The PP2A-associated Protein α4 Plays a Critical Role in the Regulation of Cell Spreading and Migration*

Received for publication, April 13, 2007, and in revised form, August 3, 2007. Published, JBC Papers in Press, August 10, 2007, DOI 10.1074/jbc.M703159200

Mei Kong1‡, Thi V. Bui‡, Dara Ditsworth15, Josh J. Gruber15, Dmitry Goncharov4, Vera P. Krymskaya*, Tullia Lindsten41, and Craig B. Thompson‡§

From the 1† Abramson Family Cancer Research Institute, 2Department of Cancer Biology, 3Pulmonary, Allergy, and Critical Care Division, 4Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Compared with kinases, the role of protein phosphatases in regulating biological functions is less well understood. Here we show that α4, a non-catalytic subunit of the protein phosphatase 2A, plays a major role in the control of cell spreading, migration, and cytoskeletal architecture. Fibroblasts lacking α4 were impaired in their ability to spread and migrate compared with wild-type cells, whereas enforced expression of α4 promoted cell spreading and migration. These effects were not restricted to fibroblasts. Using a T cell-specific α4 transgenic mouse model, increased α4 expression was found to increase lymphocyte motility and chemotaxis. Elevated α4 expression results in an increase in the GTP-bound state of Rac1, and GTP-bound Rac1 was dramatically reduced in α4-deficient cells. A constitutively active mutant of Rac1 rescued the defects of cell spreading and migration caused by α4 deletion, while inhibition of Rac1 blocked the ability of α4 to promote cell migration. Together, these data define a novel role for the protein phosphatase 2A regulatory subunit α4 in the regulation of cell spreading and migration.

Cell migration is important in a variety of biological and pathological processes, including embryonic development, wound healing, angiogenesis, inflammation, tumor invasion, and metastasis (1). Identifying the genes involved in the regulation of cell migration may lead to a better understanding of how cell migration contributes to these physiologic and pathologic events. It is generally accepted that the driving force for cell movement is provided by the dynamic reorganization of the actin cytoskeleton, directing protrusion at the front of the cell and retraction at the rear (2). Cytoskeletal dynamics and cell adhesion are controlled by small GTPases of the Rho family, in particular RhoA, Rac, and Cdc42 (1, 3). Among these, Rac induces actin polymerization and integrin adhesion complex assembly at the cell periphery, leading to membrane protrusion, and is essential for cell spreading and migration (4). The activation of Rac can be mediated by stimulation of both growth factor and integrin receptors and requires GTP/GDP exchange factors (5). Moreover, the cooperative action of other signaling components is required to promote coordinated assembly and disassembly of actin filaments, particularly protein kinases, including focal adhesion kinase, Src, Crk, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase, as has been reviewed elsewhere (6). In addition to kinases, it has been shown that several protein phosphatases are involved in the regulation of cell migration and spreading, such as phosphatase and tensin homolog (7), Src homology 2-containing protein tyrosine phosphatase-2 (8), protein tyrosine phosphatase α (9), and protein phosphatase 2A (PP2A) (10), indicating that protein phosphatases could play an equally important role in the control of cell motility. Unlike kinases, the activity and specificity of serine/threonine phosphatases is governed largely by their associated proteins (11).

The α4 protein, also known as immunoglobulin-binding protein (IgBP1), is a PP2A-, PP4-, and PP6-associated protein. It was initially identified as a component of receptor signal transduction complexes in mammalian B and T lymphocytes and was later determined to be broadly expressed (12, 13). PP2A is the major soluble serine/threonine phosphatase in mammalian cells and is predominantly found as a trimeric complex formed by the catalytic subunit (PP2Ac), core A scaffolding subunit, and any of the over 12 variable B components. Binding of α4 to PP2Ac displaces PP2Ac from A and B subunits due to an overlap in the binding site on PP2Ac (14). Several groups have reported that interaction of α4 with PP2Ac alters both the enzymatic activity and its substrate specificity (15–17). Its yeast homologue, Tap42, is a PP2A regulatory subunit that functions in target of rapamycin (TOR)-dependent nutrient sensing (18). In mammalian cells, the association of α4 with PP2A can be regulated by growth factor signals and modulators of the mammalian TOR pathway, such as rapamycin (15, 19, 20). To date, the downstream targets and physiological roles of α4 have not been established.

