Integrin-dependent translocation of p160^{ROCK} to cytoskeletal complex in thrombin-stimulated human platelets

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p160^{ROCK} is a protein serine/threonine kinase that binds to GTP-Rho and is activated by this binding. We have recently found that the expression of p160^{ROCK} induces focal adhesions and stress fibres in HeLa cells, whereas a dominant-negative form of this kinase suppresses Rho-induced formation of these structures, suggesting that this kinase is a downstream target of Rho in this process [Ishizaki, Naito, Fujisawa, Maekawa, Watanabe, Saito and Narumiya (1997) FEBS Lett. **404**, 118–124]. To find out the mode of action of p160^{ROCK}, we developed immunoblotting with an anti-p160^{ROCK} antibody and investigated the subcellular localization of p160^{ROCK} during platelet aggregation. In resting human platelets, more than 90 % of p160^{ROCK} was present in the Triton X-100-soluble fraction. When platelets were stimulated with thrombin, approx. 10 % of p160^{ROCK} was translocated to the Triton X-100-insoluble fraction. This translocation was detected as early as 20 s after stimulation and reached a maximum at 5 min; it was suppressed by the addition of EDTA or an Arg-Gly-Asp-Ser peptide (RGDS), both of which inhibit integrin αIIbβ3-mediated platelet aggregation. Using [^{32}P]P_i-loaded platelets, we found that p160^{ROCK} was phosphorylated in response to stimulation by thrombin. This phosphorylation, however, was not affected by the addition of EDTA and RGDS. These results suggest that p160^{ROCK} translocates to cytoskeleton in a manner dependent on integrin ligation and works in an early stage of cytoskeletal reorganization in thrombin-stimulated platelets.

INTRODUCTION

The adhesion of cell to substratum is a process mediated by cell-surface integrins ligated with extracellular matrix proteins such as fibronectin and fibrinogen [1]. Ligated integrins are clustered and bind to many cytoskeletal proteins and signalling molecules to form a complex called focal adhesions, to which thick actin bundles named stress fibres are bound. These processes are evoked by the concerted actions of the intracellular signalling pathway and the interaction between integrin and matrix protein. The small GTPase Rho works as a molecular switch in the intracellular pathway leading to integrin activation and formation of focal adhesions and stress fibres [2]. Similar integrin-mediated adhesion is observed in cell–cell adhesion such as the adhesion of lymphocytes to endothelial cells and the aggregation of blood platelets. The former adhesion is performed by lymphocyte integrin, LFA-1, binding to the intercellular adhesion molecule (ICAM) on the endothelial cell surface, and the latter reaction is mediated by platelet integrin αIIbβ3 binding to soluble ligands such as fibrinogen, thus linking platelets to each other. Both of these adhesion processes, like the adhesion of fibroblasts to substrate, are mediated by Rho GTPase [3,4]. The molecular mechanism of this Rho action was not known until recently because of a lack of knowledge on downstream effectors of Rho. Using an overlay assay with guanosine 5′[γ-[^{35}S]thio]triphosphate–Rho, we isolated a novel serine/threonine protein kinase as a potential Rho target from human blood platelets, and cloned its cDNA [5]. This kinase, named p160^{ROCK}, consists of multiple domains, a kinase domain in the N-terminus followed by a long coiled-coil region in the middle, then a pleckstrin homology region and a Cys-rich zinc finger at the C-terminus. The Rho-binding domain localizes at the C-terminal end of the coiled-coil structure; replacement of Ile-1009 by Ala in this region abolished Rho binding [6]. We recently found that introduction of wild-type and mutants of p160^{ROCK} induced focal complexes and stress fibres in HeLa cells [7]. Moreover, Val^{14}-Rho-induced formation of focal adhesions and stress fibres was inhibited by coexpression with dominant-negative p160^{ROCK}, which is defective in both kinase and Rho-binding activities. These results indicate that p160^{ROCK} works downstream of Rho and is involved in integrin-mediated cell adhesion. However, how and where in the cell p160^{ROCK} exerts its action remains unknown, although there has been a report suggesting membrane translocation of ROKα, a p160^{ROCK} isoenzyme, on cell stimulation [8]. In the present study we used human blood platelets as an assay system for evaluating a change in the intracellular localization of p160^{ROCK} on cell activation, particularly in relation to reorganized cytoskeleton. Platelets seem a good model because they undergo reorganization of cytoskeletal elements in response to various stimuli, resulting in an aggregation reaction [9]. The platelet cytoskeleton consists of two actin-based structures. One is called the membrane skeleton. It coats the inner surface of the platelet membrane and is associated with the major platelet integrin αIIbβ3. The other is composed of cytoplasmic actin filaments that are mainly involved in contractile events. This cytoskeletal reorganization can be conveniently analysed by simple cell fractionation techniques; the filamentous actin and associated proteins, called the cytoskeletal fraction, are sedimented from Triton X-100 extracts by low-speed centrifugation, whereas the membrane skeleton can be separated from the soluble fraction by centrifugation at 100000 ***g***. It has been reported that many actin-binding proteins as well as signalling molecules involved in cell adhesion are redistributed to the cytoskeletal fraction during platelet aggregation [10]. These

Abbreviation used: RGDS, Arg-Gly-Asp-Ser peptide.

