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Rapid Ca^{2+}-mediated activation of Rap1 in human platelets

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Rap1 is a small, Ras-like GTPase whose function and regulation are still largely unknown. We have developed a novel assay to monitor the active, GTP-bound form of Rap1 based on the differential affinity of Rap1GTP and Rap1GDP for the Rap binding domain of RalGDS (RBD). Stimulation of blood platelets with α-thrombin or other platelet activators caused a rapid and strong induction of Rap1 that associated with RBD *in vitro*. Binding to RBD increased from undetectable levels in resting platelets to >50% of total Rap1 within 30 s after stimulation. An increase in the intracellular Ca^{2+} concentration is both necessary and sufficient for Rap1 activation since it was induced by agents that increase intracellular Ca^{2+} and inhibited by a Ca^{2+}-chelating agent. Neither inhibition of translocation of Rap1 to the cytoskeleton nor inhibition of platelet aggregation affected thrombin-induced activation of Rap1. In contrast, prostaglandin I_2 (PGI_2), a strong negative regulator of platelet function, inhibited agonist-induced as well as Ca^{2+}-induced activation of Rap1. From our results, we conclude that Rap1 activation in platelets is an important common event in early agonist-induced signalling, and that this activation is mediated by an increased intracellular Ca^{2+} concentration.

Keywords: calcium/GTPase/platelet/Rap1/thrombin

Introduction

Rap1A and Rap1B are two members of the Ras family of small GTPases. Rap1A was first identified both by its homology to Ras and as the product of a cDNA that induces flat revertants of K-*ras*-transformed cells (K*rev*-1). Rap1B is a very close relative of Rap1A, differing in only nine amino acids predominantly in the C-terminal part of the protein (Noda, 1993). The functional difference between the two proteins is unclear and in most studies no discrimination between the two has been made. Various functions of Rap1 have been described, some of which are related to the ability of Rap1 to counteract the function of Ras. For instance, introduction of the active, GTP-bound form of Rap1 into fibroblasts inhibits Ras-mediated activation of MAP kinase (Cook *et al.*, 1993). In addition, constitutively active Rap1 can inhibit Ras-mediated induction of germinal vesicle breakdown in *Xenopus* oocytes (Campa *et al.*,1991). *In vitro* Rap1 binds regulators ($p120^{RasGAP}$; Frech *et al.*, 1990; Hata *et al.*, 1990) and effectors (Raf1, RalGDS; Spaargaren and Bischoff, 1994; Nassar *et al.*, 1995; Wittinghofer and Herrmann, 1995) of Ras, suggesting that the inhibition of Ras function is due to sequestration of Ras effectors by Rap1. Interestingly, cAMP, an inhibitor of Ras signalling in various cell types (Burgering *et al.*, 1993; Cook *et al.*, 1993; Wu *et al.*, 1993), has been reported to induce the activation of exogenously expressed epitope-tagged Rap1 in a Ras-transformed fibroblast (Altschuler *et al.*, 1995). However, not all effects of Rap1 point to an inhibition of Ras signalling and indicate that Rap1 has a separate function in various cell types. For instance, in Swiss 3T3 fibroblasts, constitutively active Rap1 induces DNA synthesis (Yoshida *et al.*, 1992). In neutrophils, Rap1 is found in association with the NADPH oxidase system (Quinn *et al.*, 1989), and introduction of Rap1 into neutrophil-like HL60 cells results in a stimulation of this oxidase (Gabig *et al.*, 1995). Also, in blood platelets, Rap1 may play an important role, since Rap1, in particular Rap1B, is highly expressed (Torti and Lapetina, 1994).

Platelets are anuclear cell fragments that adhere to sites of injury in a blood vessel and aggregate to stop bleeding. Adhesion and aggregation are accompanied by a profound change in the morphology of platelets due to remodelling of the actin-based cytoskeleton (Hartwig, 1992; Fox, 1993). Thrombin is the most potent stimulator of platelets, but a large number of other activators has been described (Kroll and Schafer, 1989; Siess, 1989). When platelets are activated, Rap1 is translocated to the platelet cytoskeleton (Fischer *et al.*, 1990, 1994). This translocation is a relatively slow process that depends on aggregation of platelets. In addition, it has been reported that Rap1 associates with $p120^{RasGAP}$ and $PLC\gamma_1$ after platelet stimulation (Torti and Lapetina, 1992). Also, prostaglandin I_2 (PGI_2), a strong negative regulator of platelet function, affects Rap1 by inducing the phosphorylation of a serine residue at the C-terminal end of Rap1 (Kawata *et al.*, 1989; Siess *et al.*, 1990). This phosphorylation induces a translocation of Rap1 from the plasma membrane to the cytosol (Lapetina *et al.*, 1989).

Despite all these results, it is still unclear what the function of Rap1 is and in which signal transduction pathway Rap1 is involved. This is partly due to the lack of information on signals that may activate endogenous Rap1, i.e. induce the conversion of the inactive GDP-bound form of the protein to the active GTP-bound form. Unfortunately, no antibodies are available that can immunoprecipitate Rap1 to measure the ratio of GTP:GDP bound to Rap1. Therefore, we have developed an alternative, indirect assay to measure Rap1 activation. This assay is based on the differential affinity of Rap1GTP versus Rap1GDP for the Rap binding domain of RalGDS

(RBD) as shown by Wittinghofer and co-workers (Herrmann *et al.*, 1996): whereas the Rap binding domain of RalGDS binds to the GTP-bound form of Rap1 with a very high affinity (K_D =10 nM), the affinity for the GDP-bound form is undetectably low. Here we report that in platelets α-thrombin stimulation results in a dramatic increase of Rap1 which can associate with RBD *in vitro*, indicating an increase of Rap1 in the GTP-bound state. We have investigated this activation further and found that most, if not all, activators of platelet function activate Rap1, whereas the negative regulator PGI_2 inhibits Rap1 activation. Furthermore, we show that an increase in the intracellular Ca^{2+} concentration is both necessary and sufficient to activate Rap1. Finally, we show that Rap1 activation occurs independently of, and probably prior to, platelet aggregation and the association of Rap1 with the cytoskeleton. From these results, we conclude that Rap1 is activated rapidly and strongly by Ca^{2+}-mediated signalling after platelet stimulation, suggesting a critical role for Rap1 in platelet activation.

