ZRP-1 controls Rho GTPase-mediated actin reorganization by localizing at cell-matrix and cell-cell adhesions

Chen-Yu Bai[1,*], Miho Ohsugi[1,*], Yoshinori Abe[2] and Tadashi Yamamoto[1,‡]

[1]Division of Oncology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
[2]Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki 211-8533, Japan
*These authors contributed equally to this work
‡Author for correspondence (e-mail: tyamamot@ims.u-tokyo.ac.jp)

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Summary

Focal adhesion protein ZRP-1/TRIP6 has been implicated in actin reorganization and cell motility. The role of ZRP-1, however, remained obscure because previously reported data are often conflicting one another. In the present study, we examined roles of ZRP-1 in HeLa cells. ZRP-1 is localized to the cell-cell contact sites as well as to cell-matrix contact sites in HeLa cells. RNA-interference-mediated depletion of ZRP-1 from HeLa cells revealed that ZRP-1 is essential not only for the formation of stress fibers and assembly of mature focal adhesions, but also for the actin reorganization at cell-cell contact sites and for correct cell-cell adhesion and, thus, for collective cell migration. Impairment of focal adhesions and stress fibers caused by ZRP-1 depletion has been associated with reduced tyrosine phosphorylation of FAK. However, maturation of focal adhesions could not be recovered by expression of active FAK. Interestingly, stress fibers in ZRP-1-depleted cells were ameliorated by exogenous expression of RhoA. We also found that total Rac1 activity is elevated in ZRP-1-depleted cells, resulting in abnormal burst of actin polymerization and dynamic membrane protrusions. Taken together, we conclude that that ZRP-1 plays a crucial role in coupling the cell-matrix/cell-cell-contact signals with Rho GTPase-mediated actin remodeling by localizing at cell-matrix and cell-cell contact sites.

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Key words: Actin reorganization, Focal adhesion, Cell-cell adhesion, Collective cell migration, Rho family GTPase

Introduction

The actin cytoskeleton provides mechanical support for the cell, determines cell shape, enables cell movement, and participates in cell-matrix and cell-cell adhesions. It is well established that the actin cytoskeleton is regulated by Rho GTPases. The three prototypic Rho GTPases, RhoA, Rac1 and Cdc42, are best known for their effects on the actin cytoskeleton. Activation of RhoA, Rac1 and Cdc42 leads to assembly of actin stress fibers, protrusive actin-rich lamellipodia and protrusive actin-rich filopodia, respectively (Etienne-Manneville and Hall, 2002). When a cell is migrating, it extends membrane protrusions or lamellipodia through a Rac-dependent mechanism. At the leading edge of an extended protrusion, focal complexes containing integrin receptors and cytoskeletal proteins are formed in a Rac-dependent manner (Nobes and Hall, 1999). A Rho-dependent mechanism then promotes growth of the focal complex into the focal adhesion (Greenwood and Murphy-Ullrich, 1998; Nobes and Hall, 1999). Thus, focal adhesion provides a tension point for contractile forces to move the cell forward (Horwitz et al., 1999).

Focal adhesions are specialized regions of cell adhesion to the extracellular matrix, where integrin receptors associate with a number of structural and signaling proteins to form a link with the actin cytoskeleton (Craig and Johnson, 1996; Jockusch et al., 1995; Schaller et al., 1994). These integrin-associated proteins include focal adhesion kinase (FAK), Src family kinases and scaffolding proteins, such as p130Cas, paxillin and vinculin (Jockusch et al., 1995). The importance of these molecules has been underscored by the results of gene knockout/knockdown experiments (Hagel et al., 2002; Ilic et al., 1995; Yano et al., 2004; Honda et al., 1998; Honda et al., 1999; Klinghoffer et al., 1999). Further, many of the integrin-associated proteins are tyrosine phosphorylated after integrin engagement to activate integrin-dependent signaling pathways. The activated signaling molecules play crucial roles in regulating multiple events, including cell adhesion and cell migration. For example, after integrin activation, FAK is autophosphorylated at Tyr397 (Cary et al., 1996), which serves as a binding site for Src family members. The FAK-Src complex then mediates phosphorylation of paxillin and p130Cas, which participate in the regulation of Rho family GTPases (DeMali et al., 2003). Tyr-phosphorylated p130Cas recruits the adaptor protein Crk into the developing focal complex (DeMali et al., 2003). The p130Cas-Crk complex activates Rac1 trough interaction with the DOCK180-ELMO guanine nucleotide exchange factor (GEF) (Brugnera et al., 2002; Kiyokawa et al., 1998a; Kiyokawa et al., 1998b; Sakai et al., 1994).

Like cell-matrix adhesions, cell-cell adhesions are

intimately associated both physically and functionally with the actin cytoskeleton. Cadherins, which are adhesion receptors, associate with the actin cytoskeleton through β-catenin and α-catenin. Following cadherin engagement, Rac1 is activated and regulates the actin cytoskeleton necessary for the formation of stable cell-cell adhesions (Kovacs et al., 2002). During embryonic development and carcinoma invasion, cells often show collective migration, a type of migration in which the assembly and disassembly of cell-matrix adhesions occur together with that of cell-cell adhesions. Coordination of these two different modes of adhesion is important in the migration process. However, the mechanisms and molecules involved in this cross-talk remain unknown.

ZRP-1 (zyxin-related protein 1), also known as TRIP6 (thyroid receptor-interacting protein 6), is another component of focal adhesions. ZRP-1 belongs to a subset of LIM-domain-containing proteins that include the prototypic members zyxin, Ajuba and lipoma-preferred partner (LPP) (Li et al., 2003; Marie et al., 2003; Petit et al., 2003), all of which localize to focal adhesions and cell-cell adhesions. All members of this family contain two distinct regions: a C-terminal half containing three LIM domains and a proline-rich N-terminal half containing one or two nuclear export sequences (Wang and Gilmore, 2001). There are conflicting reports regarding the effect of depletion and overexpression of ZRP-1 on cell motility, F-actin organization and focal-adhesion assembly. In 10T1/2 cells, overexpression of ZRP-1 slows cell migration (Yi et al., 2002). Depletion or overexpression of ZRP-1 decreases LPA-induced cell migration of ovarian carcinoma SKOV3 cells (Xu et al., 2004), whereas depletion of ZRP-1 in lung carcinoma A549 cells or epidermoid carcinoma A431 cells resulted in an increase of the cell migration rate induced by wounding (Guryanova et al., 2005). Deletion of ZRP-1 in human umbilical vein endothelial cells (HUVECs) leads to reduction of F-actin fibers, (Sanz-Rodriguez et al., 2004) and, in contrast, depletion from A549 or A431 cells leads to enhanced focal adhesion and stress fiber formation (Guryanova et al., 2005). In either case, ZRP-1 is involved in the assembly/disassembly cycle of focal adhesion, actin reorganization and cell migration. Several ZRP-1 interactors have been reported to bind to the LIM domains of ZRP-1. Some ZRP-1 interactors are involved in actin and/or focal adhesion assembly, and include the adaptor protein RIL, the tyrosine phosphatase hPTP1E (Cuppen et al., 2000; Murthy et al., 1999), OpaP (Williams et al., 1998), endoglin (Sanz-Rodriguez et al., 2004) and supervillin (Takizawa et al., 2006). Other ZRP-1-interacting proteins, such as the members of Cas family (Yi et al., 2002) and the LPA_2 receptor (Xu et al., 2004), are associated with cell motility. However, the impact of overexpressed or depleted ZRP-1 on the integrin-dependent signaling pathways, including the tyrosine phosphorylation status of focal-adhesion-associated signaling proteins and Rho GTPase activities, was not clear.

Here, we examined the roles of ZRP-1 in HeLa cervical carcinoma cells by RNA interference (RNAi)-mediated gene knockdown. We found that, in addition to cell-matrix contact sites, ZRP-1 also localized to the cell-cell contact sites and plays a crucial role in regulating Rho GTPase-mediated actin reorganization during formation of the cell-matrix and cell-cell adhesions.

