<table>
<thead>
<tr>
<th>Title</th>
<th>Sequential activation of Rap1 and Rac1 small G proteins by PDGF locally at leading edges of NIH3T3 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Takahashi, Motonori / Rikitake, Yoshiyuki / Nagamatsu, Yuichi / Hara, Tetsuya / Ikeda, Wataru / Hirata, Ken-ichi / Takai, Yoshimi</td>
</tr>
<tr>
<td>Citation</td>
<td>Genes to Cells, 13(6): 549-569</td>
</tr>
<tr>
<td>Issue date</td>
<td>2008-04-21</td>
</tr>
<tr>
<td>Resource Type</td>
<td>Journal Article / 学術雑誌論文</td>
</tr>
<tr>
<td>Resource Version</td>
<td>author</td>
</tr>
<tr>
<td>DOI</td>
<td>10.1111/j.1365-2443.2008.01187.x</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/90000795">http://www.lib.kobe-u.ac.jp/handle_kernel/90000795</a></td>
</tr>
</tbody>
</table>

PDF issue: 2018-11-24
Sequential Activation of Rap1 and Rac1 Small G Proteins by PDGF Locally at Leading Edges of NIH3T3 Cells

Motonori Takahashi¹², Yoshiyuki Rikitake¹², Yuichi Nagamatsu¹, Tetsuya Hara¹², Wataru Ikeda³, Ken-ichi Hirata², and Yoshimi Takai¹³

¹Division of Molecular and Cellular Biology, Department of Biochemistry and Molecular Biology, ²Division of Cardiovascular Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, 650-0017, ³Department of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, Suita, 565-0871, Japan.

Running Title: Small G protein cascade in cell movement

Address correspondence to:
Yoshimi Takai
Division of Molecular and Cellular Biology
Department of Biochemistry and Molecular Biology
Kobe University Graduate School of Medicine
Kobe, 650-0017, Japan,
Tel. +81-78-382-5410;
Fax. +81-78-382-5419;
E-Mail: ytakai@med.kobe-u.ac.jp
Abstract

Moving cells form protrusions, such as filopodia and lamellipodia, and focal complexes at leading edges, which eventually enhance cell movement. The Rho family small G proteins, Rac1, Cdc42, and RhoA, are involved in the formation of these leading edge structures. We investigated the role of another small G protein Rap1 in the platelet-derived growth factor (PDGF)-induced formation of leading edge structures and cell movement. Upon stimulation of NIH3T3 cells by PDGF, leading edge structures were formed and Necl-5, integrin αVβ3, and PDGF receptor were accumulated at leading edges. Rap1, upstream regulators of Rap1 such as Crk and C3G, and a downstream effector RalGDS, were accumulated at peripheral ruffles over lamellipodia. Overexpression of Rap1GAP, which inactivates Rap1, and knockdown of Rap1 inhibited the PDGF-induced formation of leading edge structures, accumulation of these molecules, and cell movement. In addition, Rap1 activation subsequently induced accumulation of Rac1, Vav2, and PAK at peripheral ruffles, which was inhibited by Rap1GAP and knockdown of Rap1. These results indicate that Rap1, activated by PDGF, is recruited to leading edges and that Rac1 is thereby locally activated at peripheral ruffles. This process is pivotal for the PDGF-induced formation of leading edge structures and cell movement.
Introduction

Moving cells dynamically form special structures at leading edges, which are necessary for cell movement: these structures include protrusions, such as filopodia and lamellipodia, ruffles, focal complexes, and focal adhesions. These structures are formed by reorganization of the actin cytoskeleton, which is regulated by the action of each member of the Rho family small G proteins: lamellipodia and ruffles are formed by the action of Rac1; filopodia are formed by the action of Cdc42; and focal complexes are formed by the actions of Rac1 and Cdc42 (Ballestrem et al. 2001; Rottner et al. 1999). The formation of these structures is inhibited by the action of RhoA. Focal complexes are transformed to focal adhesions by inactivation of Cdc42 and Rac1 and activation of RhoA. Focal adhesions are associated with stress fibers, formation of which is enhanced by the action of RhoA. The activities of these small G proteins are cooperatively regulated by growth factor receptors and integrins, but the molecular mechanisms underlying the coordinated regulation of the activities of these small G proteins remain unknown.

Integrins are cell-matrix adhesion molecules that are essential for cell movement and regulate cell movement in cooperation with cell surface receptors for chemoattractants. Integrins form cell-matrix junctions called focal complexes and adhesions. Focal complexes are smaller in size than focal adhesions and are formed at contact sites between protrusions and matrix, at leading edges. Focal adhesions are formed at sites other than leading edges by the action of RhoA. Ruffles randomly attach to matrix and some form new focal complexes; the pre-existing focal complexes are transformed to focal adhesions by inactivation of Cdc42 and Rac1 and activation of RhoA. Of the many integrins, integrin $\alpha_V\beta_3$ forms focal complexes and is up-regulated in many cancer cells (Guo & Giancotti 2004).

We recently found that Necl-5 forms a complex with integrin $\alpha_V\beta_3$, which enhances its clustering and the subsequent formation of focal complexes at leading edges of moving NIH3T3 cells in response to platelet-derived growth factor (PDGF) (Amano et al. 2008; Ikeda et al. 2004; Minami et al. 2007). Necl-5 was originally identified as the poliovirus receptor (PVR)/CD155 in humans (Koike et al. 1990; Mendelsohn et al. 1989), and as the product of a gene (Tage4) that is up-regulated in colon carcinomas in rodents (Chadeneau et al. 1994). PVR/CD155 has also been shown to be up-regulated in many human cancer cells (Gromeier et al. 2000; Masson et al. 2001). This molecule, with four nomenclatures, is named Necl-5. Although it remains unknown how the Necl-5-integrin complex is clustered at leading edges or how cells determine the direction of movement, we recently found that PDGF receptor forms a ternary complex with Necl-5 and integrin...
αβ3, and that this complex is clustered at leading edges in response to PDGF (Amano et al. 2008; Ikeda et al. 2004; Minami et al. 2007).

Rap1, a member of the Ras family of small G proteins, has been recognized as an important regulator of cell proliferation, differentiation, and adhesion (Bos et al. 2001). Rap1 is known to be activated in response to a variety of stimuli, including growth factors and G protein-coupled receptor agonists, and to transmit signals to several downstream effectors. Small G proteins are activated by guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP and allow GTP to bind, and are inactivated by GTPase-activating proteins (GAPs), which stimulate hydrolysis of GTP to GDP (Etienne-Manneville & Hall 2002). GEFs for Rap1 include Epac (de Rooij et al. 1998; Kawasaki et al. 1998), DOCK4 (Yajnik et al. 2003), C3G (Gotoh et al. 1995), and PDZ-GEF (de Rooij et al. 1999; Ohtsuka et al. 1999), while GAPs for Rap1 consist of Rap1GAP family proteins (Polakis et al. 1991; Mochizuki et al. 1999), and SPAL family proteins, such as SPA-1 (Kurachi et al. 1997) and E6-TP1α/SPAR/SPAL (Gao et al. 1999). Although recent studies suggested that Rap1 is implicated in the migration of vascular endothelial cells and lymphocytes (Durand et al. 2006; Fujita et al. 2005), the precise localization of Rap1 that controls crosstalk between Rap1 and the Rho family small G proteins, including Rac1, during cell movement is still unclear. We attempted to investigate how activated Rap1 regulates the PDGF-induced formation of leading edge structures and cell movement. In addition, we examined whether activated Rap1 is recruited to leading edges and how recruited Rap1 promotes the local activation of Rac1 at leading edges, which is critical for the formation of leading edge structures.
Results