Results

α-Thrombin induces rapid activation of Rap1

Freshly isolated human platelets were stimulated with α-thrombin for various time periods and lysed. Rap1 was precipitated with the polyhistidine-tagged Rap binding domain of RalGDS (RBD) bound to nickel beads and identified by Western blotting using a monoclonal antibody directed against Rap1. This antibody is specific for Rap1, and does not recognize Ras or R-ras (not shown). A rapid and strong increase in the amount of Rap1 that bound to RBD *in vitro* was observed (Figure 1A). This increase was visible within 5 s after thrombin addition and reached its maximum level at ~30 s. At this latter time point, >50% of total Rap1 could be precipitated with RBD (Figure 1B). Since RBD associates exclusively with the GTP-bound form of Rap1 *in vitro*, with no detectable affinity for the GDP-bound form of Rap1 (Herrmann *et al.*, 1996), we conclude that α-thrombin induces a rapid conversion of the majority of Rap1 to the GTP-bound, active state.

Treatment of platelets with agonists other than α-thrombin (Benton *et al.*, 1982; Kroll and Schafer, 1989; Siess, 1989; Inazu *et al.*, 1991) also caused activation of Rap1; the thrombin receptor-activating peptide (TRAP), collagen, the thromboxane A_2 (TxA_2) analogue U46619, ADP and platelet-activating factor (PAF) all induced rapid and strong Rap1 activation (Figure 2). Wheat germ agglutinin and lysophosphatidic acid (LPA) treatment also resulted in Rap1 activation (data not shown). From these results, we conclude that Rap1 is activated rapidly and strongly by signalling events common to most, if not all platelet agonists.

Rap1 activation occurs independently of secretion and platelet aggregation

During thrombin-induced platelet activation, TxA_2 and ADP are released by platelets. Both act in positive feedback loops to enhance further the ongoing platelet activation (Kroll and Schafer, 1989; Siess, 1989). As TxA_2 and ADP induce Rap1 activation, we investigated whether thrombin-induced Rap1 activation is a consequence of these processes. Treatment of platelets with indomethacin inhibits TxA_2 formation (Shen and Winter, 1977); addition of phosphoenolpyruvate and pyruvate kinase (PEP:PK) scavenges ADP, thus inhibiting ADP signalling (Ammit and O'Neill, 1991; Van Willigen *et al.*, 1996). Addition of both inhibitors together, at concentrations causing complete inhibition of both pathways, resulted in only a partial inhibition of Rap1 activation (Figure 3). From these results, we conclude that although both TxA_2 and ADP do contribute to thrombin-induced activation of Rap1, they are not essential for thrombin to induce Rap1.

Previously, it was shown that Rap1 translocates to the platelet cytoskeleton in a mainly aggregation-dependent manner (Fischer *et al.*, 1994). We therefore investigated the effects of disruption of the cytoskeleton and inhibition of platelet aggregation on thrombin-induced Rap1 activ-

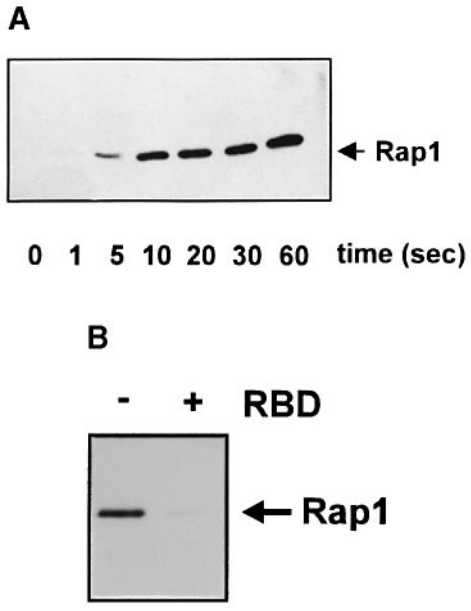

Fig. 1. α-Thrombin stimulation of blood platelets results in increased binding of Rap1 to RBD *in vitro*. (**A**) Platelets were stimulated with 0.25 U/ml α-thrombin for the indicated time periods and lysed. Rap1 was precipitated with his-tagged RBD bound to Ni^{2+}-NTA–agarose beads, and identified with a monoclonal antibody directed against Rap1 after SDS–PAGE and Western blotting. (**B**) Platelet lysate was incubated with either Ni^{2+}-NTA–agarose beads (–) or his-tagged RBD bound to Ni^{2+}-NTA–agarose beads (+). After removal of the beads, the supernatant was separated by SDS–PAGE, and Rap1 was detected by immunoblotting with a Rap1-specific monoclonal antibody.