Results

ZRP-1 is localized to focal adhesions and cell-cell adhesions

We generated rabbit polyclonal antibodies against a peptide corresponding to amino acid residues 10 to 25 of human ZRP-1. The affinity-purified anti-ZRP-1 antibody specifically recognized a ~50 kDa protein in western blots of HeLa cell lysates (Fig. 1A). We used this antibody to examine the localization of ZRP-1 in HeLa cells by immunofluorescence microscopy. As shown in Fig. 1B, ZRP-1 colocalized with vinculin at focal adhesions of the basal cell surface, as reported previously (Wang et al., 1999; Yi et al., 2002). In addition, in semi-confluent cells, ZRP-1 overlapped with N-cadherin at cell-cell contact sites near the apical surface (Fig. 1C). Interestingly, these proteins became less prominent at cell-cell contacts as the cells grew to confluence (Fig. 1D). ZRP-1 at focal adhesions and cell-cell contacts was absent upon ZRP-1 depletion (see below), confirming the specificity of the protein staining. These results indicate that ZRP-1 is localized specifically at cell-matrix and cell-cell adhesion sites.

ZRP-1 appears at the leading edge during the process of cell migration

It has been reported that overexpression or suppression of ZRP-1 in various cell lines resulted in increased or decreased cell motility, respectively (Yi et al., 2002; Xu et al., 2004; Guryanova et al., 2005). To track the behavior of ZRP-1 in migrating cells, we established A6 cell lines that constitutively express enhanced green fluorescent protein (EGFP)-tagged ZRP-1. Immunofluorescent staining of EGFP-ZRP-1-expressing cells with anti-vinculin antibody revealed clear colocalization of EGFP-ZRP-1 with vinculin at focal adhesions (Fig. 2A), indicating that localization of EGFP-ZRP-1 mirrors

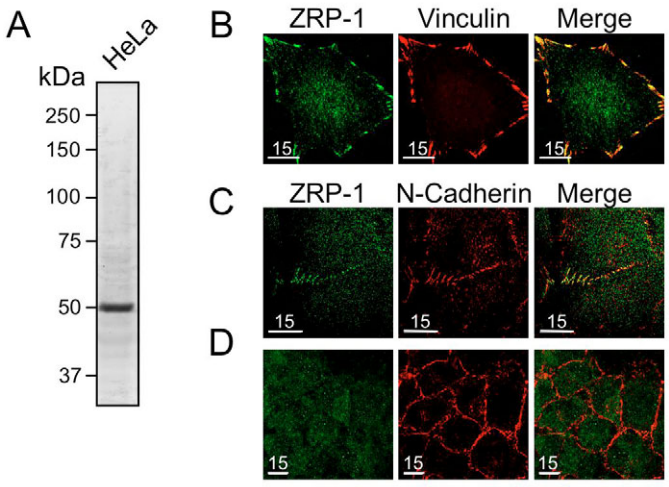

Fig. 1. ZRP-1 is localized to focal adhesions and also to cell-cell adhesions. (A) Characterization of anti-ZRP-1 polyclonal antibody. HeLa cell lysates were analyzed by western blotting with anti-ZRP-1 antibody. (B) Localization of ZRP-1 at focal adhesions. HeLa cells were stained with antibodies against ZRP-1 (left) and vinculin (middle). A merged image is shown on the right. Bars, 15 μm. (C) Localization of ZRP-1 at cell-cell contacts. Semi-confluent HeLa cells (upper panel) or saturation-density HeLa cells (lower panel) were stained with antibodies against ZRP-1 (left) and N-cadherin (middle). Merged images are shown on the right. Bars, 15 μm.

that of endogenous ZRP-1. We then grew the EGFP-ZRP-1-expressing A6 cells to confluence and introduced wounds in the culture by using a micropipette tip to induce cell migration. At 6 hours after wounding, EGFP-ZRP-1-expressing cells at wound sites were imaged at 5-minute intervals. We found that in migrating cells, EGFP-ZRP-1 signals were present continuously at the extending leading edge and that some signals grew into focal adhesions (Fig. 2B and supplementary material Movie 1), suggesting that ZRP-1 is involved in the process of focal adhesion and cell migration.

Depletion of ZRP-1 abrogates the assembly of actin stress fibers and maturation of focal adhesions

Previous reports showed that suppression of ZRP-1 expression alters organization of the actin cytoskeleton, though the results obtained were conflicting depending on the cell types used (Sanz-Rodriguez et al., 2004; Guryanova et al., 2005). To obtain clearer view on the role of ZRP-1, we examined the effect of ZRP-1 depletion on the organization of actin filaments in HeLa cells. We introduced one of three different ZRP-1-specific small interfering RNAs (siRNAs; ZRP-1-siRNA1, ZRP-1-siRNA2 and ZRP-1-siRNA3) together with a 21-nucleotide irrelevant RNA (control-siRNA) as a control into HeLa cells. Western blotting analyses showed that the expression of ZRP-1 was effectively suppressed by any of the ZRP-1-siRNAs (Fig. 3A and supplementary material Fig. S1A). Consistent with the data from HUVECs (Sanz-Rodriguez et al., 2004), staining with Rhodamine-labeled phalloidin revealed that majority of ZRP-1-depleted HeLa cells lacked actin stress fibers (Fig. 3B and supplementary material Fig. S1B). At the leading edge of an extended lamellipodial protrusion, focal complexes containing paxillin and FAK are formed initially. Then, focal complexes grow to mature focal adhesions as other focal adhesion proteins, such as vinculin, are recruited. Because focal adhesions are required for the formation of actin stress fibers and because ZRP-1 is localized at focal adhesions, we then examined whether maturation of focal adhesions was altered after ZRP-1 depletion. For this purpose, control cells and ZRP-1-depleted cells at the wound sites were fixed, and stained for paxillin and FAK. Although paxillin signals were present within the lamellipodia-like protrusions in both control and ZRP-1-depleted cells (Fig. 3C and supplementary material Fig. S1C), individual paxillin signals in ZRP-1-depleted cells were small even at the inner side of the protrusion. Immunostaining with anti-FAK antibody yielded the same result (Fig. 3D and supplementary material Fig. S1D). In addition, as shown in Fig. 3C and supplementary material Fig. S1C, signals of ZRP-1 in lamellipodia of a motile control cell showed less overlap with that of paxillin compared with that of vinculin (Fig. 1B, Fig. 2A). These facts suggested that the recruitment and accumulation of ZRP-1 to focal adhesions occurs later than the recruitment of paxillin. Therefore, we tentatively concluded that ZRP-1 is not essential for the formation of focal complexes but is necessary for the growth and maturation of focal complexes into focal adhesions, and the assembly of actin stress fibers in HeLa cells.

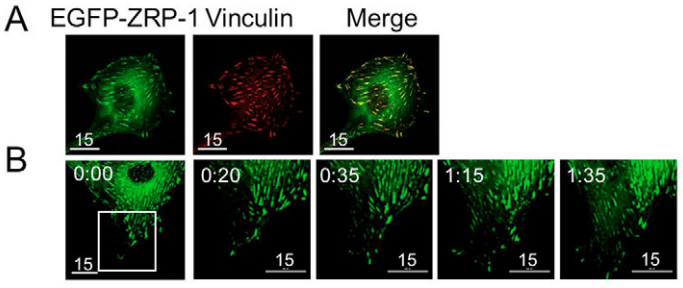

Fig. 2. ZRP-1 appears at the leading edges of migrating cells. (A) *Xenopus* A6 cells constitutively expressing EGFP-ZRP-1 (left) were fixed and stained with anti-vinculin antibody (middle). A merged image is shown on the right. (B) Behavior of ZRP-1 in migrating living cells. A6 cells constitutively expressing EGFP-ZRP-1 were grown to confluence and wounded. After 6 hours, cells were imaged at 5-minute intervals. The arrowheads indicate the appearance of EGFP-ZRP-1 at the tip of the leading edge. Bars, 15 μm.