Necessity of activation of Rap1 for PDGF-induced formation of leading edge structures

We first examined whether Rap1 is necessary for the PDGF-induced formation of lamellipodia, ruffles, and focal complexes and cell movement in NIH3T3 cells. For analysis of cell movement, NIH3T3 cells were sparsely plated on µ-slide VI flow dishes pre-coated with vitronectin, an extracellular matrix protein that binds to integrin αVβ3 (van der Flier & Sonnenberg 2001), starved of serum, and directionally stimulated by PDGF. Time-lapse microscopy in wild-type NIH3T3 cells revealed that leading edges were formed as cells moved forward in response to PDGF (Fig. 1Aa). Most wild-type NIH3T3 cells became polarized and formed protrusive lamellipodia at leading edges toward the higher concentrations of PDGF (Fig. 1B, a and d, and 1C). They formed peripheral ruffles over lamellipodia and focal complexes under the ruffles. Focal adhesions were formed at sites to the rear of leading edges. The immunofluorescence signals for Necl-5, PDGF receptor, and integrin β3 were concentrated and co-localized at peripheral ruffles of leading edges in the middle section of the cells (Fig. 1Bb and 1C). The signal for F-actin was observed as stress fibers and at peripheral ruffles. In the basal section of the cells, the signals for Necl-5 and integrin β3, but not that for PDGF receptor, were observed at focal complexes under ruffles. The signal for integrin β3, but not that for Necl-5, was observed at focal adhesions. Essentially the same results were obtained when cells were stained with integrin αV instead of integrin β3 (unpublished data). These results are consistent with our earlier observations (Amano et al. 2008; Ikeda et al. 2004; Minami et al. 2007).

Expression of GFP-tagged Rap1GAP (GFP-Rap1GAP), which inactivates Rap1 (Rubinfeld et al. 1991), resulted in a failure to develop definite leading edges and the formation of peripheral ruffles in the direction of higher concentrations of PDGF and in a significant inhibition of the formation of focal complexes (Fig. 1Ab and 1Bd). Less than half of GFP-Rap1GAP-expressing cells showed the formation of lamellipodia and peripheral ruffles, although more than 80% of control NIH3T3 cells became polarized and displayed the formation of lamellipodia (Fig. 1Bd). Neither the signals for Necl-5 nor PDGF receptor was concentrated at any regions in non-polarized cells, although the signal for integrin β3 was concentrated at focal adhesions (Fig. 1B, a and c). The signal for F-actin was observed as stress fibers.

To further confirm the role of Rap1 in the PDGF-induced formation of leading edge structures, we examined the effect of knockdown of Rap1. We used 2 sets of siRNAs for Rap1A and Rap1B (Rap1 siRNA-I and -II). Western blot analysis confirmed that co-transfection of Rap1
siRNA-I and -II, but not of non-silencing control siRNA, decreased Rap1 protein expression by >70% without silencing Ras (Fig. 2Aa). Transfection of Rap1 siRNA-I or Rap1 siRNA-II resulted in a significant inhibition of the formation of definite leading edges and peripheral ruffles in the direction of higher concentrations of PDGF (Fig. 2B, b and c, and Cd). As compared to control siRNA-transfected cells, the formation of peripheral ruffles was significantly reduced in Rap1-knockdown cells. Rap1-knockdown cells significantly inhibited the formation of focal complexes (Fig. 2C, a and b, and 2D, b and c). Neither the signal for Necl-5 nor PDGF receptor was concentrated at any regions in non-polarized cells, although the signal for integrin β3 was concentrated at focal adhesions. The signal for F-actin was observed as stress fibers. When non-silencing control siRNA was transfected, the signals for Necl-5, integrin β3, and PDGF receptor were concentrated at peripheral ruffles over lamellipodia of leading edges (Fig. 2Da). The signal for F-actin was observed as stress fibers and at peripheral ruffles. The expression of GFP-Rap1, which was resistant to Rap1 siRNA-I (Fig. 2Ab), restored the impairment of the PDGF-induced formation of leading edge structures (Fig. 2C, a, c, and d, and 2Dd). Taken together, these results indicate that activation of Rap1 is necessary for the PDGF-induced formation of leading edge structures.

**Necessity of activation of Rap1 for PDGF-induced cell movement**

We then examined the effects of overexpression of Rap1GAP and knockdown of Rap1 on random and directional cell movement. We first compared the random movement activity of NIH3T3 cells transiently expressing GFP-Rap1GAP or knockdown of Rap1 with that of the control cells using the phagokinetic track motility assay on colloidal gold-coated coverslips in the presence of serum containing PDGF as a major chemoattractant. GFP-Rap1GAP-expressing NIH3T3 cells significantly reduced cell movement activity as compared to control GFP-expressing NIH3T3 cells (10,060 ± 480 µm² versus 4,870 ± 1,020 µm², P<0.05) (Fig. 3Aa). Similarly, Rap1-knockdown NIH3T3 cells significantly reduced random cell movement as compared to control siRNA-transfected cells (11,280 ± 830 µm² versus 5,300 ± 140 µm², P<0.05) (Fig. 3Ab). These results indicate that activation of Rap1 is required for random cell movement.

We next compared the PDGF-induced directional movement activity of GFP-Rap1GAP-expressing NIH3T3 cells or Rap1-knockdown NIH3T3 cells with that of the control cells using the Boyden chamber assay. The movement of GFP-Rap1GAP-expressing NIH3T3 cells was approximately 40% slower than that of control GFP-expressing NIH3T3 cells. Likewise, Rap1-knockdown NIH3T3 cells showed over 60% reduction of chemotaxis as compared with that
of control cells, indicating the involvement of Rap1 in chemotaxis (Fig. 3B, a and b). Collectively, these results indicate that activation of Rap1 is important for both PDGF-induced random and directional cell movement.

**Activation of Rap1 in response to PDGF**

We then performed a pull-down assay to examine whether Rap1 is indeed activated in response to PDGF in NIH3T3 cells. Because we could not practically prepare sufficient cells for the pull-down assay from the cells cultured on µ-slide VI flow dishes, NIH3T3 cells were plated on dishes pre-coated with vitronectin, starved of serum, and cultured in the medium in the presence or absence of PDGF. The pull-down assay revealed that the amount of GTP-bound Rap1 was increased in response to PDGF within 1 min and then declined rapidly thereafter (Fig. 4A). The effect of PDGF on this increase in GTP-bound Rap1 was concentration-dependent (Fig. 4B). These results indicate that Rap1 is transiently activated in response to PDGF.

We then confirmed the effect of Rap1GAP on the activity of Rap1. In this experiment, to detect clearly the effect of Rap1GAP, GFP-tagged Rap1 was co-expressed with GFP-Rap1GAP, and GFP-Rap1 from transfected cells was then selectively detected by subsequent anti-GFP blotting of precipitated GTP-bound Rap1. When GFP was expressed, the amount of GTP-bound Rap1 was increased in response to PDGF, however, when GFP-Rap1GAP was expressed, the amount of GTP-bound Rap1 was greatly decreased in the presence and absence of PDGF and notably, the PDGF-induced increase in the amount of GTP-bound Rap1 was completely absent (Fig. 4C). Thus, expression of GFP-Rap1GAP completely inhibited the PDGF-induced activation of Rap1.