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results suggest that this fraction represents not only cytoskeletal apparatus but also a part of the signal transduction machinery that is related to cell-adhesive events.

Here we demonstrate, by immunoblotting with an antibody against p160^{ROCK}, that a part of p160^{ROCK} is translocated to the cytoskeletal fraction in aggregating platelets. This association of p160^{ROCK} with the cytoskeleton is mediated by αIIbβ3 ligand occupancy. In contrast, p160^{ROCK} is phosphorylated by thrombin stimulation in an aggregation-independent manner.

EXPERIMENTAL

Materials

[^{32}P]P_i (8500 Ci/mmol) was purchased from Dupont–New England Nuclear. ^{125}I-labelled protein A (100 mCi/mg) and ^{125}I-labelled protein G (15 mCi/mg) were obtained from ICN Biochemicals. Human thrombin, hirudin, Arg-Gly-Asp-Ser (RGDS) and apyrase were from Sigma. (15*S*)Hydroxy-11α,9α-(epoxymethano)prosta-(5*Z*,13*E*)dienoic acid (U-46619) was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). EDTA and indomethacin were obtained from Nakarai Tesque (Kyoto, Japan). Calyculin A was from Wako (Osaka, Japan). Protein A–Sepharose 4LB was purchased from Pharmacia Biotech. Anti-peptide antibodies against the N-terminal portions of p160^{ROCK} were raised by Research Genetics as described previously [5]. Antiserum 20 490 was raised against acetylated Ser^{2}-Thr-Gly-Asp-Ser-Phe-Glu-Thr-Arg-Phe-Glu-Lys-Met-Asp^{15}-Cys and antiserum 20 486 was raised against Met^{14}-Asp-Asn-Leu-Leu-Arg-Asp-Pro-Lys-Ser-Glu-Val-Asn-Ser-Asp^{28}-Cys. Monoclonal JK-5 anti-Rho GDI antibody was a gift from Y. Takai (Osaka University, Osaka, Japan). A buffy coat fraction of human blood was kindly supplied by Kyoto Red Cross Blood Center.

Preparation and activation of washed human platelets

Venous blood collected from healthy adult volunteers, or a buffy coat fraction, was mixed with 1/10 vol. of acid/citrate/dextrose and centrifuged at 130 ***g*** for 10 min to obtain platelet-rich plasma. Prostaglandin I_2 was then added at 1 μM to this fraction and the mixture was centrifuged at 1000 ***g*** for 10 min. The supernatant was decanted and platelet pellets were resuspended in 5 ml of citrated saline [13 mM sodium citrate/140 mM NaCl/30 mM glucose (pH 7.0)] containing 1 unit/ml apyrase. The suspension was again centrifuged at 1000 ***g*** for 10 min; platelets were finally suspended in Tyrode's/Hepes buffer containing 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM Na_2HPO_4, 5.6 mM glucose and 10 mM Hepes/NaOH, pH 7.4. The density of platelets was adjusted to $(1–2)\times10^9$ platelets/ml (washed platelets). Washed platelets were stirred in an aggregometer at 37 °C and activated by the addition of 1 unit/ml thrombin unless otherwise stated. In some experiments, washed platelets were preincubated for 3 min at 37 °C with 1 mM RGDS, 10 mM EDTA or 10 μM indomethacin. Treatment with prostaglandin I_2 during the preparation of washed platelets did not affect either the subsequent analysis or the results obtained, because essentially the same results were obtained with platelets prepared without this treatment.

Solubilization of platelets and fractionaiton of platelet lysates

Washed platelets were lysed with an equal volume of 2 × lysis buffer [2 % (v/v) Triton X-100/100 mM Tris/HCl (pH 7.4)/10 mM EGTA/2 mM 2-mercaptoethanol/2 mM PMSF/20 μg/ml leupeptin/100 mM benzamidine]. The cytoskeletal fraction was sedimented by centrifugation of the lysate at 8000 ***g*** for 10 min. The membrane skeletal fraction was isolated from the 8000 ***g*** supernatant by centrifugation at 100000 ***g*** for 30 min. The cytoskeletal fraction and membrane skeletal fraction were washed with 1 × lysis buffer, before use. For SDS/PAGE analysis, these fractions were solubilized in 1 × Laemmli sample buffer, and the supernatant (the Triton X-100-soluble fraction) was mixed with 0.25 vol. of 5 × Laemmli sample buffer. These samples were boiled for 3 min and subjected to SDS/PAGE [6 % or 8 % (w/v) gel].