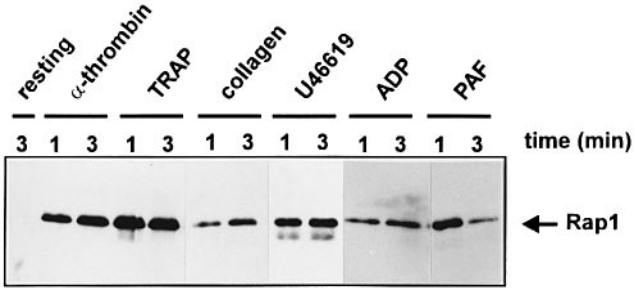

Fig. 2. Rap1 activation is induced by different platelet agonists. Platelets were stimulated for 1 and 3 min with α-thrombin (0.1 U/ml), TRAP (10 μM), collagen (5 μg/ml), the thromboxane A_2 analogue U46619 (1 μM), ADP (10 μM) and PAF (200 nM). Resting platelets were treated with buffer for 3 min. Rap1 was isolated and identified as indicated in the legend to Figure 1.

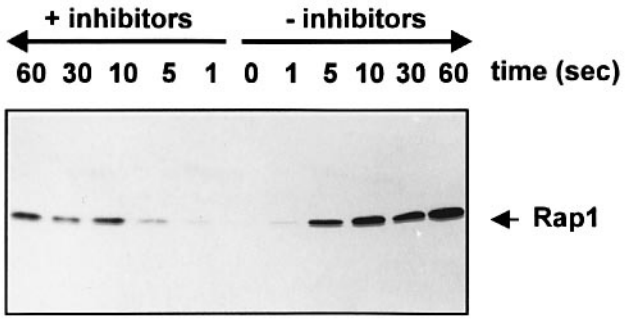

Fig. 3. Rap1 activation occurs independently of both TxA_2 formation and ADP secretion. In the lanes marked '+ inhibitors' platelets were incubated with 30 μM indomethacin for 10 min to inhibit TxA_2 formation. In the last minute, the ADP scavenger system phosphoenolpyruvate:pyruvate kinase (PEP:PK) was added at a final concentration of 0.28 mM PEP and 3 U/ml PK. Subsequently, the platelets were incubated with 0.1 U/ml α-thrombin for the indicated time periods, and Rap1 was isolated and detected as indicated in the legend to Figure 1. Control platelets (– inhibitors) were treated with 0.1 U/ml α-thrombin, only.

ation. Treatment of platelets with cytochalasin D prevents activation-dependent actin polymerization and remodelling of the cytoskeleton (Dash *et al.*, 1995). This treatment totally prevented the translocation of Rap1 to the cytoskeleton (Figure 4A). Cytochalasin D, however, did not affect thrombin-induced activation of Rap1 (Figure 4B), showing that Rap1 activation occurs independently of translocation to the cytoskeleton. Furthermore, in platelets that were not allowed to aggregate by the addition of the RGDS peptide, which blocks integrin function (Hynes, 1992; Calvete, 1994), Rap1 activation was not impaired (Figure 5). Also, inhibition of aggregation by not stirring the platelet suspension did not affect α-thrombin-induced activation of Rap1 (data not shown). From these results, we conclude that Rap1 activation occurs independently of Rap1 translocation to the cytoskeleton and of platelet aggregation.

Increase in intracellular Ca^{2+} is necessary and sufficient for Rap1 activation

One of the signalling events shared by platelet agonists is the activation of phospholipase C (PLC), which releases diacylglycerol (DAG) to activate protein kinase C (PKC), and inositol-1,4,5-triphosphate ($InsP_3$) to mobilize Ca^{2+} from intracellular stores (Kroll and Schafer, 1989; Siess, 1989). Figure 6A demonstrates that inhibition of PLC by U73122 (Okamoto *et al.*, 1995) strongly reduced thrombin-induced activation of Rap1, suggesting that either PKC or Ca^{2+} is an important factor in Rap1 regulation. We therefore tested whether an increase in the intracellular Ca^{2+} concentration is important for the activation of Rap1. Inhibition of the cytosolic Ca^{2+} increase by chelation of intracellular Ca^{2+} with BAPTA-AM (Watson *et al.*, 1995) completely blocked activation of Rap1 by α-thrombin. On the other hand, increasing the concentration of intracellular Ca^{2+} with ionomycin (Cavallini and Alexandre, 1994; Doni *et al.*, 1994) or thapsigargin (Authi *et al.*, 1993; Cavallini *et al.*, 1995) strongly activated Rap1 (Figure 6A). This indicates that an increased level of Ca^{2+} is both necessary and sufficient to activate Rap1. The Ca^{2+} effect is not due simply to Ca^{2+}-induced production and release