**Involvement of Crk and C3G in the PDGF-induced activation of Rap1**

It was reported that the PDGF-induced activation of Rap1 is mediated at least by the adaptor protein Crk and the GEF for Rap1, C3G, in porcine aortic endothelial cells (Yokote et al. 1998). We first confirmed that Crk and C3G are involved the PDGF-induced activation of Rap1 in NIH3T3 cells. In this experiment, to detect clearly the effects of dominant-negative mutants of CrkI (CrkI-W169L) and C3G (C3G-ΔCD), GFP-tagged Rap1 was co-expressed with CrkI-W169L or C3G-ΔCD, and GFP-Rap1 from transfected cells was then selectively detected by subsequent anti-GFP blotting of precipitated GTP-bound Rap1. Transfection of CrkI-W169L or C3G-ΔCD completely inhibited the PDGF-induced activation of Rap1, as estimated by the pull-down assays (Fig. 4D, a and b). These results indicate that the PDGF-induced activation of Rap1 is dependent on Crk and C3G.
Activation of Rap1 at leading edges where PDGF receptor is clustered with Necl-5 and integrin αvβ3

We previously showed that PDGF receptor is clustered with Necl-5 and integrin αvβ3 at peripheral ruffles over lamellipodia of leading edges of moving NIH3T3 cells (Amano et al. 2008; Ikeda et al. 2004; Minami et al. 2007). We then examined whether Rap1, Crk, and C3G are recruited to these clustered sites in response to PDGF. NIH3T3 cells were sparsely plated on µ-slide VI flow dishes pre-coated with vitronectin, starved of serum, and directionally stimulated by PDGF for up to 60 min. Time-lapse fluorescence microscopy in GFP-Rap1-transfected NIH3T3 cells revealed that GFP-Rap1 came to accumulate at leading edges as leading edges were formed when cells moved forward in response to PDGF (Fig. 5A, a and b). Consistently, in the absence of PDGF, the immunofluorescence signal for Rap1 did not accumulate at any regions and no significant accumulation of the signal for Rap1 was observed within 10 min of stimulation with PDGF (Fig. 5Ba). On the other hand, 30 min after stimulation with PDGF, Rap1 accumulated at peripheral ruffles over lamellipodia of leading edges and co-localized with the signal for PDGF receptor in association with the formation of peripheral ruffles at leading edges. Co-localization of the signals for Rap1 and PDGF receptor was observed for up to 60 min following PDGF treatment (data not shown). The signals for Crk, C3G, and RalGDS, a downstream effector protein of Rap1, were also observed at peripheral ruffles over lamellipodia of leading edges and co-localized with that for Rap1 (Fig. 5B, b-d).

We then examined the effect of inhibition of Rap1 activity by overexpression of Rap1GAP or knockdown of Rap1 on the PDGF-induced recruitment of Rap1 and upstream regulators and a downstream effector of Rap1 to leading edges. Inactivation of Rap1 by overexpression of Rap1GAP prevented the formation of leading edge structures and the PDGF-induced accumulation of Rap1 as well as upstream regulators and a downstream effector of Rap1 at peripheral ruffles over lamellipodia of leading edges (Fig. 5C, a-d). Likewise, Rap1-knockdown cells showed similar phenotypes as a failure of the formation of leading edge structures and the PDGF-induced accumulation of Rap1 as well as upstream regulators and a downstream effector of Rap1 at peripheral ruffles over lamellipodia of leading edges (Fig. 5D, a-d). These phenotypes were restored by the expression of siRNA-resistant GFP-Rap1 (Fig. 5D, a-d).Taken together, these results indicate that Rap1 and upstream regulators and a downstream effector of Rap1 are recruited to leading edges in a PDGF-dependent manner, resulting in the formation of leading edge structures, and suggest that Rap1 is locally activated at peripheral ruffles over lamellipodia of leading edges where PDGF receptor is clustered with Necl-5 and integrin αvβ3.
Activation of Rac1 downstream of Rap1 at leading edges

It has been shown that Rac1 is activated at leading edges and is necessary for the formation of lamellipodia, ruffles, and focal complexes (Arthur et al. 2004; Pertz et al. 2006). NIH3T3 cells were sparsely plated on µ-slide VI flow dishes pre-coated with vitronectin, starved of serum, and directionally stimulated by PDGF for up to 60 min as described above. The pattern of the immunofluorescence signal for Rac1 was quite similar to that for Rap1. Time-lapse fluorescence microscopy in GFP-Rac1-transfected NIH3T3 cells revealed that GFP-Rac1 came to accumulate at leading edges as leading edges were formed when cells moved forward in response to PDGF (Fig. 6A, a and b). In the absence of PDGF, Rac1 did not accumulate at any regions within 10 min, whereas 30 min after stimulation with PDGF, the signal for Rac1 accumulated at peripheral ruffles over lamellipodia of leading edges and co-localized with that for PDGF receptor (Fig. 6Ba). Co-localization of the signal for Rac1 with that for PDGF receptor continued for up to 60 min (data not shown). The signals for Vav2, an upstream regulator of Rac1, and PAK, a downstream effector of Rac1, were also observed at peripheral ruffles over lamellipodia of leading edges and co-localized with that of Rac1 (Fig. 6B, b and c).

To test the possibility that Rap1 regulates activation of Rac1 at peripheral ruffles over lamellipodia of leading edges, we examined the effects of overexpression of Rap1GAP and knockdown of Rap1 on the PDGF-induced accumulation of the signal for Rac1 at peripheral ruffles. Whereas transfection of GFP did not affect the localization of the signal for Rac1, transfection of cells with GFP-Rap1GAP inhibited the accumulation of the signal for Rac1 at peripheral ruffles (Fig. 6C, a-c). Similarly, Rap1-knockdown cells failed to display the PDGF-induced accumulation of Rac1 and an upstream regulator and a downstream effector of Rac1 at peripheral ruffles over lamellipodia of leading edges (Fig. 6D, a-c). In contrast, the expression of siRNA-resistant GFP-Rap1 reversed these phenotypes (Fig. 6D, a-c).

Taken together, these results indicate that Rac1 and an upstream regulator and a downstream effector of Rac1 are recruited to leading edges in a PDGF-dependent manner, resulting in the formation of leading edge structures, and suggest that Rac1 is locally activated at peripheral ruffles over lamellipodia of leading edges where PDGF receptor is clustered with Necl-5 and integrin αVβ3. These results also indicate that the recruitment of activated Rap1 to leading edges is required for the PDGF-induced activation of Rac1 at leading edges, which is critical for the formation of leading edge structures such as ruffles and lamellipodia.
Involvement of Vav2 in the Rap1-mediated activation of Rac1

We examined the mechanistic implication of Rap1 for the PDGF-induced activation of Rac1. Knockdown of Rap1 abrogated the PDGF-induced activation of Rac1 in NIH3T3 cells (Fig. 7Aa), whereas expression of a constitutively active mutant of Rap1 (Rap1-CA) in HEK293 cells induced Rac1 activation (Fig. 7Ab). These results clearly demonstrated that Rac1 locates downstream of Rap1.