The yeast homologue of α4, Tap42, has been implicated in the distribution of actin via a Rho GTPase-dependent mechanism (21). In studying the conditional deletion of α4 in mouse embryonic fibroblasts (MEFs), we observed that the α4-deleted cells adopt an altered morphology, appearing to be less spindle-shaped compared with control fibroblasts.4 This observation prompted us to investigate the role of α4 in cell adhesion

* This work was supported in part by a grant from the NCI, National Institutes of Health (to C. B. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be solely to indicate this fact.

† Recipient of a postdoctoral fellowship from the Leukemia and Lymphoma Society and American Association for Cancer Research.

‡To whom correspondence should be addressed: Abramson Family Cancer Research Inst., 450 BRBII/III, 421 Curie Blvd., Philadelphia, PA 19104. Tel.: 215-746-5515; Fax: 215-746-5511; E-mail: craig@mail.med.upenn.edu.

§M. Kong, unpublished data.

3 The abbreviations used are: PP2A, protein phosphatase 2A; MEF, mouse embryonic fibroblast; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FN, fibronectin; SDF, stromal cell-derived factor; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; TOR, target of rapamycin; ER, estrogen receptor.

4 M. Kong, unpublished data.
and motility in mammalian cells. Using α4 knock-out or overexpressing cells, we demonstrate here that α4 plays a novel role in cell spreading and motility by influencing the activity of the cellular GT-Pase Rac. The results demonstrate that the protein phosphatase 2A binding partner α4 is a non-redundant regulator of cell spreading and migration.

EXPERIMENTAL PROCEDURES

**Cell Lines and DNA Constructs**—MEFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. α4<sup>Δlox</sup>-immortalized MEFs stably expressing Bcl-xL were generated and infected as previously described (19). Lentiviral supernatant expressing Cre-estrogen receptor (ER) fusion protein was a gift from Dr. as previously described (19). Mouse α4 cDNA was generated by reverse transcription PCR using the primers 5'-TGC GAA TTC GCC ACC ATG GAT TAC AAC GAT GAC GAC GAT AAC GCA GCG TCT GAA GAC GAG TTA CTG-3' and 5'-TGG GGA TCC TCA GCT CAT GTT CTG GTT GCC GTA G-3'. Rac1V12 was a gift from Dr. Margaret M. Chou (University of Pennsylvania) and has been subcloned into the LPC retrovirus vector. Virus was generated and stable cell lines were generated as previously described (19). Lentiviral supernatant expressing Cre-estrogen receptor (ER) fusion protein was a gift from Dr. Eric Brown (University of Pennsylvania).

**Generation of α4 Transgenic Mice and Isolation of T Cells**—α4 transgenic mice were generated by oocyte injection of a wild-type α4 transgene with a FLAG tag cloned into the EcoR I-Sall sites of a plasmid containing the CD2 promoter/enhancer, kindly provided by Dr. Paul E. Love (National Institutes of Health, Bethesda, MD). T cells were purified by negative selection as described before (22) (StemCell Technologies, Vancouver, BC, Canada). Purified T cells were cultured at 37 °C in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Gemini), 10 units/ml penicillin/streptomycin (Invitrogen), 50 μm β-mercaptoethanol (Sigma), and 10 mM HEPES (Invitrogen).

**Cell Spreading**—Fibroblasts were starved in DMEM with 0.1% FBS overnight and collected by trypsinization. Equal numbers of cells were replated onto dishes coated with or without fibronectin (FN) (10 μg/ml). Cells were allowed to spread for the indicated times. Photographs were taken using phase-con- trast with a 20× objective (TE300; Nikon). The percentage of spreading cells was then determined. Spread cells were defined as cells with extended processes, lacking a rounded morphology, and not phase-bright, whereas nonspread cells were rounded and phase-bright.

**Wound Healing Assay**—Fibroblasts were grown in 60-mm culture dishes until confluent and were cultured in DMEM containing 0.1% FBS overnight. The cell monolayer was wounded with a linear scratch by a sterile 200-μl pipette tip. The wounded area was defined in each image by positioning lines in correspondence to the original scratch. The same wounded areas were photographed using phase-con- trast with a 10× objective at 0 or 6 h after scratch on an inverted microscope (TE300; Nikon) with or without inhibitors. Okadaic acid, fos- tricin, and Rac1 inhibitor 553502 were all from Calbiochem. Quantitative analysis of the wound closure was calculated by counting the number of cells per 9 × 10<sup>4</sup>-μm<sup>2</sup> wound area at 6 h using Metamorph software. Time-lapse microscopy was performed in the micro-incubator (model CSIM; Harvard Apparatus) with constant 37 °C temperature on an inverted microscope (Nikon Eclipse TE2000-E) equipped with a digital video camera (model Evolution QEi; Media Cybernetics) with 10× objective for 12 h. Images were taken every 5 min in the phase-contrast and were analyzed using Image-Pro Plus 5.1 software (Media Cybernetics).