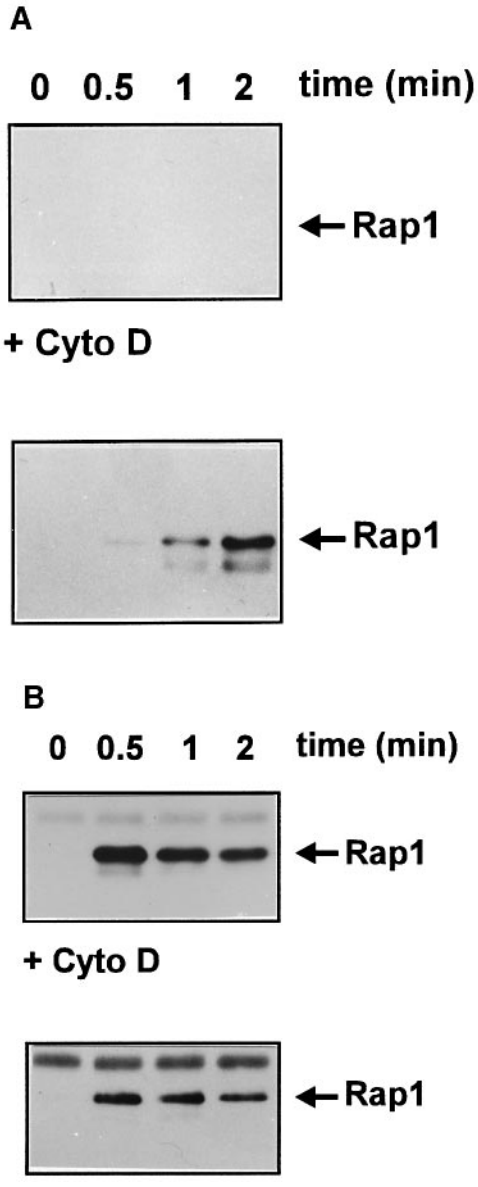

Fig. 4. Inhibition of actin polymerization prevents Rap1 translocation but does not affect thrombin-induced activation of Rap1. Platelets were treated with cytochalasin D (Cyto D, 5 μg/ml) (top) or DMSO (bottom) 5 min prior to stimulation with 0.2 U/ml α-thrombin for the indicated time periods under aggregating conditions (stirring). (**A**) Cytoskeletal proteins were isolated as described in Materials and methods. Amounts of protein representing 3×10^7 platelets were used for protein immunoblot analysis with a monoclonal antibody directed against Rap1. (**B**) Rap1 was precipitated with RBD and analysed as indicated in the legend of Figure 1. The band present above Rap1 is caused by aspecific binding to the Ni^{2+}-NTA–agarose beads.

of TxA_2 or secretion of ADP, since inhibitors of both signalling events, indomethacin and PEP:PK, caused only a slight reduction of Ca^{2+}-induced Rap1 activation (Figure 6A). Moreover, this reduction is probably due to the slightly lower Ca^{2+} level induced by thapsigargin and ionomycin in the presence of indomethacin and PEP:PK (data not shown).

Inhibition of PKC by several inhibitors, i.e. staurosporin, bisindolylmaleimid and calphostin C (Tamaoki, 1991; Toullec *et al.*, 1991), did not at all or only marginally affect the activation of Rap1 by α-thrombin (Figure 6B). Consistent with this, activation of PKC by the phorbol

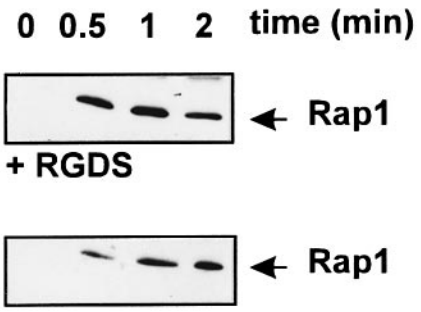

Fig. 5. Inhibition of platelet aggregation does not affect thrombin-induced activation of Rap1. Platelets were treated with 0.1 U/ml α-thrombin under aggregating conditions for the indicated time periods in the presence (+RGDS) or absence of 100 μM of the aggregation-inhibitory peptide RGDS added 1 min prior to activation. Rap1 was precipitated with RBD and analysed as indicated in the legend to Figure 1.

ester phorbol 12-myristate 13-acetate (PMA) (Kroll and Schafer, 1989; Siess, 1989) activated Rap1 only slightly and after a prolonged period of time (Figure 6B). From these results, we conclude that Rap1 activation is mediated by an increase in intracellular Ca^{2+}, which is both necessary and sufficient, whereas activation of PKC does not play an essential role.

Prostaglandin I_2 (PG I_2) inhibits thrombin- and Ca^{2+}-induced Rap1 activation

PGI_2, a potent inducer of cAMP production, is a strong antagonist of platelet activation (Kroll and Schafer, 1989; Siess, 1989). We therefore tested the effect of PGI_2 on thrombin-induced activation of Rap1. Figure 7A shows that addition of PGI_2 inhibited thrombin-induced activation of Rap1 completely. Furthermore, when platelets were first treated with thrombin and subsequently with PGI_2, the initial activation of Rap1 was followed by a rapid down-regulation (Figure 7B). Interestingly, the activity of Rap1 (again) correlated with the level of intracellular Ca^{2+} (Figure 7C). However, Ca^{2+}-induced activation of Rap1 by thapsigargin and ionomycin was also fully blocked by PGI_2 (Figure 7D), even though the intracellular Ca^{2+} levels induced by thapsigargin or ionomycin were still high in the presence of PGI_2 (data not shown; Siess and Lapetina, 1989; Nakamura *et al.*, 1995). These results show that Rap1 activity is under tight negative control of PGI_2, which acts both upstream and downstream of Ca^{2+}.

PGI_2 activates adenylate cyclase resulting in an increase in the cAMP level. It is intriguing that Rap1 is an *in vivo* substrate for cAMP-dependent protein kinase A (PKA) (Kawata *et al.*, 1989; Siess *et al.*, 1990). This phosphorylation, which results in a lower electrophoretic mobility of Rap1 (Siess *et al.*, 1990; Siess and Grünberg, 1993), occurs only slowly and was still very limited 2 min after PGI_2 treatment of the platelets (Figure 8A; Grünberg *et al.*, 1995). At this time point, however, PGI_2 had already completely inhibited Rap1 activity (see Figure 7A). Furthermore, α-thrombin was able to activate phosphorylated Rap1 apparently normally. This was shown by pre-treating platelets with PGI_2 for various times, followed by thrombin stimulation: whereas platelets became fully responsive to thrombin stimulation within 60 min after PGI_2 addition (due to the instability of PGI_2), Rap1 phosphorylation remained high (Figure 8A; Siess and Grünberg, 1993; Grünberg *et al.*, 1995). Under these conditions, thrombin activated the phosphorylated, shifted form of Rap1 (Figure 8B). We conclude, therefore, that the PGI_2-induced inhibition of Rap1 activation is not caused by phosphorylation of the GTPase.