To determine whether Vav2 was involved in the Rap1-mediated activation of Rac1, we tested the effect of a dominant negative mutant of Vav2 (Vav2-DN). Expression of Vav2-DN effectively inhibited the PDGF-induced activation of Rac1 in NIH3T3 cells as well as the Rap1-CA-dependent activation of Rac1 in HEK293 cells (Fig. 7B, a and b). These results demonstrated that Vav2 mediates the Rap1-induced activation of Rac1. Collectively, these results indicate that Rap1-Vav2 is involved in the PDGF-induced activation of Rac1.
Discussion

Rap1 is involved in a variety of cellular processes such as cell proliferation, adhesion, and polarization through downstream effectors (Ohba et al. 2001; Shimonaka et al. 2003). Rap1 binds to several effector proteins such as B-Raf (Vossler et al. 1997), RalGDS (Ikeda et al. 1995), and afadin/AF-6 (Boettner et al. 2000). In the present study, we showed for the first time that Rap1 activity is necessary for the PDGF-induced formation of lamellipodia, ruffles, and focal complexes at leading edges as well as cell movement in NIH3T3 cells. Inhibition of Rap1 activity by overexpression of Rap1GAP or knockdown of Rap1 resulted in a failure of the cells to undergo the PDGF-induced formation of these leading edge structures. In Rap1GAP-overexpressing and Rap1-knockdown cells, none of the signals for Necl-5, integrin β3, and PDGF receptor were concentrated at any regions of the cell membrane. In addition, morphological analysis of Rap1GAP-overexpressing and Rap1-knockdown cells suggests that Rap1 may be required for the establishment of polarity at the leading edges, as cell spreading is still exhibited in Rap1GAP-overexpressing and Rap1-knockdown cells, but spreading lacks directionality/polarity. This is consistent with the results of the recent paper showing the critical role of Rap1 in the establishment of polarity in T-cells (Gerard et al. 2007). In this paper, Par polarity protein complex is activated downstream of Cdc42 during Rap1-induced T cell polarization and the activation of the Par complex leads to activation of Rac1 through Tiam1, which then regulates actin remodeling required for T cell polarization. This may lead us to allow for the possible association of Rap1 with the Par complex during the PDGF-induced formation of leading edge structures in NIH3T3 cells.

In agreement with the observations that Rap1GAP-overexpressing and Rap1-knockdown NIH3T3 cells failed to form leading edge structures in response to PDGF, Rap1GAP-overexpressing and Rap1-knockdown NIH3T3 cells showed a significant impairment of cell movement. Compared with GFP-expressing control NIH3T3 cells, Rap1GAP-overexpressing NIH3T3 cells showed an approximately 50% reduction in random cell movement and a nearly 40% decrease in directional cell movement, as determined by the phagokinetic track motility assay and the Boyden chamber assay, respectively. Likewise, Rap1-knockdown NIH3T3 cells showed an approximately 50% reduction in random cell movement and a >60% decrease in directional cell movement as compared with control NIH3T3 cells. Hence, activation of Rap1 is needed for cell movement. It was previously shown that a constitutively active mutant of Rap1 suppressed cell motility of NBT-II rat bladder carcinoma cells (Valles et al. 2004). Indeed, we observed that
Rap1-CA did not increase, but rather decreased directional cell movement by 14% as estimated by the Boyden chamber assay (unpublished data). Thus, it is likely that coordinated regulation of dynamic activation and inactivation of Rap1, but not persistent activation of Rap1, is important for cell movement.

It was reported that Rap1 localizes mainly at perinuclear regions, such as the Golgi apparatus and late endosomes, and partly at the plasma membrane (Beranger et al. 1991). Signaling initiated by diverse extracellular stimuli is capable of activating Rap1. Ligands for receptor tyrosine kinases, such as PDGF, epidermal growth factor, and nerve growth factor, and G protein-coupled receptor agonists like lysophosphatidic acid, rapidly induce activation of Rap1 (Zwartkruis et al. 1998). However, it has not been determined how activation of Rap1 regulates the formation of leading edge structures and cell movement. We showed here by the pull-down assay that PDGF rapidly induces activation of Rap1 in NIH3T3 cells sparsely plated on vitronectin-coated dishes and that this effect was transient; activation of Rap1 was observed only during the 1 min following stimulation with PDGF. Furthermore, immunofluorescence examinations demonstrated that Rap1 is locally activated by PDGF at leading edges. In terms of Rap1 activation by PDGF, Zwartkruis et al. reported a critical role of phospholipase C (PLC)-mediated signaling (Zwartkruis et al. 1998). They demonstrated that the PDGF-induced activation of Rap1 was inhibited by U73122, an inhibitor of PLC, and BAPTA-AM, an intracellular calcium chelator, in Rat-1 fibroblasts. However, they did not show whether PLC-mediated signaling is important for the activation of perinuclear Rap1 or membrane Rap1. Yokote et al. (Yokote et al. 1998) reported that Crk/C3G forms a complex with PDGF receptor. They showed that Crk interacts with the α-chain of PDGF receptor in response to PDGF and then forms a complex with C3G. Although the formation of this ternary complex occurs at the plasma membrane and may contribute to the activation of Rap1, they did not show whether Crk/C3G regulates the PDGF-induced activation of Rap1 at the plasma membrane. In the present study, we demonstrated by the pull-down assay that the PDGF-induced activation of Rap1 is suppressed in NIH3T3 cells transiently expressing a dominant negative mutant of Crk or C3G. We also showed accumulation of Crk and C3G at peripheral ruffles over lamellipodia, where Rap1, PDGF receptor, and RalGDS, a downstream effector of Rap1, accumulated. These results strongly suggest that, upon stimulation with PDGF, Crk and C3G may be recruited to PDGF receptor at peripheral ruffles, followed by the recruitment and activation of Rap1, which leads to the recruitment of RalGDS. These results also indicate that the activation of Rap1 in response to PDGF locally occurs at leading edges. We demonstrated, for the first time, that this local activation of Rap1 at leading edges is
critical for the formation of leading edge structures in response to PDGF, because overexpression of Rap1GAP, which inactivates Rap1, and knockdown of Rap1 inhibited the accumulation of upstream regulators and a downstream effector of Rap1, namely PDGF receptor, Crk, C3G, and RalGDS, at peripheral ruffles, and thereby resulted in a failure of the cells to develop leading edge structures. The pull-down assay showed that activation of Rap1 was seen at 1 min following stimulation with PDGF, whereas immunofluorescence examinations revealed that accumulation of Rap1 at peripheral ruffles over lamellipodia was observed 30 min after stimulation with PDGF. The delayed response to PDGF in immunofluorescence experiments is likely due to the time required for PDGF to reach the cells on µ-slide VI flow dishes and for the cells to become polarized.

Rho family small G proteins have been suggested to be critical for directional cell movement, but the mechanisms underlying coordinated regulation of their activities at local sites, in response to extracellular stimuli, are still unclear. It is also unknown where activation of Rap1, which regulates crosstalk between Rap1 and the Rho family small G proteins during cell movement, occurs. In the present study, we demonstrated that suppression of Rap1 activity by overexpression of Rap1GAP or knockdown of Rap1 did inhibit the PDGF-induced directional movement, suggesting that Rap1 also regulates directionality of cell movement. Recently, a role for Rac1 in determining the directionality of cell movement has been proposed; increasing Rac1 activity promotes random migration, whereas decreasing Rac1 activity induces directional migration (Pankov et al. 2005). Rap1 signaling has a crosstalk with Rho family G proteins, including Rac1 and RhoA (Maillet et al. 2003; Yamada et al. 2005). It was shown that Rap1, through Rac GEFs such as Vav2 and Tiam1, regulates membrane protrusions at the cell periphery during spreading (Arthur et al. 2004). We showed here that Rac1, PDGF receptor, Vav2 (a Rac GEF), and PAK (a Rac effector) were accumulated at peripheral ruffles over lamellipodia of leading edges, suggesting the local activation of Rac1 at peripheral ruffles. Impairment of the accumulation of Rac1 and an upstream regulator and a downstream effector of Rac1 at peripheral ruffles in Rap1GAP-overexpressing cells and Rap1-knockdown cells indicates that the recruitment of activated Rap1 to leading edges is required for the PDGF-induced local activation of Rac1 at leading edges. Inhibition of activation of Rac1 may be responsible for the impaired formation of leading edge structures in Rap1GAP-overexpressing cells and Rap1-knockdown cells. Taken together, the results of the present study indicate that the Rap1-dependent local activation of Rac1 at leading edges controls directional cell movement. Moreover, the Rap1-dependent local activation of Rac1 at leading edges is required for the PDGF-induced formation of leading edge structures during directional cell movement.
A crosstalk between Rap1 and RhoA at leading edges during cell movement is poorly understood. We showed here that overexpression of Rap1GAP enhanced the accumulation of integrin to focal adhesions. Identical results were obtained in Rap1-knockdown cells. These results appear to reflect activation of RhoA in NIH3T3 cells in which Rap1 is inactivated by overexpression of Rap1GAP or knockdown of Rap1. Consistently, we showed here that stress fiber formation was enhanced in Rap1GAP-overexpressing NIH3T3 cells as well as Rap1-knockdown NIH3T3 cells. Further studies are necessary to investigate whether Rap1 may regulate RhoA activity at leading edges as well and, if so, to clarify the molecular mechanism by which Rap1 regulates RhoA activity.
Experimental Procedures