Immunoblotting

The transfer of separated proteins to a nitrocellulose membrane and blocking of the membrane were performed as described [11]. The membrane was incubated for 4 h with antibodies in Tris-buffered saline (TBS) [25 mM Tris/HCl/136 mM NaCl/2.6 mM KCl (pH 7.4)] containing 0.5 % BSA. The membrane was then washed three times in TBS containing 0.05 % Tween-20 and incubated for 1 h with either ^{125}I-labelled protein A or ^{125}I-labelled protein G in TBS. After being washed with TBS containing 0.05 % Tween-20, the membrane was dried and subjected to analysis with a Bioimage Analyzer (Fuji BAS2000).

^{32}P-labelling of washed platelets and immunoprecipitation of p160^{ROCK}

Washed platelets (2×10^9/ml) were incubated for 2 h at room temperature with 0.5 mCi/ml [^{32}P]P_i. The platelets were pretreated with or without 1 mM RGDS for 3 min at 37 °C, activated by 1 unit/ml thrombin with stirring and lysed by the addition of an equal volume of 2 × RIPA buffer [0.2 % SDS/2 % (w/v) sodium deoxycholate/50 mM Tris/HCl (pH 7.4)/4 mM EGTA] containing 0.2 μM calyculin A and the mixture of protease inhibitors described above. In one experiment the Triton X-100-insoluble cytoskeletal fraction was first prepared from labelled platelets after 1 min of stimulation and then solubilized in 100 μl of 1 × RIPA buffer by sonication for 10 s twice. Samples were kept on ice for 10 min and were centrifuged at 10000 ***g*** for 15 min at 4 °C. Supernatants were precleared by incubation with Protein A–Sepharose 4LB conjugated with preimmune serum for 4 h at 4 °C. Immunoprecipitation was performed by incubating cleared lysates with Protein A–Sepharose 4LB conjugated with antibodies against p160^{ROCK} for 3 h at 4 °C. The immunocomplex was washed three times with 1 × lysis buffer containing 200 mM NaCl and then solubilized with an equal volume of 2 × Laemmli sample buffer. Samples were boiled and then analysed by SDS/PAGE [8 % (w/v) gel]. The gel was dried and exposed to an X-ray film for autoradiography.

RESULTS

Characterization of anti-p160^{ROCK} antibodies

We previously isolated a novel serine/threonine kinase p160^{ROCK} as a GTP-Rho-binding protein from platelet homogenate [5], and have recently shown that this kinase works as a downstream target of Rho, mediating the formation of focal adhesions and stress fibres in cultured cells [7]. However, it remains unclear how endogenous p160^{ROCK} is regulated spatially and temporally to induce the above-described cytoskeletal rearrangement. To examine these issues we raised anti-p160^{ROCK} antibodies against two N-terminal peptides of p160^{ROCK} and analysed the behaviour of endogenous p160^{ROCK} during platelet aggregation. Figure 1 shows the specificity of the antibodies that we used in this study. We used two antisera, 20 490 and 20 486, raised against the sequences of residues 2–15 and 14–28 respectively. These sequences are not conserved in a p160^{ROCK} isoenzyme, ROCK-

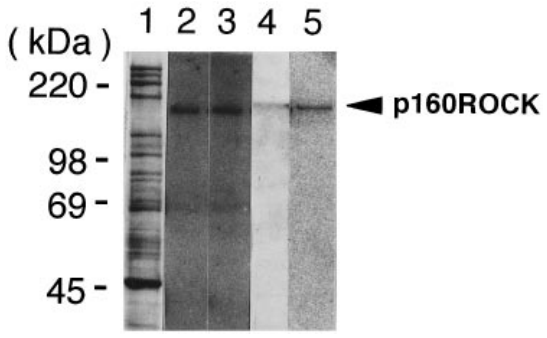

Figure 1 Immunoblot analysis of p160^{ROCK} in human platelet lysates

Total lysates from washed resting platelets (10^9/ml) (lanes 1–3) and p160^{ROCK} purified from platelet lysates (lanes 4 and 5) were subjected to SDS/PAGE [8% (w/v) gel] and either stained with Coomassie Brilliant Blue (lanes 1 and 4) or probed with anti-p160^{ROCK} antibodies 20 490 (lanes 2 and 5) or 20 486 (lane 3) as described in the Experimental section. The positions of molecular mass standards are shown at the left.

II/ROKα/Rho-kinase [12]. These antibodies reacted well with p160^{ROCK} purified from platelet homogenates (Figure 1, lanes 4 and 5; and results not shown). When the total homogenates were probed, both antibodies detected a single band at the same mobility, which corresponded to that of p160^{ROCK} in the homogenate. This band was not seen with preimmune sera of two antibodies (results not shown). These results verified that antibodies 20 490 and 20 486 specifically recognize p160^{ROCK} in human blood platelets.