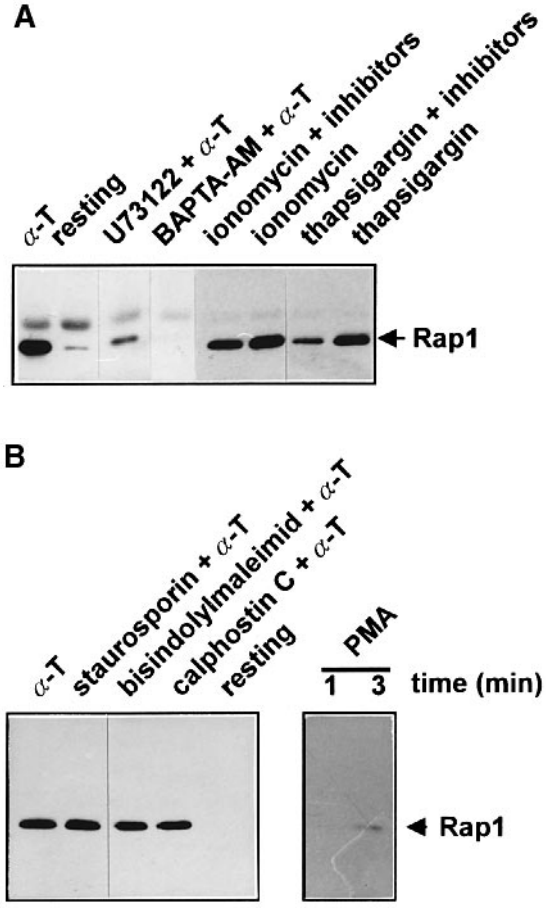

Fig. 6. Role of PLC, Ca^{2+} and PKC in α-thrombin-induced Rap1 activation. (**A**) U73122 and BAPTA-AM inhibit Rap1 activation, whereas ionomycin and thapsigargin stimulate it. Platelets were incubated with either the PLC inhibitor U73122 (1 μM, 3 min) or the intracellular Ca^{2+} chelator BAPTA-AM (30 μM, 30 min) prior to 1 min of thrombin (0.1 U/ml) stimulation. In addition, platelets were activated in the absence of thrombin with either ionomycin (100 nM) + $CaCl_2$ (1 mM) or thapsigargin (100 nM), both for 1 min. In the lanes labelled 'ionomycin + inhibitors' and 'thapsigargin + inhibitors' platelets were pre-treated with inhibitors of TxA_2 production and ADP signalling (as indicated in the legend to Figure 2) prior to 1 min of treatment with either ionomycin + $CaCl_2$ or thapsigargin. Rap1 was precipitated with RBD and analysed as indicated in the legend to Figure 1. The band present above Rap1 on the blot is caused by aspecific binding of protein to the Ni^{2+}-NTA–agarose beads. (**B**) Inhibition or activation of PKC do not significantly affect Rap1 activity. Platelets were incubated with the PKC inhibitors staurosporin (1 μM, 5 min), bisindolylmaleimid (5 μM, 1 min), calphostin C (5 μM, 5 min) prior to a 1 min thrombin stimulation (0.1 U/ml) (left panel). In the right panel, platelets were stimulated with the PKC-activating phorbol ester PMA (10 nM) for 1 and 3 min. Rap1 was precipitated with RBD and analysed as indicated in the legend to Figure 1.

Discussion

We have developed a novel assay to identify the active GTP-bound state of Rap1. This assay is based on the observation that the GTP-bound form of Rap1 associates

with the RBD of RalGDS with high affinity *in vitro*, whereas no interaction can be detected with the GDP-bound form of Rap1. When we incubated cell lysates of resting and α-thrombin-stimulated human blood platelets with polyhistidine-tagged RBD bound to Ni^{2+}-NTA–agarose beads, we observed a large amount of Rap1 associated with RBD only in stimulated platelets. Based on the *in vitro* binding affinity, we conclude that this increase is due to an induction of the GTP-bound form of Rap1 by α-thrombin. Alternatively, Rap1 might be constitutively GTP-bound but complexed to a factor in resting platelets that prevents it from associating with RBD. Stimulation with thrombin would then lead to dissociation of this factor, rendering Rap1 available for RBD binding. To discriminate between the two possibilities, the ratio of GDP and GTP bound to Rap1 in platelets needs to be measured. Unfortunately, suitable antisera for Rap1 immunoprecipitation, which are essential for such an experiment, are still not available. Irrespective of this, in functional terms there may be no difference between the two mechanisms, as both result in free GTP-bound, active Rap1.