Plasmid constructions
Expression vectors for GFP-tagged Rap1 (pEGFP-Rap1), GFP-tagged Rap1GAP (pEGFP-Rap1GAP), GFP-tagged Rap1-CA (pEGFP-Rap1-CA), GFP-tagged Rac1 (pEGFP-Rac1), Myc-tagged Rac1 (pEF-BOS-Myc-Rac1), and a Myc-tagged dominant negative mutant of Vav2 (pClneo-Myc-Vav2-DN) were prepared as described (Fukuyama et al. 2005; Yamada et al. 2005). Expression vectors for a FLAG-tagged dominant-negative mutant of CrkI (pIRM21-FLAG-CrkI-W169L) and a dominant-negative mutant of C3G (pCAGGS-C3G-ΔCD) were kindly provided by Dr. M. Matsuda (Kyoto University, Kyoto, Japan).

Cell culture, transfection, and siRNA experiments
NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum. For transient expression experiments, cells were transfected with various expression vectors using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. For knocking down of Rap1, we obtained 3 sets of Stealth RNAis for Rap1A and Rap1B (Invitrogen) and 2 sets of them (Rap1 siRNA-I and -II) effectively induced knockdown of Rap1 in NIH3T3 cells. Cells were transfected with Stealth RNAi using Lipofectamine RNAiMAX (Invitrogen). Stealth RNAi negative control (Invitrogen) was used as a control. Forty-eight hrs after transfection, cells were subjected to each experiment.

Antibodies and reagents
Rat anti-Necl-5 monoclonal antibody (mAb) and rabbit anti-Vav2 polyclonal antibody (pAb) were prepared as described (Ikeda et al. 2003; Kodama et al. 2000). Hamster anti-integrin αV and β3 mAbs (BD Biosciences), rabbit anti-PDGF receptor mAb (Abcam), mouse anti-actin mAb (Chemicon International), rabbit anti-Rap1 pAb (Santa Cruz Biotechnology), mouse anti-Rap1 mAb (BD Biosciences), mouse anti-Ras mAb (BD Biosciences), mouse anti-Rac1 mAb (Upstate Biotechnology), mouse anti-Crk mAb (Santa Cruz Biotechnology), mouse anti-C3G mAb (Santa Cruz Biotechnology), rabbit anti-RalGDS pAb (Santa Cruz Biotechnology), rabbit anti-PAK pAb (Santa Cruz Biotechnology), rabbit anti-GFP pAb (Medical and Biological Laboratories), rabbit anti-Myc-tag pAb (Cell Signaling), mouse anti-Myc-tag mAb (Cell Signaling), and rhodamine-phalloidin (Molecular Probes) were purchased from commercial sources. Horseradish peroxidase-conjugated secondary Abs were purchased from Amersham Biosciences. Fluorophore
(FITC, Cy3, and Cy5)-conjugated secondary Abs were purchased from Jackson Immuno Research. Human recombinant PDGF-BB was purchased from PEPROTECH. Vitronectin was purified from human plasma (Kohjinbio) as described (Yatohgo et al. 1988).

**Directional stimulation by PDGF**

To generate a concentration gradient of PDGF, a μ-Slide VI flow (uncoated; Ibidi) was used (Minami et al. 2007). In brief, the μ-Slide VI flow has six parallel channels, which were coated with 5 µg/ml vitronectin, according to the manufacturer’s protocol. Cells were plated at a density of 5 x 10³ cells per square centimeter, cultured for 16 hrs, and starved of serum with DMEM containing 0.5% bovine serum albumin (BSA) for 1 hr. A concentration gradient of PDGF was applied using DMEM containing 0.5% BSA and 30 ng/ml PDGF according to the manufacturer’s protocol. PDGF was applied from the bottom side in each figure image. After incubation for the indicated periods of time, cells were fixed with acetone/methanol (1:1) or 4% paraformaldehyde, incubated with 1% BSA in phosphate-buffered saline (PBS), and then incubated with 20% BlockAce (Dainihon Sumitomo Seiyaku) in PBS (Fujito et al. 2005). Fluorescent signals were visualized using a confocal laser scanning microscope (LSM510 META, Carl Zeiss). The number of the cells which form leading edge structures was blindly counted.

**Time-lapse fluorescence microscopy**

Time-lapse fluorescence microscopy was performed as follows. NIH3T3 cells, transfected with pEGFP-Rap1GAP, pEGFP-Rap1, or pEGFP-Rac1, were sparsely plated on vitronectin-coated glass μ-Slide VI flow, cultured in the serum-containing medium for 18 hrs, and then starved of serum with DMEM containing 0.5% BSA for 1 hr. A concentration gradient of PDGF was applied as described above. Fluorescent signals were visualized by a confocal laser scanning microscope. Differential interference contrast (DIC) and fluorescence images were recorded in intervals of 2 min.

**Phagokinetic track motility assay**

A uniform carpet of gold particles was prepared on glass coverslips as described (Albrecht-Buehler 1977). In brief, colloidal gold-coated coverslips were placed in 35-mm dishes and cells were plated at a density of 2 x 10³ cells/35-mm dish. Cells were starved of serum with DMEM supplemented with 0.5% BSA for 1 hr and then cultured for 18 hrs. The samples were fixed with 3.7% formaldehyde and cell motility was analyzed by measuring the areas free of gold particles.
around a single cell. At least 30 independent samples in each experiment were analyzed to
determine the areas.

**Boyden chamber assay**

Cell migration was investigated using the Boyden chamber assay as described (Ikeda et al. 2004). In brief, Falcon cell culture inserts with translucent PET membranes (8.0-µm pores, Becton Dickinson Labware) were coated with 5 µg/ml vitronectin for 1 hr and then blocked with 1% BSA at 37 °C for 30 min. NIH3T3 cells, which had been starved of serum with DMEM supplemented with 0.5% BSA for 1 hr, were detached with 0.05% trypsin and 0.53 mM EDTA, and then treated with a trypsin inhibitor (Sigma-Aldrich). Cells were then re-suspended in DMEM supplemented with 0.5% BSA and plated at a density of 5 x 10⁴ cells/insert. Cells were cultured at 37 °C for 4 hrs in the presence of 30 ng/ml PDGF-BB. PDGF-BB was added only to the bottom well in order to generate a concentration gradient. The inserts were washed with PBS and the cells were fixed with 3.7% formaldehyde. Cells which had not migrated were removed by wiping the top of the membrane with a cotton swab. The number of GFP-positive cells in five randomly chosen fields per insert was counted by fluorescence microscopic examination and calibrated by transfection efficiency, which was determined by the ratio of GFP-positive cells to total cells plated on the microslides.