ZRP-1-depleted cells exhibit aberrant actin cytoskeleton dynamics

We next examined the dynamics of actin organization. We sequentially transfected expression vectors for EGFP-actin and ZRP-1-siRNA1 or control-siRNA into HeLa cells, and subjected the transfected cells to time-lapse observations. Consistent with the results of Rhodamine-phalloidin staining, 58.2% (57 of 98) of ZRP-1-siRNA-treated cells showed perturbed actin filament rearrangement with reduced or no actin stress fiber formation, whereas only 12.9% (11 of 85) of control cells exhibited such a reduction in actin stress fiber formation (Fig. 4A,B and supplementary material Movies 2A, 2B). Interestingly, 83.3% (10 of 12) of the ZRP-1-siRNA-treated cells with few or no actin stress fibers exhibited reoccurring bursts of actin polymerization, which were rarely observed in control cells (1 of 5 cells) (Fig. 4A,C and supplementary material Movies 2A, 2B). EGFP only did not give rise to such filamentous signals in ZRP-1-siRNA treated cells (supplementary material Movie 8). In addition, staining with Rhodamine-labeled phalloidin confirmed the aberrant and intense actin polymerization in ZRP-1-depleted HeLa cells (supplementary material Fig. S2). The reoccurring bursts of actin polymerization appeared randomly either at the cell periphery or at the center of the cell and then spread to the cell periphery, accompanied by membrane protrusions in some cases (Fig. 4A and supplementary material Movies 2A, 2B). These data suggest that ZRP-1 is not only required for formation of focal adhesions and actin stress fibers, but that it also plays a broader role in the regulation of the actin cytoskeleton.

ZRP-1 is involved in the formation of cell-cell adhesions

As shown in Fig. 1C, ZRP-1 also localized to cell-cell adhesion sites. This raised the possibility that ZRP-1-depletion causes defects in the formation of cell-cell adhesions. Time-lapse observations of EGFP-actin-expressing control cells grown under semi-confluent conditions revealed that parallel lines of actin filaments were assembled continuously between neighboring cells (Fig. 5A, upper panels and supplementary material Movie 3A). By contrast, actin filaments between neighboring ZRP-1-depleted cells were disordered and tangled (Fig. 5A, lower panels and supplementary material Movie 3B). Moreover, we often observed that, instead of forming adhesions with or departing from neighboring attached cells, ZRP-1-depleted cells migrated under or over neighboring cells and kept moving (Fig. 5B and Movie 4). These results suggest that ZRP-1 depletion perturbs organization of actin filaments

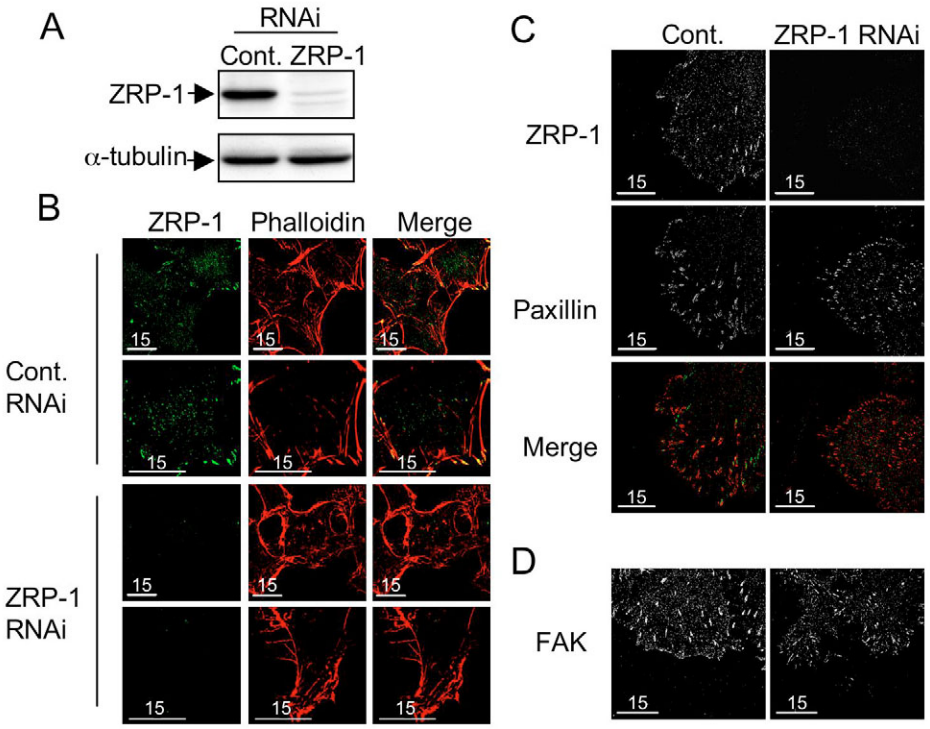

Fig. 3. ZRP-1-depleted cells lack actin stress fibers and mature focal adhesions. (A) siRNA-mediated suppression of ZRP-1 expression. Lysates of HeLa cells treated with control-siRNA or ZRP-1-siRNA for 72 hours were immunoblotted with anti-ZRP-1 antibody (upper panel) or anti-α-tubulin antibody (lower panel, loading control). (B) HeLa cells transfected with control-siRNA or ZRP-1-siRNA were fixed and stained with anti-ZRP-1 antibody and Rhodamine-phalloidin. The lower panels show magnified views. (C,D) HeLa cells transfected with or without ZRP-1 siRNA were grown to confluence and wounded. Ten hours later, cells at wound sites were fixed and stained with anti-ZRP-1 and anti-paxillin (C) or anti-FAK (D) antibodies. Bars, 15 μm.

at cell-cell contact sites, thereby abolishing correct communication between neighboring cells.

To examine further the effects of ZRP-1 depletion on the formation of cell-cell contacts, we induced formation of cell-cell adhesions in control and ZRP-1-depleted cells by changing the Ca^{2+} concentration in the culture medium from low to high (Vasioukhin et al., 2000). Three hours later, when cell-cell adhesions had actively reformed, cells were fixed and stained with Rhodamine-phalloidin or anti-N-cadherin antibody. In control cells, consistent with the time-lapse observation of EGFP-actin-expressing cells, actin filaments aligned at cell-cell contact sites (Fig. 5C, upper panels), and ZRP-1 and N-cadherin colocalized on those actin filaments (Fig. 5D, upper panels). By contrast, such organized actin filaments connecting neighboring cells were almost absent in ZRP-1-depleted cells (Fig. 5C lower panels, 5E). Only N-cadherin was visible at sites where cell membranes of adjacent cells were randomly attached to each other (Fig. 5D, lower panels, 5F). There was no obvious reduction in N-cadherin expression levels in ZRP-1-depleted cells