Translocation of p160^{ROCK} to the cytoskeleton during platelet aggregation

Using these antibodies we examined subcellular localization of this kinase during platelet aggregation. Platelet aggregation was evoked by the addition of 1 unit/ml thrombin, which reached a maximum at 5 min (Figure 2A). Samples were taken at 0, 20, 60 and 300 s after the stimulation, then extracted with 1.0% Triton X-100 to yield the Triton-soluble, membrane skeletal and cytoskeletal fractions. As repeatedly reported [9], the accumulation of cytoskeletal proteins such as filamin, talin, myosin heavy chain, α-actinin and actin in the cytoskeletal fraction was already evident at 20 s with the concomitant loss of these proteins in the membrane skeleton (Figure 2B). In resting platelets, most p160^{ROCK} was present in the Triton X-100-soluble fraction and the rest was in the membrane skeletal fraction, whereas no immunoreactivity was found in the cytoskeletal fraction. When the radioactivities in the immunoblot were quantified, 93% of total p160^{ROCK} was present in the Triton X-100-soluble fraction

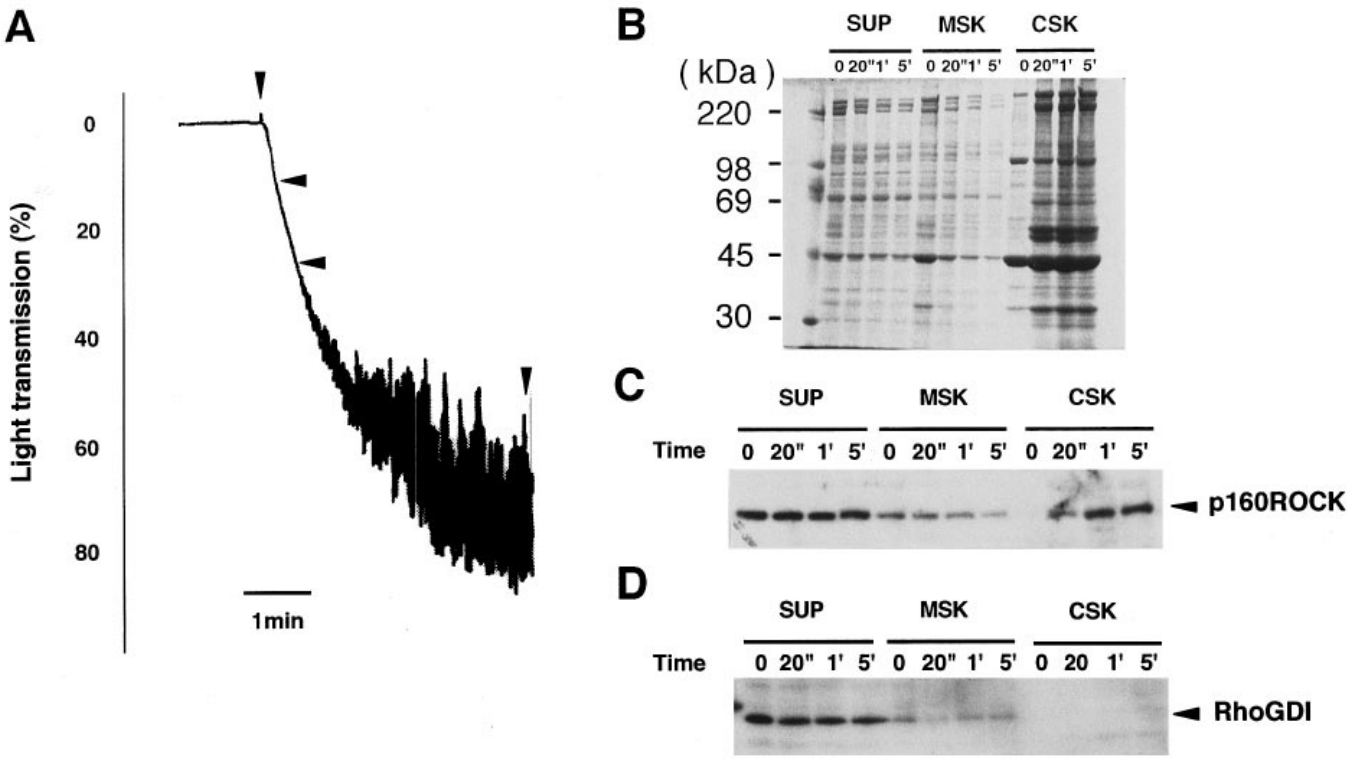

Figure 2 Translocation of p160^{ROCK} to the Triton X-100-insoluble fraction during platelet aggregation