**Pull-down assays for Rap1 and Rac1**

Pull-down assays were performed as described (Fukuhara et al. 2004). In brief, cells were plated on vitronectin-coated dishes and cultured overnight. After a 1-hr serum starvation, cells were treated with PDGF and then subjected to the pull-down assays for Rap1 and Rac1.

**Statistical analysis**

All data are expressed as the mean ± SEM. Statistical analysis was performed by unpaired Student's t test. A P value of < 0.05 was considered statistically significant.
References

Fujito, T., Ikeda, W., Kakunaga, S., et al. (2005) Inhibition of cell movement and proliferation by


Acknowledgements

We thank Dr. M. Matsuda (Kyoto University, Kyoto, Japan) for providing us with plasmids. This work was supported by grants-in-aid for Scientific Research, for Cancer Research, and for Initiatives for Attractive Education in Graduate Schools: “Program of Raising Young Research Leaders in Biomedical Sciences” from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (2006, 2007).
Figure 1. Necessity of activation of Rap1 for the PDGF-induced formation of leading edge structures.  
A, time-lapse fluorescence microscopy of PDGF-stimulated NIH3T3 cells cultured on µ-slide dishes. NIH3T3 cells were transfected with GFP-tagged Rap1GAP and directionally stimulated by PDGF. Times after the PDGF application are shown in min.  

a, NIH3T3 cells;  
b, NIH3T3 cells transiently expressing GFP-Rap1GAP.  

Scale bars, 20 µm.  

B, inhibition of the PDGF-induced formation of leading edge structures by overexpression of Rap1GAP.  

a-c, immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. Cells were cultured in the presence of PDGF for 30 min and stained with various combinations of the anti-PDGF receptor mAb, the anti-integrin β3 mAb, the anti-Necl-5 mAb, and rhodamine-phalloidin (for F-actin).  

Arrow heads, leading edges; insets, higher magnification images of leading edges; scale bars, 20 µm. The results shown are representative of three independent experiments.  

a, low magnification images;  
b, NIH3T3 cells;  
c, NIH3T3 cells transiently expressing GFP-Rap1GAP;  
d, quantitative analysis of leading edge structures in wild-type and GFP-Rap1GAP-expressing NIH3T3 cells. To assess the leading edge formation, the signal for F-actin, a major component of peripheral ruffles, was observed. At least 100 cells in each type of cells were blindly counted and classified into four categories: “lamellipodia with ruffles”, “lamellipodia without ruffles”, “thin and angular shape”, and “round shape”. The results shown are the means ± S.E. of three independent experiments.  

C, immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. Cells were stained with the anti-PDGF receptor mAb, the anti-integrin β3 mAb, and the anti-Necl-5 mAb, and rhodamine-phalloidin (for F-actin).  

Arrow heads, leading edges; insets, higher magnification images of leading edges; scale bars, 20 µm. The results shown are representative of three independent experiments.

Figure 2. Necessity of activation of Rap1 for the PDGF-induced formation of leading edge structures.  

A, knockdown of Rap1 by transfection of Rap1A and Rap1B siRNAs. The cell lysates of NIH3T3 cells untransfected or transfected with the indicated siRNAs and plasmids were subjected to Western blotting using the anti-Rap1 pAb, the anti-Ras mAb, and the anti-actin mAb.  

a, NIH3T3 cells transfected with one set of Rap1A and Rap1B siRNAs (Rap1 siRNA-I), the other set of Rap1A and Rap1B siRNAs (Rap1 siRNA-II), or non-silencing control siRNA.  

b, NIH3T3 cells co-transfected with Rap1 siRNA-I or non-silencing control siRNA and with GFP or GFP-Rap1.  

B, time-lapse fluorescence microscopy of PDGF-stimulated NIH3T3 cells cultured on
vitronectin-coated µ-slide dishes. NIH3T3 cells were transfected with Rap1 siRNA-I, Rap1 siRNA-II, or non-silencing control siRNA, or co-transfected with Rap1 siRNA-I and siRNA-resistant GFP-Rap1. Times after the PDGF application are shown in min. **Arrow heads,** leading edges; **scale bars,** 20 µm.  

**a**, NIH3T3 cells transfected with non-silencing control siRNA;  
**b**, NIH3T3 cells transfected with Rap1 siRNA-I;  
**c**, NIH3T3 cells transfected with Rap1 siRNA-II;  
**d**, NIH3T3 cells transfected with Rap1 siRNA-I and GFP-Rap1.  

**C and D,** inhibition of the PDGF-induced formation of leading edge structures by knockdown of Rap1. **Ca-Cc,** immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. Cells were transfected with Rap1 siRNA-I and GFP-Rap1 and stained with various combinations of the anti-PDGF receptor mAb, the anti-integrin β3 mAb, the anti-Necl-5 mAb, and rhodamine-phalloidin (for F-actin). **Arrow heads,** leading edges; **insets,** higher magnification images of leading edges; **scale bars,** 20 µm. The results shown are representative of three independent experiments.  

**a,** low magnification images;  
**b,** NIH3T3 cells transfected with Rap1 siRNA-I;  
**c,** NIH3T3 cells transfected with Rap1 siRNA-I and GFP-Rap1.  

**Cd,** quantitative analysis of leading edge structures in NIH3T3 cells transfected with Rap1A and Rap1B siRNAs or non-silencing control siRNA. NIH3T3 cells were transfected with Rap1 siRNA-I, Rap1 siRNA-II, or non-silencing control siRNA, or co-transfected with Rap1 siRNA-I and siRNA-resistant GFP-Rap1. The leading edge formation was assessed as described in the legend of Fig. 1Bd.  

**D,** immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. NIH3T3 cells were transfected with Rap1 siRNA-I, Rap1 siRNA-II, or non-silencing control siRNA, or co-transfected with Rap1 siRNA-I and siRNA-resistant GFP-Rap1.  

**a,** NIH3T3 cells transfected with control siRNA;  
**b,** NIH3T3 cells transfected with Rap1 siRNA-I;  
**c,** NIH3T3 cells transfected with Rap1 siRNA-II;  
**d,** NIH3T3 cells transfected with Rap1 siRNA-I and GFP-Rap1GAP.

**Figure 3.** **Necessity of activation of Rap1 for the PDGF-induced cell movement. A,** inhibition of random cell movement by Rap1GAP or knockdown of Rap1. NIH3T3 cells transiently expressing GFP-Rap1GAP or GFP, or transfected with Rap1 siRNA-I or non-silencing control siRNA were starved of serum for 1 hr and incubated on colloidal gold-coated coverslips coated with vitronectin in the culture media for 18 hrs.  

**a,** GFP-Rap1GAP- or GFP-expressing cells;  
**b,** Rap1 siRNA-I- or non-silencing control siRNA-transfected cells. **Scale bar,** 50 µm.  

Bars in the quantitative analysis represent the areas free of gold particles around a single cell ($n = 30$) and are expressed as the means ± S.E. of three independent experiments. *, $P<0.05$.  

**B,**
inhibition of directional cell movement by Rap1GAP or knockdown of Rap1. NIH3T3 cells transiently expressing GFP-Rap1GAP or GFP, or transfected with Rap1 siRNA-I or non-silencing control siRNA were starved of serum and incubated on cell culture inserts coated with vitronectin in the presence of 30 ng/ml of PDGF in the bottom wells for 4 hrs. The GFP-positive migrated cells were counted. a, GFP-Rap1GAP- or GFP-expressing cells; b, Rap1 siRNA-I or non-silencing control siRNA-transfected cells. *, \( P<0.05 \). The results shown are the means ± S.E. of six independent experiments.