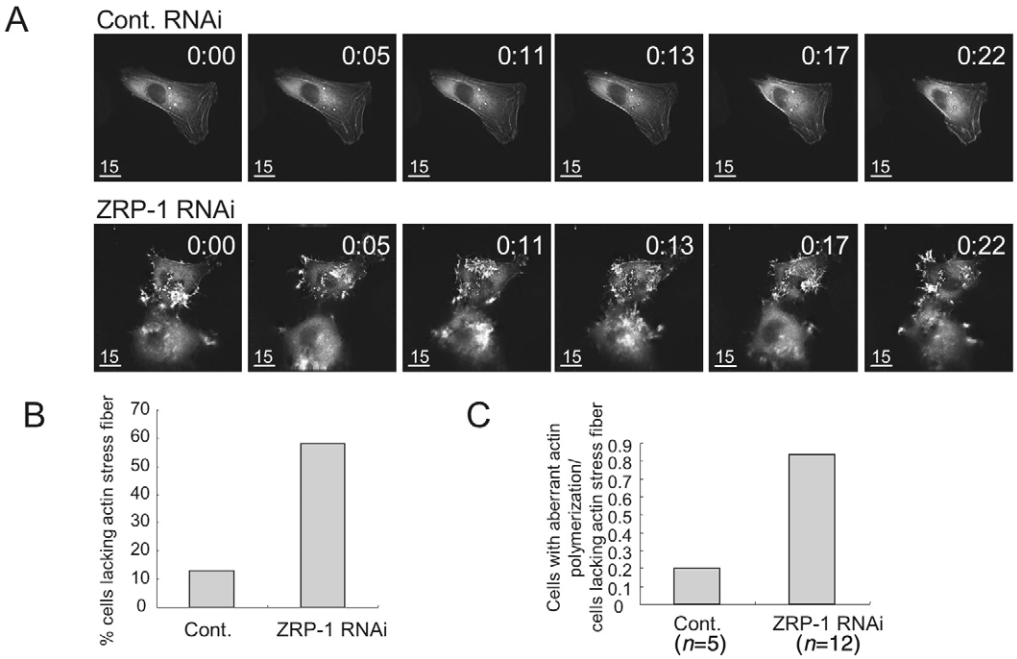

Fig. 4. ZRP-1-depleted cells exhibit aberrant actin dynamics. (B) HeLa cells were treated with control siRNA (upper panel) or ZRP-1 siRNA (lower panel) for 24 hours. Cells were then transfected with EGFP-actin expression vector. Forty-eight hours after vector transfection, cells were imaged at 1-minute intervals (A). Bars, 15 μm. (B) Percentages of cells lacking actin stress fibers. (C) Among the cells lacking actin stress fibers, the emergence rates of cells displaying aberrant actin polymerization were measured. *n* indicates the number of cells examined.

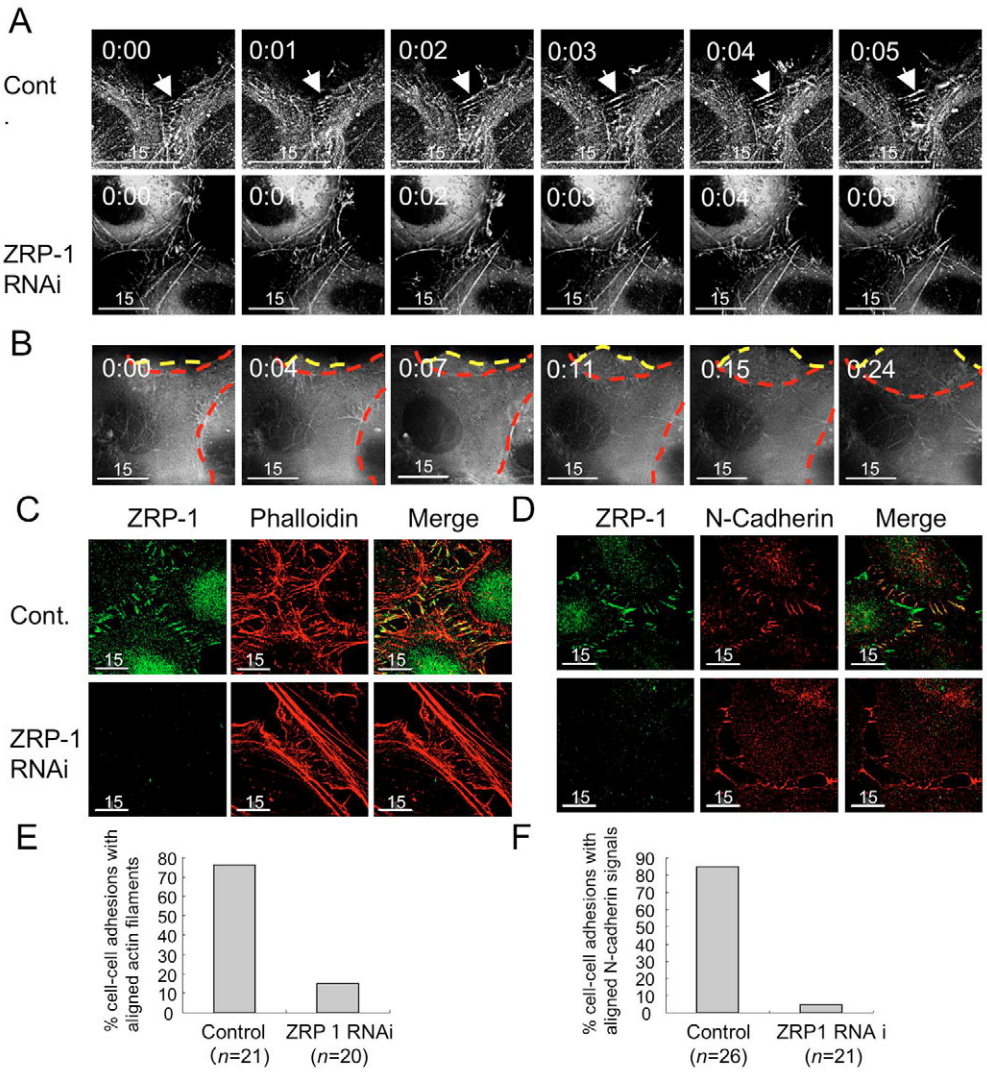

Fig. 5. ZRP-1-depleted cells fail to form N-cadherin-based cell-cell adhesions. (A) Actin dynamics at cell-cell contacts. Control cells (lower panel) and ZRP-1-depleted cells (upper panel) were transfected with EGFP-actin expression vector as described in Fig. 4. Cell-cell contact regions were then imaged at 1-minute intervals. Arrowheads indicate parallel actin filaments that connect neighboring cells. (B) ZRP-1-depleted cells transfected with EGFP-actin were imaged at 1-minute intervals. Red and yellow dashed lines outline two moving cells. (C,D) Three hours after switching the Ca^{2+} concentration of cell growth medium from low to high, both control cells (upper panels in C,D) and ZRP-1-depleted cells (lower panels in C,D) were fixed and stained with antibodies against ZRP-1 and phalloidin (C) or N-cadherin (D). Merged images are shown on the right of C and D. Bars, 15 μm. (E,F) Percentages of cell-cell adhesion with aligned actin filaments and N-cadherin signals. *n* indicates the number of cell-cell adhesions examined.

(supplementary material Fig. S3). These results suggest that ZRP-1 plays a role in the control of actin filament organization upon cell-cell attachment and, therefore, in the formation of N-cadherin-mediated cell-cell adhesions.

ZRP-1 is required for collective cell migration

HeLa cells show collective migration, a type of migration that requires continuous assembly and disassembly of cell-matrix or cell-cell contacts and constant remodeling of the actin cytoskeleton. Because ZRP-1-depleted cells showed abnormalities in focal adhesion, cell-cell adhesion and actin reorganization, we examined whether migration of HeLa cells was affected by ZRP-1 depletion. siRNA-treated cells were replated at a saturating density and wound-healing assay was carried out. The healing processes at various time points (4, 7, 10 and 16 hours) after wounding are shown in Fig. 6A, and the mean percentage of 'healed' (closed-up) wounded area at each time point is shown in Fig. 6B. ZRP-1-depleted cells displayed a marked enhancement of wound repair compared with control cells. By 10 hours after wounding, ZRP-1-depleted cells had virtually closed the wound, whereas control cells had only migrated to cover 20-30% of the wounded area. The time-lapse observations of the cells facing the wound edge showed that most migrating ZRP-1-depleted cells were detached from each other and had migrated out from the cell sheet (Fig. 6A and supplementary material Movie 5B). These cells migrated into the wound site by extending lamellipodia-like protrusions in random directions. Some ZRP-1-depleted cells extended excessively long protrusions, resulting in extremely elongated cell bodies followed by cell-adhesion collapse and cell death (supplementary material Movie 5B). By contrast, control cells rarely formed such abnormal protrusions and exhibited typical collective migration (supplementary material Movie 5A). These observations indicate that ZRP-1 is necessary for collective migration of HeLa cells. Thus, ZRP-1 seems to play an important role in the coordinated control of cell-matrix and cell-cell adhesions during cell migration.