The assay was used to monitor the activation of Rap1 in platelets. We observed that all agonists tested that activate platelets also activate Rap1. This could imply that Rap1 is either involved in a signalling pathway common to all of these agonists or that Rap1 activation is a secondary event, induced by positive feedback loops (TxA_2 release and ADP secretion) or by platelet aggregation, for instance. However, inhibition of both TxA_2 production and ADP signalling as well as aggregation did not affect α-thrombin-induced activation of Rap1 significantly. This strongly suggests that Rap1 activation is one of the early events in platelet activation. The observed translocation of Rap1 to the cytoskeleton is not involved in the activation of Rap1, since it occurs much later than Rap1 activation. Furthermore, inhibition of actin polymerization by cytochalasin D, which totally blocked Rap1 association with the cytoskeleton, did not inhibit Rap1 activation. Therefore, we conclude that Rap1 is involved in a signalling pathway common to all agonists tested. A common denominator for all these agonists is the increase in intracellular Ca^{2+} by mobilization of Ca^{2+} from internal stores and by influx of extracellular Ca^{2+}. Indeed, we found that increasing the concentration of intracellular Ca^{2+} with either ionomycin or thapsigargin induced Rap1

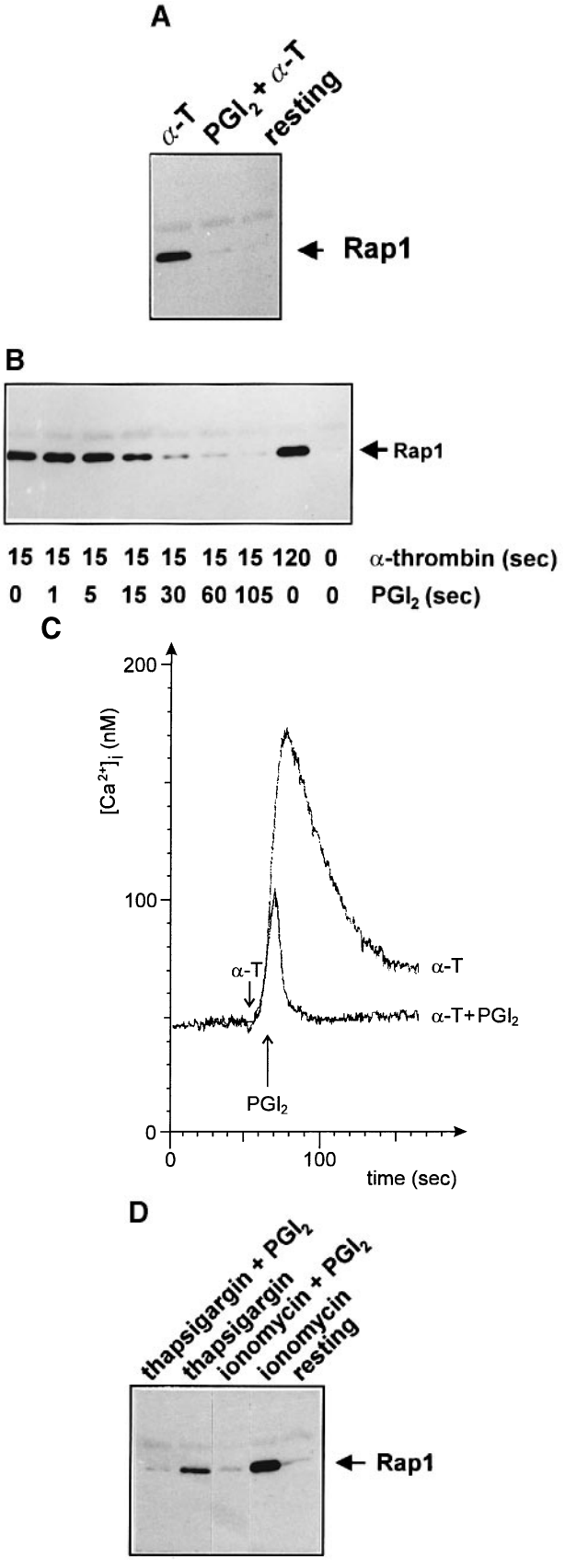

Fig. 7. Effects of PGI_2 treatment of platelets on Rap1 activation and cytosolic Ca^{2+} levels. (**A**) PGI_2 pre-treatment prevents Rap1 activation by α-thrombin. Platelets were treated with thrombin (α-T, 0.1 U/ml), in the presence or absence of PGI_2 added 2 min earlier at 10 ng/ml. Rap1 was precipitated with RBD and analysed as indicated in the legend to Figure 1. (**B**) PGI_2 treatment of α-thrombin-activated platelets results in a time-dependent reduction of Rap1 activity. Platelets were treated with α-thrombin (0.1 U/ml) for 15 s to activate Rap1. PGI_2 (10 ng/ml) was then added for the indicated times. Controls were either left untreated or were activated with α-thrombin for 120 s to serve as a positive control for the activity state of Rap1 at the end of the experiment. Rap1 was precipitated with RBD and analysed as indicated in the legend to Figure 1. (**C**) PGI_2 reverses the α-thrombin-induced rise in the cytosolic Ca^{2+} level. The intracellular Ca^{2+} level was monitored in Fura-2-loaded platelets as described in Materials and methods. α-Thrombin (0.1 U/ml) was added at the indicated time point (α-T), 15 s later PGI_2 (10 ng/ml) was added (indicated with PGI_2) and incubation was continued for another 90 s. (**D**) PGI_2 pre-treatment of platelets prevents Rap1 activation by the cytosolic Ca^{2+}-elevating agents thapsigargin and ionomycin. Platelets were incubated either with or without PGI_2 (10 ng/ml) for 2 min. Subsequently, either ionomycin (100 nM) + $CaCl_2$ (1 mM) or thapsigargin (100 nM) was added for 1 min. Rap1 was precipitated with RBD and analysed as indicated in the legend to Figure 1.