(**A**) Aggregation curve. Washed platelets (10^9/ml) were stimulated by 1 unit/ml thrombin. Aggregation was monitored by light transmission. Aliquots were taken for extraction at the times indicated by arrowheads. (**B**) Protein staining of subcellular fractions during aggregation. Platelet lysates were fractionated as described in the Experimental section, and fractionated samples were subjected to SDS/PAGE [8% (w/v) gel] and stained with Coomassie Brilliant Blue. The positions of molecular mass standards are shown at the left. (**C**) Subcellular distribution of p160^{ROCK} during aggregation. (**D**) Subcellular localization of Rho GDI during aggregation. The fractionated samples were subjected to SDS/PAGE [6% (w/v) gel] and transferred to nitrocellulose membranes, which were then immunostained with either anti-p160^{ROCK} antibody 20 490 (**C**) or with anti-RhoGDI monoclonal antibody (**D**) as described in the Experimental section. A one-eighth equivalent fraction was used for the analysis of the soluble fraction compared with the cytoskeletal and membrane skeletal fractions. A representative result of five independent experiments is shown. Abbreviations in (**B**–**D**): SUP, Triton X-100-soluble fraction; MSK, membrane skeletal fraction; CSK, cytoskeletal fraction; minutes and seconds are denoted by $'$ and $''$ respectively.

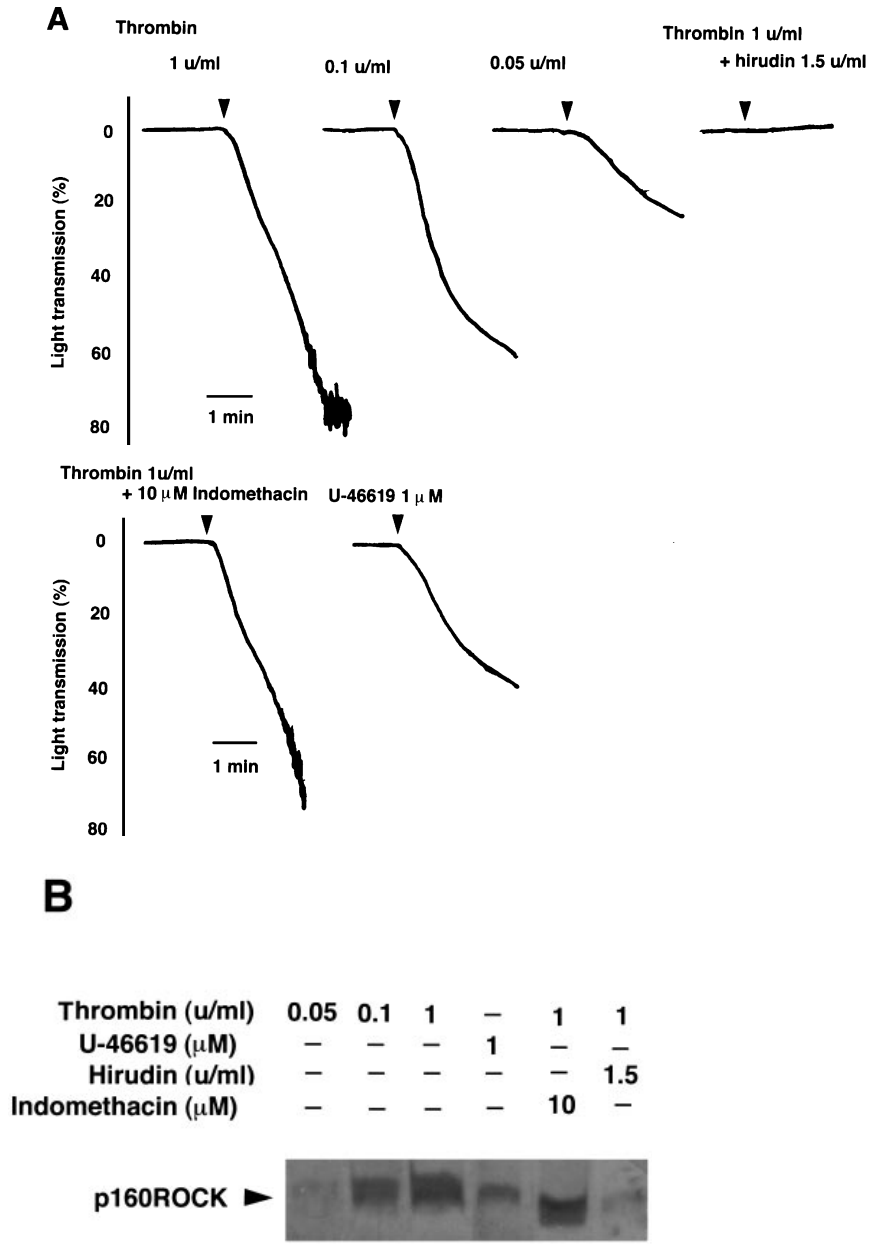

Figure 3 Aggregation-dependent translocation of p160^{ROCK} to the cytoskeletal fraction