ZRP-1-depletion alters the Tyr phosphorylation status of focal adhesion proteins

At focal adhesions, FAK is a primary mediator of the signal transduction pathway from integrin in the control of Rho family GTPases. It has recently been reported that, in HeLa cells, depletion of FAK or mislocalization of FAK caused by paxillin depletion results in a reduced number of robust focal

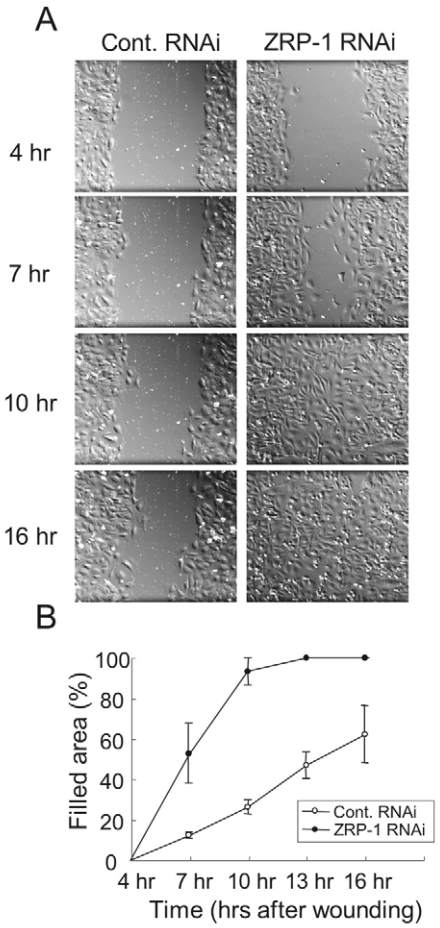

Fig. 6. ZRP-1-depleted cells show altered collective cell migration. HeLa cells treated with control siRNA or ZRP-1 siRNA were subjected to wound-healing assay as described in Materials and Methods. Four hours after wounding, cells were imaged at 1-minute intervals for 12 hours. (A) Images of ZRP-1-depleted cells and control cells at 4, 7, 10 and 16 hours after wounding. (B) Percentages of the wound area that has been filled in by migrating cells at each time point. The mean percentages of three independent experiments are shown. Black and white circles indicate ZRP-1-depleted cells and control cells, respectively; error bars indicate standard deviations.

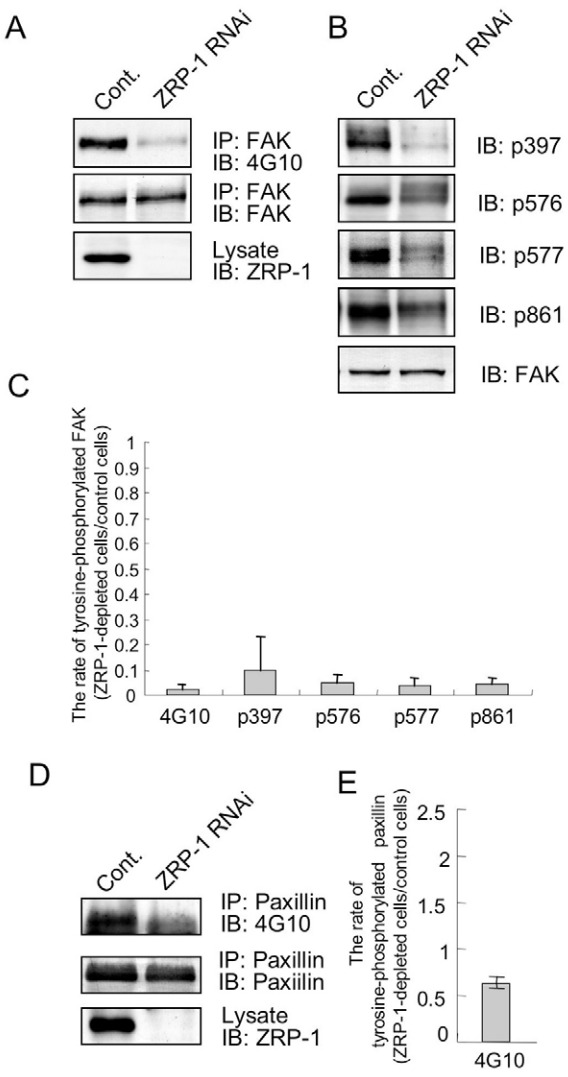

Fig. 7. Phosphorylation of focal adhesion kinase (FAK) and paxillin is suppressed in ZRP-1-depleted cells. (A) Endogenous FAK was immunoprecipitated from control or ZRP-1-depleted HeLa cell lysates. Tyr phosphorylation of FAK was detected by a phosphotyrosine antibody (4G10). (B) Endogenous FAK immunoprecipitated from control or ZRP-1-depleted HeLa cells was analyzed with the indicated phosphorylation site-specific antibodies. (C) The relative levels of total and site-specific (Tyr397, Tyr576, Tyr577, Tyr861) Tyr phosphorylation of FAK in ZRP-1-depleted cells as compared with those in control cells. (D) Endogenous paxillin was immunoprecipitated and analyzed as in (A). (E) The relative levels of total tyrosine phosphorylation of paxillin in ZRP-1-depleted cells as compared with those in control cells.

adhesions, loss of N-cadherin-mediated cell-cell adhesions in collective cell migration and upregulation of Rac1 at cell periphery (Yano et al., 2004). Phosphorylation of Tyr397 and Tyr861 of FAK was necessary to rescue these FAK knockdown phenotypes (Yano et al., 2004). Given that the FAK-depleted and ZRP-1-depleted HeLa cells share many – but not all – phenotypes, we examined FAK expression and Tyr phosphorylation levels of FAK and paxillin in ZRP-1-depleted cells. ZRP-1 depletion did not reduce FAK and paxillin expression levels but the total tyrosine phosphorylation levels of both proteins were markedly decreased (Fig. 7A,C,D,E). When further analyzed with phosphorylation-site-specific antibodies, we found that the levels of phosphorylated Tyr397 and Tyr861 of FAK were decreased in ZRP-1-depleted cells (Fig. 7B,C). Consistently, phosphorylation of Tyr576 and Tyr577, which facilitates maximal FAK activation and phosphorylation of Tyr861 (Schlaepfer and Mitra, 2004), were also reduced (Fig. 7B,C). These results indicate that FAK activity is downregulated upon ZRP-1 depletion.

Because FAK activity largely depends on the status of cell adhesion, we next examined whether a reduction of FAK phosphorylation is the reason for impaired maturation of focal adhesion, or whether suppressed FAK phosphorylation is a result of impaired maturation of focal adhesion. For this purpose, we overexpressed FAK in ZRP-1-depleted HeLa cells. As shown in supplementary material Fig. S4, even when constitutively active FAK was overexpressed, ZRP-1-depleted cells still displayed

immature focal adhesion as well as impaired stress fibers. These results imply that downregulation of FAK activity is not the sole cause but rather the result of impaired focal adhesion and stress fiber formation in ZRP-1depleted cell. We speculate that other factors required for maturation of focal adhesion are inactivated and/or activated FAK is sequestered away from focal contacts by ZRP-1 depletion.