**Figure 4. Activation of Rap1 in response to PDGF.** Rap1 activity measured by the pull-down assay. A, NIH3T3 cells were plated on vitronectin-coated dishes, starved of serum, and cultured in the presence of 15 ng/ml PDGF for the indicated periods of time. B, NIH3T3 cells were plated on vitronectin-coated dishes, starved of serum, and cultured in the presence of indicated concentrations of PDGF for 1 min. Cell lysates were subjected to the pull-down assay followed by Western blotting using the anti-Rap1 pAb. C, inhibition of Rap1 by Rap1GAP. NIH3T3 cells, co-transfected with GFP-tagged Rap1 and either of GFP-Rap1GAP or GFP, were plated on vitronectin-coated dishes, starved of serum, and then cultured in the presence of 15 ng/ml PDGF for 1 min. Cell lysates were subjected to the pull-down assay followed by Western blotting using the anti-GFP pAb. D, involvement of the Crk-C3G complex in the PDGF-induced activation of Rap1. NIH3T3 cells, co-transfected with GFP-tagged Rap1 and one of a dominant-negative mutant of CrkI (CrkI-W169L), C3G (C3G-ΔCD), or vector alone (Mock), were plated on vitronectin-coated dishes, starved of serum, and then cultured in the presence of 15 ng/ml PDGF for 1 min. Cell lysates were subjected to the pull-down assay followed by Western blotting using the anti-GFP pAb. a, CrkI-W169L- or Mock-transfected NIH3T3 cells; b, C3G-ΔCD- or Mock-transfected NIH3T3 cells. The results shown are representative of three independent experiments.

**Figure 5. Activation of Rap1 at leading edges where PDGF receptor clustered with Necl-5 and integrin \( \alpha V \beta 3 \).** A, time-lapse fluorescence microscopy of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. NIH3T3 cells were transfected with GFP-Rap1. Times after the PDGF application are shown in min. a, DIC images; b, fluorescence images of GFP-Rap1. Arrow heads, leading edges; scale bars, 20 µm. B, immunofluorescence images of NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. NIH3T3 cells were cultured in the presence of PDGF for the indicated periods of time and stained with various combinations of the
indicated Abs. Images displayed at the bottom are higher magnification images of the areas surrounded with dotted squares.  

A, the anti-Rap1 mAb and the anti-PDGF receptor mAb;  
B, the anti-Rap1 pAb and the anti-Crk mAb;  
C, the anti-Rap1 pAb and the anti-C3G mAb;  
D, the anti-Rap1 mAb and the anti-RalGDS pAb.  

C, inhibition of the PDGF-induced accumulation of Rap1 by Rap1GAP. NIH3T3 cells, untransfected or transfected with GFP-Rap1GAP, were cultured in the presence of PDGF for 30 min and stained with various combinations of the indicated Abs.  
A, the anti-Rap1 mAb and the anti-PDGF receptor mAb;  
B, the anti-Rap1 pAb and the anti-Crk mAb;  
C, the anti-Rap1 pAb and the anti-C3G mAb;  
D, the anti-Rap1 mAb and the anti-RalGDS pAb.  

D, inhibition of the PDGF-induced accumulation of Rap1 by knockdown of Rap1. NIH3T3 cells, transfected with either of Rap1 siRNA-I or Rap1 siRNA-I and GFP-Rap1, were cultured in the presence of PDGF for 30 min and stained with various combinations of the indicated Abs.  
A, the anti-Rap1 mAb and the anti-PDGF receptor mAb;  
B, the anti-Rap1 pAb and the anti-Crk mAb;  
C, the anti-Rap1 pAb and the anti-C3G mAb;  
D, the anti-Rap1 mAb and the anti-RalGDS pAb.  

**Arrow heads,** leading edges;  
**scale bars,** 20 µm.  
The results shown are representative of three independent experiments.

**Figure 6. Activation of Rac1 downstream of Rap1.**  
A, time-lapse fluorescence microscopy of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. NIH3T3 cells were transfected with GFP-Rac1. Times after the PDGF application are shown in min.  
A, DIC images;  
B, fluorescence images of GFP-Rac1.  
**Arrow heads,** leading edges;  
**scale bars,** 20 µm.  
B, immunofluorescence images of NIH3T3 cells cultured on vitronectin-coated µ-slide dishes. NIH3T3 cells were cultured in the presence of PDGF for the indicated periods of time and stained with various combinations of the indicated Abs. Images displayed at the bottom are higher magnification images of the areas surrounded with dotted squares.  
A, the anti-Rac1 mAb and the anti-PDGF receptor mAb;  
B, the anti-Rac1 mAb and the anti-Vav2 pAb;  
C, the anti-Rac1 mAb and the anti-PAK pAb.  

C, inhibition of the PDGF-induced accumulation of Rac1 by Rap1GAP. NIH3T3 cells, untransfected or transfected with GFP-Rap1GAP, were cultured in the presence of PDGF for 30 min and stained with various combinations of the indicated Abs.  
A, the anti-Rac1 mAb and the anti-PDGF receptor mAb;  
B, the anti-Rac1 mAb and the anti-Vav2 pAb;  
C, the anti-Rac1 mAb and the anti-PAK pAb.  

D, inhibition of the PDGF-induced accumulation of Rac1 by knockdown of Rap1. NIH3T3 cells, transfected with either of Rap1 siRNA-I or Rap1 siRNA-I and GFP-Rap1, were cultured in the presence of PDGF for 30 min and stained with various combinations of the indicated Abs.  
A, the anti-Rac1 mAb and the anti-PDGF receptor mAb;  
B, the
anti-Rac1 mAb and the anti-Vav2 pAb; c, the anti-Rac1 mAb and the anti-PAK pAb. *Arrow heads*, leading edges; *scale bars*, 20 µm. The results shown are representative of three independent experiments.

**Figure 7. Involvement of Vav2 in the Rap1-mediated Activation of Rac1.**  
A, activation of Rac1 downstream of Rap1.  
*a*, NIH3T3 cells, transfected with Rap1 siRNA-I or non-silencing control siRNA, were plated on vitronectin-coated dishes, starved of serum, and then cultured in the presence of 15 ng/ml PDGF for 1 min. Cell lysates were subjected to the pull-down assay followed by Western blotting using the anti-Rac1 mAb.  
*b*, HEK 293 cells, co-transfected with Myc-tagged Rac1 and either of GFP-Rap1-CA or GFP, were plated on vitronectin-coated dishes and cultured in the absence of serum. Cell lysates were subjected to the pull-down assay followed by Western blotting using the anti-Myc mAb.  
B, inhibition of Rac1 activation by Vav2-DN.  
*a*, NIH3T3 cells, co-transfected with Myc-tagged Rac1 and either of Vav2-DN or Mock, were plated on vitronectin-coated dishes, starved of serum, and then cultured in the presence of 15 ng/ml PDGF for 1 min. Cell lysates were subjected to the pull-down assay followed by Western blotting using the anti-Myc mAb.  
*b*, HEK 293 cells, transfected with Myc-tagged Rac1, either of GFP-Rap1-CA or GFP, and either of Vav2-DN or Mock, were plated on vitronectin-coated dishes and cultured in the absence of serum. Cell lysates were subjected to the pull-down assay followed by Western blotting using the anti-Rac1 mAb.
Fig. 1A Takahashi, et al.

Aa  Wild-type

Ab  Rap1GAP
**Fig. 1B.** Takahashi, et al.