(**A**) Aggregation curves. Washed platelets were stimulated with 0.05, 0.1 or 1 unit/ml thrombin, with 1 unit/ml thrombin in the presence of either 1.5 i.u./ml hirudin or 10 μM indomethacin, or with 1 μM U-46619, and aggregation curves were obtained. (**B**) Immunoblot of p160^{ROCK} in the cytoskeletal fraction. After 3 min of stimulation, the platelets were lysed and the cytoskeletal fraction was obtained and subjected to SDS/PAGE and immunoblotting with anti-p160^{ROCK} antibody as described in the Experimental section.

and approx. 6% was in the membrane skeletal fraction. During platelet aggregation, some of p160^{ROCK} was translocated to the cytoskeletal fraction in a time-dependent manner. It was evident at 20 s; approx. 10% of the total amount was found in the Triton X-100-insoluble fraction after 1 min of aggregation (Figure 2C). The amount of p160^{ROCK} in the membrane skeletal fraction decreased time-dependently by 1.5–2% at 5 min after stimulation. In contrast, Rho GDI, which is known as a negative regulator of Rho [13], resides mostly in the Triton X-100-soluble fraction during aggregation: no translocation to the cytoskeletal fraction was detected. These results indicate that p160^{ROCK} is specifically translocated to the cytoskeletal fraction during platelet aggregation.

To confirm that this translocation is dependent on aggregation and can also be caused by platelet agonists other than thrombin, we stimulated platelets with various concentrations of thrombin or with a thromboxane agonist, U-46619. Effects of the blocking of thrombin action and the involvement of arachidonate metabolism were also evaluated by the addition of hirudin and indomethacin respectively on stimulation with thrombin. As shown in Figure 3(A), thrombin evoked platelet aggregation in a concentration-dependent manner; 20%, 65%

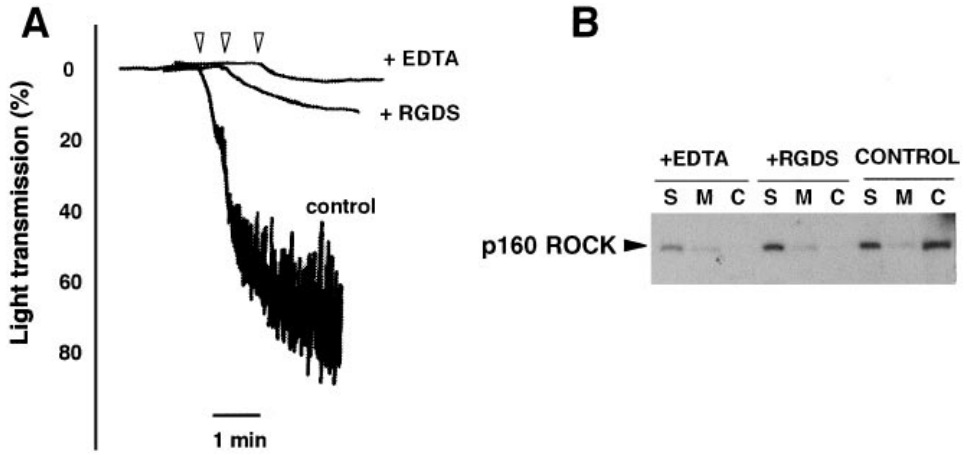

Figure 4 Effect of inhibitors of integrin ligation on translocation of p160^{ROCK}

(**A**) Platelet aggregation. Washed platelets (2×10^9/ml) were preincubated in the presence or absence of 1 mM RGDS or 10 mM EDTA and were stimulated with 1 unit/ml thrombin at times indicated by arrowheads. Aggregation was monitored as described in the legend to Figure 2. (**B**) Immunoblot of p160^{ROCK} in subcellular fractions. Platelets were extracted after 3 min of stimulation and fractionated samples were subjected to SDS/PAGE [6% (w/v) gel] and immunostained with anti-p160^{ROCK} antibody. Abbreviations: S, supernatant; M, membrane skeletal fraction; C, cytoskeletal fraction. A representative result of five independent experiments is shown.

and 85% aggregation occurred with 0.05, 0.1 and 1 unit/ml thrombin respectively. p160^{ROCK} was translocated to the cytoskeletal fraction, depending on the extent of aggregation as shown in Figure 3(B). The aggregation by thrombin was completely blocked by hirudin, but no inhibition was found with indomethacin. Consistently, the translocation of p160^{ROCK} was not affected by indomethacin treatment but was abolished by the addition of hirudin. U-46619 induced 40% aggregation and caused the translocation of the kinase, consistent with the extent of this aggregation. These results demonstrate that the translocation of p160^{ROCK} to the cytoskeletal fraction is dependent on the aggregation process itself.