Enhanced Rac1 activity caused by ZRP-1-depletion does not depend on p130Cas or Crk

Rac1 is involved in membrane ruffling and the formation of lamellipodial protrusions through activation of the Arp2/3 complex (Miki et al., 2000) and formation of de novo branched actin filaments, as well as in the formation of small focal complexes within newly formed protrusions (Rottner et al., 1999). The ZRP-1-depleted phenotype of aberrantly enhanced actin polymerization with numerous immature focal adhesions raised the possibility that Rac1 activity is upregulated in ZRP-1-depleted cells. Therefore, we first assessed levels of active GTP-bound Rac1 in lysates from ZRP-1-siRNA-treated and control cells by pull-down assay (Etienne-Manneville and Hall, 2001). Strikingly, Rac1 activity in ZRP-1-depleted cells was significantly higher than that of control cells (Fig. 8A). The mean value ± s.d. of the relative amount of GTP-bound Rac1 versus total Rac1 in ZRP-1-depleted cells to control cells in three independent experiments was 4.20±1.30 (*t*-test, $P<0.05$). To confirm that the abnormal actin cytoskeleton organization observed in ZRP-1-depleted cells was caused by increased Rac1 activity, we used a dominant-negative mutant of Rac1, Myc-Rac1T17N. We co-expressed Myc-Rac1T17N with EGFP-actin in ZRP-1-siRNA-treated and control cells. Using immunofluorescence microscopy, we confirmed that approximately 90% of EGFP-actin-expressing cells co-expressed Myc-Rac1T17N (data not shown). As shown in Fig. 8B and supplementary material Movies 6A and 6B, the continuous bursts of actin polymerization caused by ZRP-1 depletion were blocked by expression of Myc-Rac1T17N. These results indicate that Rac1 activity is enhanced by ZRP-1 depletion, which results in enhanced actin polymerization and membrane protrusion, and suggest a role of ZRP-1 in downregulation of Rac1 activity. Because integrin engagement can activate Rac1 through the formation of a p130Cas-Crk-DOCK180-ELMO Rac GEF complex and because interactions between ZRP-1 and p130Cas or Crk have been reported both in vitro and in cells (Lai et al., 2005; Yi et al., 2002), we examined the effect of ZRP-1 depletion on the phosphorylation levels of p130Cas and Crk. Phosphorylation of p130Cas and dephosphorylation of Crk are primary events in Rac1 activation mediated by p130Cas-Crk-DOCK180-ELMO (Chodniewicz and Klemke, 2004). In ZRP-1-depleted HeLa cells, however,

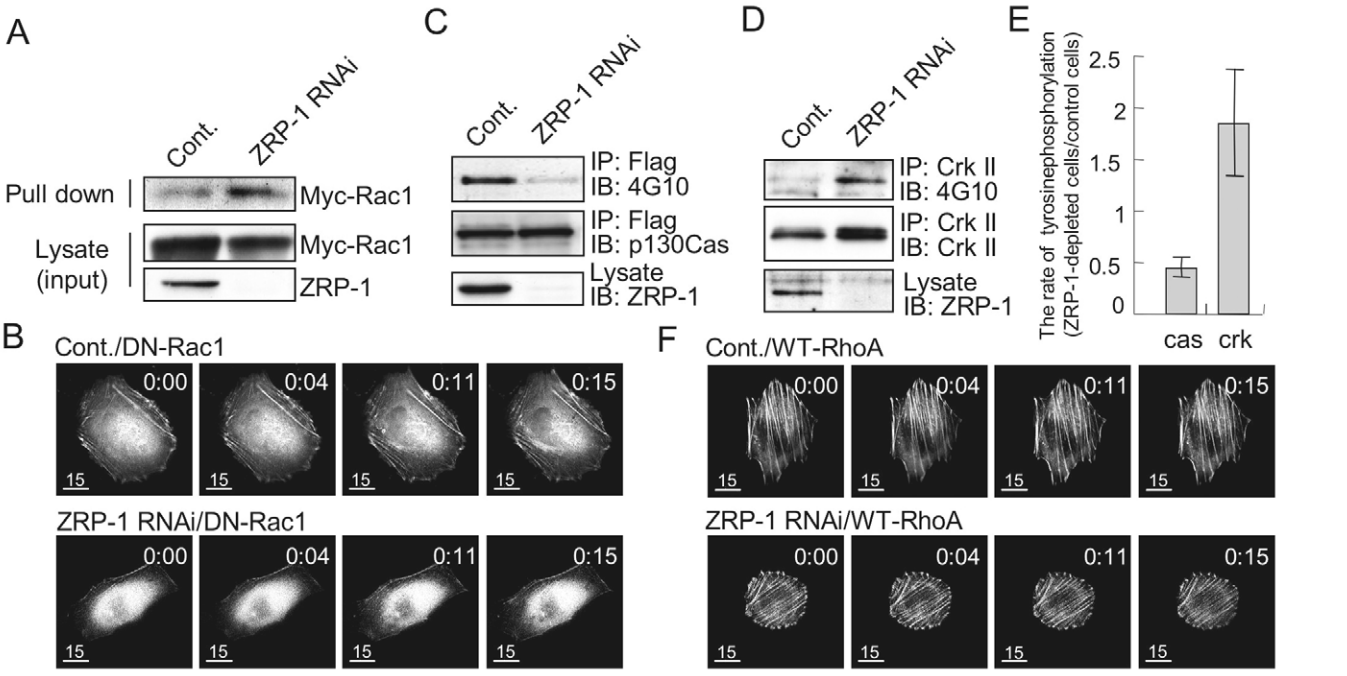

Fig. 8. Rac1 activity is enhanced by ZRP-1 depletion. (A) Rac1 pull-down assay. Lysates from HeLa cells treated with or without ZRP-1 siRNA were immunoblotted with anti-ZRP-1 antibody (lower panel) and anti-Rac1 antibody (middle panel). The upper panel shows GTP-bound Myc-Rac1 precipitated with GST-PAK CRIB. (B) Effects of expression of the dominant-negative mutant of Rac1. HeLa cells were transfected with or without Cy3-labeled ZRP-1-siRNA. Twenty-four hours later, these HeLa cells were transfected with expression vectors for Myc-DN-Rac1and EGFP-actin at a ratio of 9:1. Beginning 48 hours after vector transfection, cells were imaged at 1-minute intervals. Bars, 15 μm. (C) Exogenously expressed Flag-tagged-p1130Cas from control or ZRP-1-depleted HeLa cells was analyzed with a phosphotyrosine antibody (4G10). (D) Endogenous Crk II was immunoprecipitated and analyzed as in (C). (E) The relative levels of total Tyr phosphorylation of Flag-p130Cas and Crk II in ZRP-1-depleted cells as compared with those in control cells. (F) Effects of overexpression of wild-type RhoA. HeLa cells were transfected with or without Cy3-labeled ZRP-1-siRNA. Twenty-four hours later, these HeLa cells were transfected with expression vectors for Myc-WT-RhoA and EGFP-actin at a ratio of 9:1. Beginning 48 hours after vector transfection, cells were imaged at 1-minute intervals. Bars, 15 μm.

levels of p130Cas phosphorylated on Tyr residues was markedly reduced, whereas those of Crk were enhanced, suggesting that p130Cas-Crk-DOCK180-ELMO was suppressed by ZRP-1 depletion (Fig. 8C,D,E). Thus, p130Cas-Crk-DOCK180-ELMO is not relevant to markedly upregulate Rac1 in ZRP-1 depleted cells.

RhoA expression ameliorates stress fibers in ZRP-1 depleted cells

ZRP-1-depleted cells showed inefficient formation of focal adhesions and reduced formation of actin stress fibers. Because RhoA activity is required for both of these processes (Hall, 1998), we examined the levels of active GTP-bound RhoA. We could not detect an obvious change in total RhoA activity after ZRP-1 depletion by pull-down assay (data not shown). However, when wild-type RhoA was overexpressed in ZRP-1-depleted cells, actin stress fibers were formed at a rate similar to that of control cells and the continuous bursts of actin polymerization caused by ZRP-1 depletion were suppressed (Fig. 8F and supplementary material Movies 7A and 7B). These results indicate that, even in the absence of ZRP1, overexpressed RhoA can drive the maturation of focal adhesions from focal complexes, which suggests that ZRP-1 is not a structural component that is essential for the assembly of actin stress fibers and/or mature focal adhesions but is involved in correct regional activation of RhoA at focal complex. Taken together, our data suggest that ZRP-1 localizes to cell-matrix and cell-cell contact sites and plays a crucial role in coupling the cell-matrix/cell-cell contact signals with Rho GTPase-mediated actin remodeling, which is essential for proper cell-matrix and cell-cell contact formation.