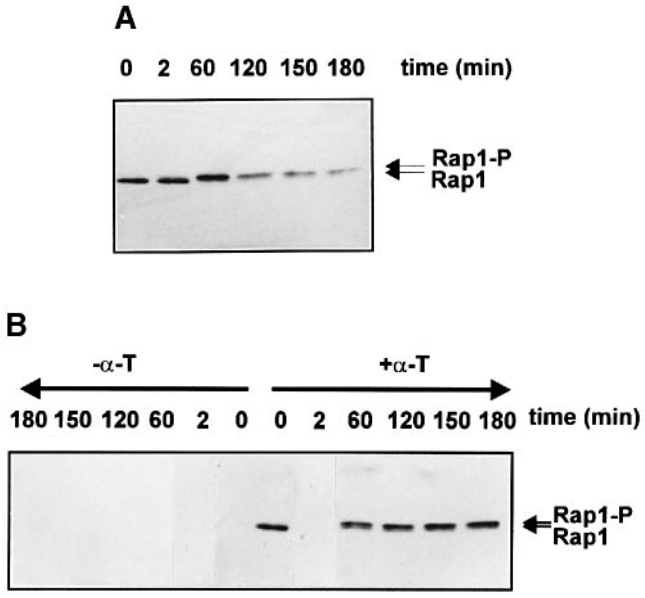

Fig. 8. PGI_2-induced phosphorylation of Rap1 does not affect Rap1 activation. (**A**) PGI_2 induces a mobility shift of Rap1. Total lysates of platelets treated with PGI_2 (20 ng/ml) for the indicated time periods were separated and analysed by protein immunoblot analysis for the presence of phosphorylated Rap1. The phosphorylated form of Rap1 shows decreased electrophoretic mobility during SDS–PAGE and can therefore be detected as a second Rap1 band on the immunoblot, indicated by Rap1-P. (**B**) α-Thrombin induces activation of phosphorylated Rap1. Platelets were treated with PGI_2 (20 ng/ml) for the indicated time periods. Subsequently, thrombin was added and the incubation was continued for another minute, followed by cell lysis (+α-T). As a control, platelets incubated with PGI_2 were not incubated with thrombin (–α-T). Rap1 was precipitated with RBD and analysed as indicated in the legend to Figure 1. Rap1-P indicates the slower migrating, phosphorylated form of Rap1. It should be noted that due to the instability of PGI_2 its inhibitory effect on Rap1 activation by α-thrombin is only seen 2 min after PGI_2 addition to the platelets. At all later time points, PGI_2 has been broken down and platelets have restored responsiveness to α-thrombin (Siess and Lapetina, 1990; Siess and Grünberg, 1993).

activation. In addition, BAPTA-AM, which chelates intracellular Ca^{2+}, inhibited thrombin-induced Rap1 activation. Apparently, Rap1 activation is mediated by a rise in intracellular Ca^{2+}. The mechanism by which the Ca^{2+} increase results in Rap1 activation, however, is unclear and currently under investigation.

PGI_2, which activates adenylate cyclase to increase the levels of cAMP in platelets, inhibits thrombin-induced activation of Rap1. This is in agreement with the observation that PGI_2 inhibited thrombin-induced increases in intracellular Ca^{2+}. However, thapsigargin- or ionomycin-induced activation of Rap1 was also inhibited by PGI_2, indicating that the antagonist also affects a factor downstream of Ca^{2+}. Although Rap1 is phosphorylated after PGI_2 treatment of platelets, it is unlikely that this phosphorylation causes the inhibition. We conclude this from the observation that (i) PGI_2-induced phosphorylation of Rap1 was a much later event than inhibition of Rap1, and (ii) thrombin could activate the phosphorylated form of Rap1. It should be noted here that the detection of the phosphorylated form of Rap1 by a mobility shift is an accurate method, as it has been shown by others that PGI_2-induced phosphorylation invariantly is accompanied by the shift in mobility (Siess *et al.*, 1990). Another candidate for mediating the PGI_2 inhibition is Rap1GAP. Phosphorylation of this protein is induced by elevated levels of cAMP, at least in insect cells and the SK-MEL-3 cell line (Polakis *et al.*, 1992; Rubinfeld *et al.*, 1992). Whether this occurs in platelets and whether it affects GAP activity is still unknown. It should be noted that phosphorylation of Rap1 correlates with the PGI_2-induced translocation of Rap1 from the plasma membrane to the cytosol (Lapetina *et al.*, 1989). This translocation may be another form of inactivation by PGI_2, which clearly interferes at a different level of Rap1-mediated signalling.

From the rapid activation of a major fraction of Rap1 and the stringent control of Rap1 activity by PGI_2, we anticipate that Rap1 plays a critical role in agonist-induced, calcium-mediated events in platelet activation. Interestingly, Ca^{2+}-induced signalling is involved in the activation of integrin αIIbβ3, resulting in the exposure of binding sites for fibrinogen and, subsequently, platelet aggregation (Kroll and Schafer, 1989; Siess, 1989). It is tempting, therefore, to speculate that Rap1 is involved in this process. Support for this possibility comes from the recent finding that R-ras, a close relative of Rap1, is involved in the activation of integrins in various cell lines (Zhang *et al.*, 1996). A function of Rap1 in the activation of integrins is not incompatible with the flat revertant phenotype of K*rev*-1/Rap1 in transformed fibroblasts.

Materials and methods

Production of his-tagged RalGDS-RBD

The cDNA encoding the 97 amino acids spanning RBD was isolated from pGEX-RGF97 (Herrmann *et al.*, 1996) as a *Bam*HI–*Xho*I fragment. The *Bam*HI site was blunted with Klenow DNA polymerase. Subsequently, the fragment was inserted into the pET-15b vector (Novagen) digested with *Nde*I (and blunted with Klenow DNA polymerase) and *Xho*I. The construct was transformed into *Escherichia coli* (strain BL21). Protein production was initiated by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to the culture. The fusion protein was affinity purified on a Ni^{2+}-NTA–agarose column (Qiagen) from the supernatant of bacteria lysed by sonication and freeze–thaw cycles in a sucrose-containing buffer.