p160^{ROCK} translocation is dependent on ligand ligation of αIIbβ3

Because several molecules translocate to the cytoskeletal fraction in an aggregation-dependent manner [9,14–16] and aggregation is mediated by platelet integrin αIIbβ3, we tested whether the ligand ligation of integrin αIIbβ3 is involved in this cytoskeletal association of p160^{ROCK}. Platelets were preincubated with RGDS or EDTA, which respectively inhibit platelet aggregation by competing with fibrinogen binding to αIIbβ3 [17] and chelate the divalent cations required for integrin ligation. When platelets were preincubated with EDTA or RGDS for 2 min, the aggregation reaction was inhibited by approx. 90% (Figure 4A). Under these conditions, p160^{ROCK} remained in the detergent-soluble fraction, indicating that the translocation of p160^{ROCK} to the cytoskeletal fraction is dependent on integrin ligation (Figure 4B).

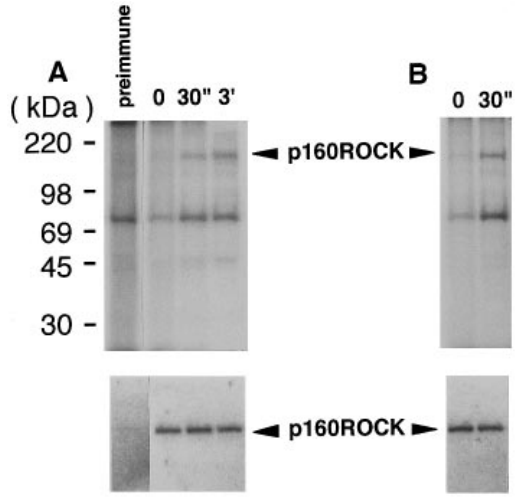

Figure 5 Phosphorylation of p160^{ROCK} during thrombin stimulation

Washed platelets (2×10^9/ml) were incubated with [^{32}P]P_i and incubated in the absence (**A**) or presence (**B**) of RGDS and stimulated by 1 unit/ml thrombin. After the indicated times, platelets were lysed and p160^{ROCK} was immunoprecipitated as described in the Experimental section. Immunoprecipitates with preimmune serum were prepared as a control. The positions of molecular mass standards are shown at the left. Immunoprecipitates were subjected to SDS/PAGE [8% (w/v) gel] and autoradiography (upper panels). Immunoprecipitates were also prepared as described above from platelets incubated without [^{32}P]P_i and subjected to immunoblot probed with anti-p160^{ROCK} antibody (lower panels). A representative result of three independent experiments is shown. Minutes and seconds are denoted by $'$ and $''$ respectively.

Phosphorylation of p160^{ROCK} during platelet aggregation

We next examined whether p160^{ROCK} is phosphorylated in thrombin-stimulated platelets and whether this phosphorylation is dependent on integrin ligation, because several kinases autophosphorylate themselves in stimulated platelets and some of them require the ligation of integrin with its ligand for this reaction [17,18]. Platelets were labelled with [^{32}P]P_i and stimulated with 1 unit/ml thrombin. Platelets were extracted with RIPA buffer at 0, 30 and 180 s after stimulation, and p160^{ROCK} was immunoprecipitated. Figure 5(A) shows an autoradiogram after SDS/PAGE of the immunoprecipitates. As shown in the bottom panel, this procedure could immunoprecipitate equal amounts of p160^{ROCK} in the lysates in these samples. Phosphorylation of p160^{ROCK} was barely found in resting platelets and was clearly detected after 30 s exposure to thrombin. The extent of this phosphorylation did not change significantly up to 3 min of

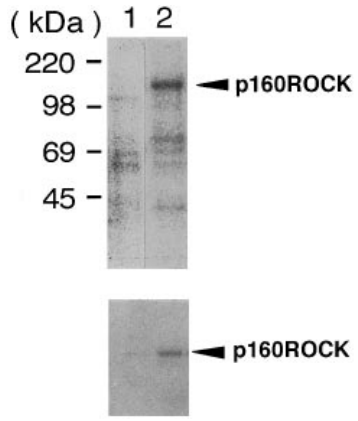

Figure 6 Phosphorylated p160^{ROCK} in the cytoskeletal fraction

The cytoskeletal fraction was obtained from [^{32}P]P_i-loaded washed platelets aggregated for 1 min and solubilized in RIPA buffer by sonication. Supernatants of the solubilized fraction were used for precipitation with anti-p160^{ROCK} antibody (lane 2) or its preimmune serum (lane1). Immunoprecipitates were then recovered and subjected to SDS/PAGE and autoradiography (upper panel). A control immunoblot analysis (lower panel) was performed as described in the legend to Figure 5. Experiments were repeated twice independently with a reproducible result. The positions of molecular mass standards are shown at the left.