Discussion

Structure and dynamics of the actin cytoskeleton are important for the regulation of cell-matrix and cell-cell adhesions and cell migration. ZRP-1/TRIP6 has been known as a focal-adhesion- and stress-fiber-associated protein (Wang and Gilmore, 2001; Yi et al., 2002; Sanz-Rodriguez et al., 2004). In this study, we show that ZRP-1 is also localized to cell-cell contact sites – like the other family members including zyxin, Ajuba, and LPP (Li et al., 2003; Marie et al., 2003; Petit et al., 2003). Interestingly, localization of ZRP-1 to the cell-cell contact sites is prominent when actin reorganization is in progress (Fig. 5A,C). Similarly, ZRP-1 accumulates at cell-matrix adhesion sites where actin is reorganized into stress fibers and the focal adhesion matures (Fig. 3C). These results suggest that ZRP-1 is localized to the cell-matrix and cell-cell contact sites when and where actin cytoskeleton is actively reorganized.

Consistent with its localization, depletion of ZRP1 from HeLa cells impairs not only stress fiber assembly and focal adhesion formation, but also actin reorganization at cell-cell adhesion sites and cell-cell contact formation. Consequently, collective cell migration after wounding is abrogated in the absence of ZRP-1. Loss of FAK and paxillin expression in HeLa cells abrogates the efficient N-cadherin-mediated cell-cell adhesion and impairs collective migration (Yano et al., 2004), which provides evidence of communication between integrin and cadherin systems. Reduced Tyr phosphorylation of FAK and paxillin caused by ZRP-1 depletion may be related to the impaired collective migration in ZRP-1-depleted HeLa cells. However, different from FAK and paxillin, ZRP-1 is localized to cell-cell contacts as well as to focal adhesions, suggesting that ZRP-1 is more directly involved in the integrin-cadherin intercommunication. At the cell-cell contact sites, ZRP-1 may participate in the cadherin-mediated signaling pathway to regulate actin organization essential for the correct formation of cell-cell adhesions.

Phosphorylation and activation of FAK are required for the formation of robust focal adhesions in HeLa cells (Yano et al., 2004). We find that ZRP-1 depletion leads to reduced Tyr phosphorylation of FAK (Fig. 7A,B,C). However, expression of a constitutively active form of FAK fails to ameliorate maturation of focal adhesions in ZRP-1 depleted HeLa cells. Moreover, ZRP-1 overexpression does not enhance levels of Tyr phosphorylation of FAK (data not shown). Interaction between FAK and ZRP-1 is not detectable in HeLa cells, and the activity of Src family kinases is not significantly altered by ZRP-1 depletion (data not shown). These results strongly suggest that reduced phosphorylation of FAK in ZRP-1-depleted cells is not the cause but the result of impaired focal adhesion assembly.

In ZRP-1-depleted cells, formation of the focal complex – an initial step of focal adhesion formation – appears normal. Therefore, ZRP-1 is dispensable for formation of the focal complex, although it is indispensable for the maturation of focal complex to focal adhesion. Maturation of focal adhesion is essential for the formation of stress fibers and, vice versa, stress fiber formation is essential for the assembly and maintenance of mature focal adhesion (Pavalko and Burridge, 1991). Thus, we speculate that, at the cell-matrix adhesion sites, ZRP-1 participates in the integrin-mediated signaling pathway that regulates stress fiber formation and maturation of focal adhesions. Coordinated sequential activation and inactivation of Rac1 and RhoA are prerequisite for the formation of stress fibers and mature focal adhesions (Rottner et al., 1999). Our study shows that exogenous expression of wild-type RhoA in ZRP-1-depleted cells promotes the formation of stress fibers, which suggests that suppression of RhoA activity is a cause for impaired stress fiber formation in ZRP-1-depleted cells. Although the total RhoA activity is not significantly altered by ZRP-1 depletion, regional Rho activity at focal contact sites might be affected.

Time-lapse observations of actin dynamics reveal that loss of stress fibers caused by ZRP-1 depletion is associated with abnormal bursts of active actin polymerization even in the absence of specific stimuli. Consistent with the dynamic changes in actin filaments, Rac1 activity is elevated in ZRP-1-depleted cells, and expression of a dominant-negative mutant of Rac1 abrogates the abnormal actin polymerization caused by ZRP-1 depletion. These results suggest a role of ZRP-1 in the downregulation of Rac1. Previous report shows that a paxillin-FAK-mediated pathway downregulates Rac1 activity, especially at the cell periphery in HeLa cells (Yano et al., 2004). However, unlike FAK-depleted or paxillin-depleted cells, ZRP-1-depleted cells exhibit significantly increased levels of Rac1 activity – as detected by Rac1 pull-down assay. Furthermore, the bursts of actin polymerization observed in ZRP-1-depleted cells occur at the center of the cell as well as at the cell periphery. Taken together, Rac1 seems to be activated not only at the cell periphery, but also throughout the cytoplasm in ZRP-1-depleted HeLa cells. Therefore, FAK downregulation is not sufficient to explain the intense activation of Rac1 observed in ZRP-1-depleted HeLa cells and it is likely that ZRP-1 depletion also

affects other pathways leading to Rac1 activation. Besides FAK activation, formation of the p130Cas-Crk-DOCK180-ELMO complex in integrin signaling contributes largely to Rac 1 activation (Chodniewicz and Klemke, 2004). ZRP-1 is proposed to modulate this pathway through association with p130Cas and Crk (Lai et al., 2005; Yi et al., 2002) and, indeed, ZRP-1 depletion alters the phosphorylation status of both proteins and leads to the dissociation of the p130Cas-Crk complex (Fig. 8C,D,E). Together with the results that phosphorylation of FAK and paxillin are also reduced upon ZRP-1 depletion, ZRP-1 depletion resulted in marked upregulation of Rac1 in the absence of integrin-mediated Rac1 activation pathway. It is interesting to note that a similar situation occurred when macrophage capacity for phagocytosis of apoptotic cells is upregulated by glucocorticoid treatment (Giles et al., 2001). In this case, macrophages show decreased phosphorylation and recruitment of paxillin and Pyk2, a kinase that shares the roles with FAK (Yano et al., 2004), to focal contacts. Those macrophages also show downregulation of p130Cas, as well as marked upregulation of Rac1 associated with active formation of lamellipodia and cellular extensions necessary for phagocytosis.

Because sustained Rac activation downregulates Rho activity in fibroblasts (Sander et al., 1999), and because a Rho inhibitor induces a phenotype suggestive of activation of Rac1 in both fibroblasts and hematopoetic cells (Moorman et al., 1999), ZRP-1 might contribute to RhoA activation through downregulation of Rac1 or vice versa. Transfection of a constitutively active form of Rac1 in HeLa cells induces bursts of actin polymerization that resembled those induced by ZRP-1 depletion. However, it does not perturbe stress fibers (supplementary material Movie 9), suggesting that high activity of Rac-1 is not sufficient to suppress stress fiber formation in HeLa cells. By contrast, expression of wild type RhoA in ZRP-1-depleted cells not only restores stress fiber formation but also represses abnormal burst of actin polymerization (Fig. 8F and supplementary material Movies 7A,B). These results suggest that RhoA directly or indirectly inactivates Rac1 in ZRP-1-depleted cell, the mechanism of which need to be further examined in future studies.

