dependent on αIIbβ3 engagement does not make it possible to draw definitive conclusions concerning the site at which negative regulation occurs. One possibility is that negative regulation occurs only in the cytosol (soluble fraction), generating monophosphorylated forms of ERK2, which are then translocated from the cytosol to the CSK. The corollary is that αIIbβ3 activates a cytosolic serine/threonine phosphatase. This mechanism would be analogous to activation of the tyrosine phosphatase PTP-1B by cleavage by a calcium-dependent protease, calpain, itself activated by αIIbβ3 engagement [24]. The other possibility is that negative regulation occurs mostly in the cytosol, but with some regulation also occurring in the CSK. In this case, one would have to postulate the existence of a phosphatase associated with, or translocated in the αIIbβ3-dependent CSK. Only 14% of total ERK2 is present in the CSK, which strongly suggests that the regulation observed in the cytosol corresponds to a large part of the regulation that occurs in the whole platelet. This was confirmed by comparing the level of ERK2-YPTP, equivalent in total lysate and the Triton X-100-soluble fraction (cytosol), in the presence and absence of RGDS (results not shown).

In conclusion, our results show that the thrombin-induced phosphorylation of the threonine^{183} and tyrosine^{185} residues of ERK2 is differentially regulated by αIIbβ3 integrin engagement. Our data provide evidence that T^{183} is dephosphorylated via a serine/threonine phosphatase in conditions of platelet aggregation and that negative regulation occurs in the cytosol. This is the first report showing a dynamic relationship between integrin engagement and selective dephosphorylation of ERK2.

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References

[1] Cobb, M.H. and Goldsmith, E.J. (1995) J. Biol. Chem. 270, 14843–14846.
[2] Lewis, T.S., Shapiro, P.S. and Ahn, N.G. (1998) Adv. Cancer Res. 74, 49–139.
[3] Haneda, M., Sugimoto, T. and Kikkawa, R. (1999) Eur. J. Pharmacol. 365, 1–7.
[4] Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S. (1998) Science 280, 1262–1265.
[5] Muda, M., Boschert, U., Smith, A., Antonsson, B., Gillieron, C., Chabert, C., Camps, M., Martinou, I., Ashworth, A. and Arkinstall, S. (1997) J. Biol. Chem. 272, 5141–5151.
[6] Muda, M., Theodosiou, A., Gillieron, C., Smith, A., Chabert, C., Camps, M., Boscher, U., Rodrigues, N., Davies, K., Ashworth, A. and Arkinstal, S. (1998) J. Biol. Chem. 273, 9323–9329.
[7] Nichols, A., Camps, M., Gillieron, C., Chabert, C., Brunet, A., Wilksbacher, J., Cobb, M., Pouyssegur, J., Shaw, J.W. and Arkinstall, S. (2000) J. Biol. Chem. 275, 24613–24621.
[8] Chajry, N., Martin, P.M., Cochet, C. and Berthois, Y. (1996) Eur. J. Biochem. 235, 97–102.
[9] Alessi, D.R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S.M. and Cohen, P. (1995) Curr. Biol. 5, 283–295.
[10] Westermarck, J., Li, S.P., Kallunki, T., Han, J. and Kahari, V.M. (2001) Mol. Cell. Biol. 121, 2373–2384.
[11] Bedecs, K., Elbaz, N., Sutren, M., Masson, M., Susini, C., Strosberg, A.D. and Nahmias, C. (1997) Biochem. J. 325, 449–454.
[12] Su, L., Zhao, Z., Bouchard, P., Banville, D., Fischer, E.H., Krebs, E.G. and Shen, S.H. (1996) J. Biol. Chem. 271, 10385–10390.
[13] Yamauchi, K., Milarksi, K.L., Sartiel, A.R. and Pessin, J.E. (1995) Proc. Natl. Acad. Sci. USA 92, 664–668.
[14] Pulido, R., Zuniga, A. and Ullrich, A. (1998) EMBO J. 17, 7337–7350.
[15] Zuniga, A., Torres, J., Fabuda, J. and Pulido, R. (1999) J. Biol. Chem. 274, 21900–21907.
[16] Saxena, M., Williams, S., Brockdorff, J., Gilman, J. and Mustelin, T. (1999) J. Biol. Chem. 274, 11693–11700.
[17] Papkoff, J.H., Chen, R.H., Blenis, J. and Forsman, J. (1994) Mol. Cell. Biol. 14, 463–472.
[18] Saklatvala, J., Rawlinson, L., Waller, R.J., Sarsfield, S., Lee, J.C., Morton, L.F., Barnes, M.J. and Farndale, R.W. (1996) J. Biol. Chem. 271, 6586–6589.
[19] Mauco, G., Artçanuthurry, V., Pidard, D., Grelac, F., Maclouf, J. and Lévy-Toledano, S. (1997) Cell Signal. 9, 117–124.
[20] Murphy, C.T. and Westwick, J. (1994) Biochem. J. 301, 531–537.
[21] Yano, Y., Sakon, M., Kambayashi, J.I., Kawasaki, T., Senda, T., Tanaka, F., Yamada, F. and Shibata, N. (1995) Biochem. J. 307, 439–449.
[22] Toyoda, H., Nakai, K., Omay, S.B., Shima, H., Nagao, M., Shiku, H. and Nishikawa, M. (1996) Thromb. Haemost. 76, 1053–1062.
[23] Ezumi, Y., Takayama, H. and Okuma, M. (1995) J. Biol. Chem. 270, 11927–11934.
[24] Frangioni, J.V., Oda, A., Smith, M., Salzman, E.W. and Neel, B.G. (1993) EMBO. J. 12, 4843–4856.

[25] Li, R.Y., Gaits, F., Ragab, A., Ragab-Thomas, J.M.F. and Chap, H. (1995) EMBO. J 14, 473–483.
[26] Falet, H., Ramos-Morales, F., Bachelot, C., Fischer, S. and Rendu, F. (1996) FEBS Lett. 383, 165–169.
[27] Edmead, C.E., Crosby, D.A., Southcott, M. and Poole, A.W. (1999) FEBS Lett. 459, 27–32.
[28] Hua, C.T., Gamble, J.R., Vadas, M.A. and Jackson, D.E. (1998) J. Biol. Chem. 273, 28332–28340.
[29] Nadal, F., Levy-Toledano, S., Grelac, F., Caen, J.P., Rosa, J.P. and Bryckaert, M. (1997) J. Biol. Chem. 272, 22381–22384.
[30] Bugaud, F., Nadal-Wollbold, F., Lévy-Toledano, S., Rosa, J.P. and Bryckaert, M. (1999) Blood 94, 3800–3805.
[31] Hunter, T. (1995) Cell 80, 225–236.
[32] Moeslein, F.M., Myers, M.P. and Landreth, G.E. (1999) J. Biol. Chem. 274, 26697–26704.
[33] [33] Brautigan, D.L. and Pinault, F.M. (1993) Mol. Cell. Biochem. 121–129.
[34] Reszka, A.A., Seger, R., Diltz, C., Krebs, E.G. and Fischer, E.H. (1995) Proc. Natl. Acad. Sci. USA 92, 8881–8885.
[35] McNichol, A., Shibou, T.S., Pampolina, C. and Israels, S.J. (2001) Thromb. Res. 103, 25–34.