incubation (Figure 5A). To investigate whether this phosphorylation is dependent on the ligation of αIIbβ3, platelets were preincubated with RGDS and then stimulated by thrombin. As shown in Figure 5(B), p160^{ROCK} was phosphorylated at the same level in these platelets as under the aggregation condition. We then examined whether the phosphorylated form of this kinase could be incorporated into the cytoskeletal fraction. We therefore disintegrated the Triton X-100-insoluble fraction obtained from aggregated platelets by the use of RIPA buffer and performed immunoprecipitation of p160^{ROCK}. As shown in Figure 6, phosphorylated p160^{ROCK} was recovered from this fraction by precipitation with anti-p160^{ROCK} antibody but not with its preimmune serum. These results indicate that p160^{ROCK} is phosphorylated in response to thrombin stimulation in an aggregation-independent manner, and that at least a part of the phosphorylated form is present in the cytoskeletal fraction.

DISCUSSION

Here we report the translocation of endogenous p160^{ROCK} into the Triton X-100-insoluble cytoskeletal fraction during platelet aggregation. This translocation is a specific event because Rho-GDI taken as a control did not show any translocation during this process, and the translocation of p160^{ROCK} is sensitive to treatment with RGDS or EDTA. The latter sensitivity indicates that p160^{ROCK} is incorporated into the cytoskeleton associated with activated integrin αIIbβ3, and not that with the granular centralization. This is consistent with the proposed role of this kinase in stimulus-induced cell adhesion [7]. It has already been reported that p160^{ROCK} is activated *in vitro* by GTP-Rho and also *in vivo* by coexpression with the activated form of Rho [5,8]. Because thrombin stimulation is supposed to activate Rho in platelets as well as in other types of cells [3,19], p160^{ROCK} is likely to be activated and then translocated. We have tested, by immunocomplex kinase assay, whether this activation involves activation of the kinase. However, we could not detect enhancement of the kinase activity under our assay conditions (A. Fujita and S. Narumiya, unpublished work). Whether this indicates little activation of the catalytic activity of this kinase during this process remains to be investigated. Although many cytoskeletal proteins translocate to the cytoskeletal fraction from the membrane cytoskeleton [9], p160^{ROCK} seems to behave differently, because an amount more than that in the membrane cytoskeleton was found in the cytoskeletal fraction and a decrease in the membrane skeletal fraction of this kinase during aggregation was small. Because p160^{ROCK} contains several domains capable of interacting with other proteins [5], we presume that its binding to GTP-Rho exposes these domains, which then bind to domains of some of the cytoskeletal proteins exposed by complex formaton with ligated integrins. In this study we have also observed that p160^{ROCK} undergoes phosphorylation in reponse to thrombin. Interestingly, this phosphorylation occurs in the presence of RGDS, suggesting that the phosphorylation occurs before, or independently of, integrin activation. Although the presence of the phosphorylated form of this kinase in the cytoskeletal fraction is not inconsistent with the idea that this phosphorylation is a prerequisite for the translocation, this point should be clarified in future studies by using other types of cell expressing a phosphorylation-defective mutant of p160^{ROCK}.

The next question is how this translocation is linked to the expression of the function of this kinase. Recently, Kimura et al. [20] reported that a p160 homologue, Rho-kinase, phosphorylates the myosin-binding subunit of myosin phosphatase and decreases its activity, suggesting that this mechanism is responsible for the induction of myosin-based contractility in the cell. p160^{ROCK} and ROKα (Rho-kinase) have been shown also to induce focal adhesions and stress fibres [7,21,22]. Focal adhesions and stress fibres are the clustered structures of ligated integrin complexes and bound filamentous actin respectively, and this clustering is proposed to occur by way of myosin contractility [23]. On the basis of these findings, it is likely that p160^{ROCK} incorporated into the cytoskeleton linked to ligated integrins has a role in these clustering events. In contrast, cell adhesion to substratum might not require these clustering events. For example, the addition of cytochalasin to inhibit actin polymerization does not suppress platelet aggregation [24] or Rho-dependent, LFA-1-mediated adhesion of cultured lymphocytes [4]. Also, the addition of various myosin light chain kinase (MLCK) inhibitors does abolish focal adhesions and stress fibres but seems not to affect cell spreading on the substratum [25]. Inactivation of Rho, in contrast, affects these processes. These results suggest that the role of Rho is more than inducing contractility in the cell adhesion process. Whether p160^{ROCK} is involved in such processes or, if so, whether it exerts this action also in association with the cytoskeleton remains to be elucidated.

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