In conclusion, our results show that ZRP-1 regulates the formation of cell adhesions to both extracellular matrix and neighboring cells, and cell migration. ZRP-1 appears to participate in these events by controlling reorganization of the actin cytoskeleton at the contact sites through downregulating Rac1 and possibly by upregulating RhoA activity. We speculate that ZRP-1 has a role in controlling the balance of Rac1 and RhoA activities during cell-matrix and cell-cell contacts formation. In this context, signaling to and from ZRP-1 might be mediated by association with previously reported and/or yet unidentified ZRP-1 interactors, which constitute the focal complex and/or N-cadherin complex (Williams et al., 1998; Murthy et al., 1999; Cuppen et al., 2000; Yi et al., 2002; Xu et al., 2004; Sanz-Rodriguez et al., 2004; Takizawa et al., 2006). This model can explain the conflicting observations about the effect of ZRP-1 depletion on focal adhesion and actin organization by differences and/or variations of factors that regulate Rho GTPases activities upon different stimuli or in different cell-types. Further studies of the precise signaling pathway from ZRP-1 to Rho GTPases and, thus, actin dynamics may improve our understanding of the mechanisms that underlay orchestrated cell-cell/cell-matrix interactions.

Materials and Methods

cDNAs, plasmids and antibodies

The cDNA fragment encoding amino acids 195-476 of ZRP-1 was isolated from a yeast two-hybrid screen for proteins interacting with LATS2 (Abe et al., 2006). The cDNA fragment encoding amino acids 1-358 of ZRP-1 was cloned from a HeLa cell cDNA library by PCR with appropriate primers. The two fragments were assembled to create the full-length ZRP-1 cDNA, encoding a 476 amino acid protein. To generate EGFP-ZRP-1 expression vector pEGFP-ZRP-1, a full-length ZRP-1 cDNA was subcloned into pEGFP-C2 (Clontech). Expression vectors for Myc-WT-Rac1, Myc-Rac1T17N, Myc-RacG12V, and Myc-WT-RhoA were gifts from Y. Takai (University of Osaka, Osaka, Japan). Expression vectors for Flag-Cas was a gift from H. Hanafusa (Osaka Bioscience Institute, Osaka, Japan). Expression vector for EGFP-actin (pEGFP-actin) was a gift from J. Yokota (National Cancer Center, Tokyo, Japan). The plasmid for GST-Cdc42/Rac-interactive binding domain of Pak (GST-CRIB) was kindly provided by C. Sasakawa (University of Tokyo, Tokyo, Japan). The plasmids for HA-FAK and HA-ΔFAK were kindly provided by D. D. Schlaepfer (The Scripps research institute, CA). Anti-ZRP-1 antibody was generated in New Zealand White rabbits against the peptide KQPEPARAPQGRAIPR (amino acids 10 to 25). Rabbits were immunized three times at 2-week intervals with subcutaneous injections of peptide conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce). The anti-ZRP-1 antibody was affinity-purified from antiserum with the immunogen peptide linked to Sulfolink gel (Pierce) as described previously (Harlow and Lane, 1988). Other antibodies used in this study included: anti-α-tubulin, anti-vinculin (Sigma), anti-paxillin, anti-N-cadherin, anti-CrkII (BD Transduction), anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-FAK, anti-phosphorylated FAKs (pY397, pY576, pY577, and pY861) (Biosource, Camarillo, CA), and anti-Rac1 (gift from T. Takenawa, University of Tokyo, Tokyo, Japan).

Cell culture and EGFP-ZRP-1-expressing stable clones

HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The *Xenopus* kidney epithelial cell line A6 was kindly provided by Y. Kiyosue (KAN Research Institute, Kyoto, Japan) and grown at 25°C without CO_2 in 50% Leibovitz's L-15 medium (Gibco BRL) supplemented with 10% FBS. To establish clonal A6 cell lines constitutively expressing EGFP-ZRP-1, cells were transfected with pEGFP-ZRP-1 using Fugene reagent (Roche) and cultured in selection medium containing 400 μg/ml G418. Selected clones were screened for EGFP-ZRP-1 expression by immunofluorescence. Positive clones were then expanded and maintained in the selection medium.

RNA interference

RNAi experiments were carried out with small interfering RNAs (siRNAs) as described previously (Elbashir et al., 2002). Target sequences were 5′-AATTGTTGCTCTGGATCGAAG-3′ (ZRP-1-siRNA), 5′-AAGCTGGTTCACGA-CATGAAC-3′ (ZRP-1-siRNA2), 5′-AAGATGCTCAGTGTGCGGTGG-3′ (ZRP-1-siRNA3) for ZRP-1 and 5′-AATTCTCCGAACGTGTCACGT-3′ for negative control siRNA. Synthetic siRNAs (JbioS, Tokyo, Japan) were transfected into HeLa cells with Oligofectamine (Invitrogen) at a final concentration of 100 nM or with Lipofectamine RNAiMAX (Invitrogen) at a final concentration of 10 nM.

Indirect immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in 0.1 M Hepes (pH 7.0) and stained with primary antibodies as specified in each experiment, and secondary antibodies coupled to Alexa Fluor-488 (Invitrogen) or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). For visualization of actin filaments, Rhodamine-conjugated phalloidin (Invitrogen) was added during the incubation with secondary antibodies. Immunofluorescence images were collected on a DeltaVision system (Applied Precision, Issaquah, WA) and then analyzed by iterative constrained deconvolution.

Time-lapse imaging of live cells

To visualize EGFP-ZRP-1 in migrating A6 cells, cells were grown to confluence and wounded with a micropipet tip. After incubation for 6 hours, cells at the wound sites were observed with the DeltaVision system. For visualizing the EGFP-actin in living cells, 24 hours after the addition of the ZRP-1-siRNA, pEGFP-actin was transfected into ZRP-1-siRNA-treated cells or control cells with or without Myc-DN-Rac1 or Myc-WT-RhoA expression vector with LipofectAMINE Plus (Invitrogen). After incubation for 48 hours, cells were observed with the DeltaVision system.

For wound-healing assay, siRNA-treated cells were replated at saturating density. After 24 hours of incubation, cells were scratched with a micropipette tip, and the culture medium was replaced with DMEM supplemented with 10% FBS. After culture for 4 hours, cell migration was observed with an Olympus IX70 inverted microscope (Olympus Optical Co., Tokyo, Japan) equipped with a cooled CCD camera (DV887DCS-BV, Andor Technology, Belfast, Northern Ireland). This imaging system was controlled with MetaMorph software (Universal Imaging, Downingtown, PA).

Rac1 pull-down assay

HeLa cells were suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% NP-40, 10% glycerol and 50 units/ml aprotinin). GST-PAK CRIB

was purified as described previously (Miki et al., 2000). GST-PAK CRIB was immobilized on glutathione Sepharose 4B beads (GE Healthcare, Giles, UK) and then incubated with cell lysate. Beads were washed with lysis buffer and suspended in SDS sample buffer. Samples were analyzed by western blot (described below) with anti-Rac1 antibody. Whole-cell lysates were also subjected to western blotting to examine the amount of Rac1.

Western analysis and immunoprecipitation

Cells were lysed in buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% NP40, 1 mM EDTA) containing 1 mM sodium orthovanadate and 50 units/ml aprotinin. After removing insoluble materials, the supernatants were incubated with protein-A–Sepharose 4B (Amersham Pharmacia Biotech) for 30 minutes at 4°C to preclear and collected after centrifugation. The precleared lysates were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore, Billerica, MA). For immunoblotting with anti-phosphorylated FAK antibodies, four membranes generated in parallel were used and then stripped, and FAK was detected with monoclonal anti-FAK antibody. As for the results of the anti-FAK immunoblot, only a representative one is shown in the bottom panel of Fig. 8B. For immunoprecipitation, the precleared lysates were incubated with anti-FAK polyclonal antibody for 1 hour at 4°C followed by protein-A–Sepharose for another 1 hour at 4°C. The immunoprecipitates were then separated by 7.5% SDS-polyacrylamide gel electrophoresis and two membranes generated in parallel were western blotted with anti-4G10 monoclonal and anti-FAK polyclonal antibodies.

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