**Ba**

GFP (green)
F-Actin (red)
PDGF receptor (blue)

GFP (green)
Integrin β3 (red)
Nec-5 (blue)

**Bb** Rap1GAP (-)

F-Actin
PDGF receptor (blue)
Merge

Integrin β3
Nec-5 (blue)
Merge

**Bc** Rap1GAP (+)

F-Actin
PDGF receptor (blue)
Merge

Integrin β3
Nec-5 (blue)
Merge

**Bd**

<table>
<thead>
<tr>
<th>Lamellipodia w/ ruffles</th>
<th>Lamellipodia w/o ruffles</th>
<th>Thin / angular</th>
<th>Round</th>
</tr>
</thead>
</table>

**Leading edge formation (%)**

<table>
<thead>
<tr>
<th>Rap1GAP (-)</th>
<th>Rap1GAP (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bar Graph</td>
<td>Bar Graph</td>
</tr>
</tbody>
</table>

Scale bars: 20 μm.
Fig. 1C  Takahashi, et al.

Middle section

Basal section
Fig. 2A Takahashi, et al.

Aa

<table>
<thead>
<tr>
<th>siRNA:</th>
<th>–</th>
<th>Rap1-I</th>
<th>Rap1-II</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rap1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ras</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ab

<table>
<thead>
<tr>
<th>GFP:</th>
<th>+</th>
<th>–</th>
<th>+</th>
<th>–</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-Rap1:</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>siRNA:</td>
<td>Control</td>
<td>Rap1-I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB: Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

→ GFP-Rap1

→ Rap1
Fig. 2B Takahashi, et al.

Ba  Control siRNA

Bb  Rap1 siRNA-I

Bc  Rap1 siRNA-II

Bd  Rap1 siRNA-I + GFP-Rap1
Fig. 2C. Takahashi, et al.

**Ca**
GFP (green) PDGF receptor (red) Integrin β3 (blue)
GFP (green) F-Actin (red) Necl-5 (blue)

**Cb**
GFP-Rap1 (-)
PDGF receptor
Integrin β3 (blue)
Merge
F-Actin
Necl-5 (blue)
Merge

**Cd**

Graph showing leading edge formation with different conditions:
- Control siRNA
- Rap1 siRNA
- Rap1 siRNA-I
- Rap1 siRNA-II
- Rap1 siRNA-I + GFP-Rap1

Legend:
- Lamellipodia w/ ruffles
- Lamellipodia w/o ruffles
- Thin / angular
- Round

**Cc**
GFP-Rap1 (+)
PDGF receptor
Integrin β3 (blue)
Merge
F-Actin
Necl-5 (blue)
Merge
Fig. 2D. Takahashi, et al.

**Da** Control siRNA

- Rap1
- F-Actin
- Necl-5 (blue)
- Merge

**Db** Rap1 siRNA-I

- Rap1
- PDGF receptor
- Integrin β3 (blue)
- Merge

- Rap1
- PDGF receptor
- Integrin β3 (blue)
- Merge
Fig. 2D. Takahashi, et al.

**Dc** Rap1 siRNA-II

<table>
<thead>
<tr>
<th>Rap1</th>
<th>F-Actin</th>
<th>Necl-5 (blue)</th>
<th>Merge</th>
</tr>
</thead>
</table>

**Dd** Rap1 siRNA-I + GFP-Rap1

<table>
<thead>
<tr>
<th>GFP-Rap1</th>
<th>F-Actin</th>
<th>Necl-5 (blue)</th>
<th>Merge</th>
</tr>
</thead>
</table>

**Legend**
- **Rap1**: Red
- **F-Actin**: Green
- **Necl-5 (blue)**: Blue
- **Integrin β3 (blue)**: Blue
- **Merge**: Overlay of all channels
Fig. 3. Takahashi, et al.

**Aa**
- GFP
- GFP-Rap1GAP

**Ba**
- Area (x 10³ µm²)
- Migrated cells (x 10³/well)

**Ab**
- Control siRNA
- Rap1 siRNA-1

**Bb**
- Area (x 10³ µm²)
- Migrated cells (x 10³/well)
Fig. 4. Takahashi, et al.

A

Time (min):
0 1 2 5 10 20 30 60
GTP-Rap1
Total Rap1

B

PDGF (ng/ml):
0 2.5 5 10 15
GTP-Rap1
Total Rap1

C

PDGF:
- + - +
GTP-GFP-Rap1
Total GFP-Rap1
GFP GFP-Rap1GAP

Da

PDGF:
- + - +
GTP-GFP-Rap1
Total GFP-Rap1
Mock Crkl-W169L

Db

PDGF:
- + - +
GTP-GFP-Rap1
Total GFP-Rap1
Mock C3G-ΔCD
Fig. 5A Takahashi, et al.

Aa  DIC

Ab  GFP·Rap1
Fig. 5B. Takahashi, et al.

<table>
<thead>
<tr>
<th>Ba</th>
<th>0</th>
<th>10</th>
<th>30 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rap1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>PDGF receptor</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Merge</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bb</th>
<th>0</th>
<th>10</th>
<th>30 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rap1</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Crk</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>Merge</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bc</th>
<th>0</th>
<th>10</th>
<th>30 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rap1</td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
</tr>
<tr>
<td>C3G</td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td>Merge</td>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bd</th>
<th>0</th>
<th>10</th>
<th>30 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rap1</td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
</tr>
<tr>
<td>RalGDS</td>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
<td><img src="image33.png" alt="Image" /></td>
</tr>
<tr>
<td>Merge</td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Fig. 5C. Takahashi, et al.

Ca: Rap1 (red), PDGF receptor (blue), GFP-Rap1GAP
Cb: Rap1 (red), Crk (blue), GFP-Rap1GAP
Cc: Rap1 (red), C3G (blue), GFP-Rap1GAP
Cd: Rap1 (red), RalGDS (blue), GFP-Rap1GAP
Fig. 5D. Takahashi, et al.
Fig. 6A Takahashi, et al.

Aa DIC

Ab GFP-Rac1
Fig. 6B. Takahashi, et al.

<table>
<thead>
<tr>
<th></th>
<th>Ba</th>
<th>0</th>
<th>10</th>
<th>30 (min)</th>
<th></th>
<th>Bb</th>
<th>0</th>
<th>10</th>
<th>30 (min)</th>
<th></th>
<th>Bc</th>
<th>0</th>
<th>10</th>
<th>30 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rac1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rac1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rac1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDGF receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vav2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PAK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Merge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- Rac1: Rho-associated protein kinase family member 1
- PDGF receptor: Platelet-derived growth factor receptor
- Vav2: Nuclear factor-κB inhibitor
- PAK: p21-activated kinase 1
Fig. 6C. Takahashi, et al.

Ca: Rac1 (red), PDGF receptor (blue), GFP-Rap1GAP
Cb: Rac1 (red), Vav2 (blue), GFP-Rap1GAP
Cc: Rac1 (red), PAK (blue), GFP-Rap1GAP
Fig. 6D. Takahashi, et al.
Fig. 7. Takahashi, et al.

**Aa**

<table>
<thead>
<tr>
<th>PDGF:</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP-Rac1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Rac1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Control siRNA | Rap1 siRNA

**Ab**

<table>
<thead>
<tr>
<th></th>
<th>GTP-Myc-Rac1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Myc-Rac1</td>
<td></td>
</tr>
</tbody>
</table>

GFP | GFP-Rap1-CA

**Ba**

<table>
<thead>
<tr>
<th>PDGF:</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP-Myc-Rac1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Myc-Rac1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mock | Vav2-DN

**Bb**

<table>
<thead>
<tr>
<th></th>
<th>GTP-Myc-Rac1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Myc-Rac1</td>
<td></td>
</tr>
</tbody>
</table>

GFP | GFP-Rap1-CA | GFP | GFP-Rap1-CA

Mock | Vav2-DN