Isolation and stimulation of platelets

Freshly drawn venous blood from healthy volunteers (with informed consent) was collected into trisodium citrate (0.1 vol. of 130 mM trisodium citrate). The donors claimed not to have taken any medication during the previous 10 days. The blood was centrifuged at 200 *g* for 15 min at room temperature to yield platelet-rich plasma (PRP). Then, 0.1 vol. of ACD (2.5% trisodium citrate, 1.5% citric acid, 2% D-glucose) was added to the PRP to lower the pH of the plasma to 6.5 and thus prevent platelet activation during further isolation. Platelets were purified from PRP by centrifugation at 700 *g* for 15 min at room temperature. The platelet pellet was resuspended in HEPES/Tyrode buffer (10 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 11.9 mM $NaHCO_3$, pH 7.4) containing 5 mM D-glucose at 2×10^8 platelets/ml. Platelets were left at room temperature for at least 30 min to ensure a resting state. Samples of 0.5 ml were used for the experiments. Purified platelets were incubated in a lumiaggregometer (CHRONO-LOG corporation) at 37°C. In the standard assay, incubation with agonists was without stirring. Without stirring, platelets only change their shape but do not aggregate, whereas in a stirred suspension (900 r.p.m.) platelet shape change and aggregation occur. Platelet agonists used in the study are: α-thrombin which was added to the platelets for 1 min at a final concentration of 0.1, 0.2 or 0.25 U/ml (as indicated), TRAP (6mer: SFLLRN) (10 μM), collagen (5 μg/ml), U46619 (TxA_2 analogue) (1 μM), ADP (10 μM) and PAF (200 nM). PMA was added to the platelets at a final concentration of 10 nM, thapsigargin, an inhibitor of intracellular Ca^{2+} ATPases, was used at 100 nM concentration as was ionomycin, a Ca^{2+} ionophore. In the

case of ionomycin, 1 mM $CaCl_2$ was added to the platelet suspension just prior to stimulation. PGI_2 was used at a concentration of 10 or 20 ng/ml and incubated with the platelets for 2 min (unless indicated otherwise). The PLC inhibitor U73122 (1 μM) was present during 3 min pre-incubation. The inactive component U73343 used as control had no effect on Rap1 activity (data not shown). BAPTA-AM (30 μM), an intracellular Ca^{2+} chelator, was pre-incubated with the platelet suspension for 30 min. Indomethacin (30 μM) was added to the platelets for 10 min to inhibit TxA_2 formation. The ADP scavenger PEP:PK was freshly prepared prior to every experiment and added to the platelets at a final concentration of 0.28 mM PEP and 3 U/ml PK 1 min before stimulation. PKC inhibitors were used as follows: staurosporin (1 μM), 5 min incubation, bisindolylmaleimid (5 μM), 1 min incubation, calphostin C (5 μM), 5 min incubation. Cytochalasin D was added to the platelet suspension at a concentration of 5 μg/ml 5 min prior to platelet stimulation. The peptide RGDS was used at a concentration of 100 μM and was added to the platelets 1 min prior to stimulation. RGDS binds to the ligand binding site of the platelet integrin αIIbβ3. It inhibits binding of fibrinogen to the integrin and therefore blocks platelet aggregation. RGDS does not activate outside-in signalling of integrin αIIbβ3 (Hynes, 1992; Calvete, 1994).

Rap1 activation assay using RalGDS-RBD

Platelets were lysed by addition of 1 vol. of cold 2× RIPA lysis buffer [150 mM NaCl, 100 mM Tris–HCl pH 7.4, 2% NP-40, 1% deoxycholic acid (DOC), 0.2% SDS, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM leupeptin, 2 μM aprotinin] to the platelet suspension. Lysis was performed at 4°C for 10–30 min. Lysates were clarified by centrifugation at maximal speed in an Eppendorf centrifuge for 10 min at 4°C. Five μg of RalGDS-RBD coupled to Ni^{2+}-NTA–agarose beads (Qiagen) were added to the supernatant and incubated at 4°C for 30–90 min with slight agitation. Beads were washed four times in 1× RIPA. After the final wash, Laemmli sample buffer was added to the samples. Next, proteins were fractionated by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The antibody used specifically to detect Rap1 was a monoclonal antibody directed against Rap1 (Transduction Laboratories). Immune complexes were detected by enhanced chemiluminescence (Amersham). All experiments shown here were performed at least three times with the same result to exclude donor-specific effects.

Calcium measurements

PRP was prepared as described above. ACD was added and platelets were incubated with 3 μM Fura-2-AM for 45 min at 37°C. Surplus Fura-2-AM and plasma were removed by gel filtration over a Sepharose 2B column equilibrated in HEPES/Tyrode buffer. Measurement of the cytosolic Ca^{2+} concentration was performed using a Hitachi F4500 fluorescence spectrophotometer by a dual wavelength program (excitation was measured at 340 nm and 380 nm, emission at 510 nm).

Cell fractionation

Samples of 5×10^8 platelets/ml were lysed in cold 2× CSK buffer (100 mM Tris–HCl, 20 mM EGTA, 2% Triton X-100, 2 mM sodium orthovanadate, 2 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin) after stimulation. Lysis was for at least 10 min at 4°C. Lysates were centrifuged at maximal speed in an Eppendorf centrifuge for 10 min at 4°C. The pellet containing the actin cytoskeleton of the lysed platelets was washed once with 1× CSK buffer and centrifuged as before. Laemmli sample buffer was added to the pellet. Amounts of protein representing equal numbers of platelets were used for SDS–PAGE.

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