**Transwell Cell Migration Assay**—Transwell cell migration assay of the fibroblasts was performed using the CHEMICON<sup>®</sup> QCM<sup>TM</sup> 24-well Cell Migration Assay kit following user instructions. In brief, cells were cultured overnight in DMEM containing 0.1% FBS. The next day, cells were harvested and resuspended in DMEM containing 5% bovine serum albumin, added (1.5 × 10<sup>5</sup>/300 μl) to the top of each migration chamber with an 8-μm pore size polycarbonate membrane, and allowed to migrate in the presence or absence of 10% serum into the lower chamber. After 3 or 6 h, the inserts were stained with the Cell Stain Solution (Chemicon), and cells that had not migrated were removed using a cotton swab. Migrated cells were then photographed using a microscope (MZ16FA; Leica) equipped with a digital camera (DC 500; Leica). To quantify the number of migrated cells, stained cells were subsequently extracted and detected on a standard microplate reader at 560 nm (Spectra- MAX190; Molecular Devices). For retroviral MIGRI-GFP-Cre-infected cells, non-migrated cells were removed using a cotton swab and the transwell chambers were analyzed by fluorescent microscopy using a microscope (Eclipse E300; Nikon) equipped with a 10× objective. In this way, only fluorescent (green) cells that had migrated were visualized and counted. For the T cell migration assay, purified T cells were cultured overnight before the migration assay. T cell migration was assayed in transwell plates with 5-μm pore size (Costar; Fisher, Atlanta, GA) with 600 μl of medium in the lower chambers with or without 10 ng/ml stromal cell-derived factor 1 (SDF-1) and 5 × 10<sup>4</sup> live T cells in 100 μl added to the upper chambers for 3 h at 37 °C. Cells that migrated to the lower chambers were counted using a hemocytometer.

**Immunofluorescence**—Fibroblasts were cultured in DMEM containing 0.1% FBS for 24 h and replated onto coverslips coated with FN (10 μg/ml). Cells were allowed to spread for 30 min in DMEM containing 10% serum. Cells were fixed in 4% paraformaldehyde and permeabilized for 10 min in phosphate-buffered saline containing 0.2% Triton X-100. Cells were washed with phosphate-buffered saline containing 0.02% Triton X-100 and 1.5% FBS, followed by incubation with antibodies against vinculin (Sigma-Aldrich) for 1 h at room tempera- ture. Cells were then incubated with rhodamine red or fluorescein isothiocyanate (FITC)-conjugated secondary antibod- ies (Jackson ImmunoResearch Laboratories). Images were taken using a Nikon E800 fluorescence microscope equipped with a CCD camera at 100× objective, and images were ana- lyzed using the Metamorph software package.

**Immunoprecipitation and Western Blot**—Whole cell extracts were prepared either in radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Roche Applied Science) and phosphatase inhibitor cocktails I and II (Sigma-Aldrich) or lysis buffer as described before (23). 20–30 μg of
**FIGURE 1.** α4 modulates cell spreading and focal adhesion formation. α4^flox^ MEFs were infected with MIGR1-GFP (Vec) or MIGR1-GFP-Cre (Cre). A, after 48 h of infection, cells were trypsinized and replated onto dishes coated with fibronectin (FN) (10 μg/ml) at 37 °C for 60 min and photographed by FITC microscopy. Total cellular α4 was shown by immunoblotting. *, p < 0.005 for Cre-infected cells versus Vec-infected cells using paired Student t test. B, after 48 h of infection, cells were trypsinized and replated onto dishes either coated with fibronectin (FN) (10 μg/ml) in medium containing 0.1% serum (right panels) or without FN using medium supplemented with 10% serum (left panels). Cells were photographed by phase-contrast at the times indicated. *, p < 0.0001 for Cre-infected cells at 20 h versus Cre-infected cells at 60 min using paired Student t test. **, p < 0.0001 for Cre-infected cells at 120 min versus Cre-infected cells at 30 min using paired Student t test. C, MEFs stably expressing retroviral vector (Vec) or FLAG-α4 were allowed to adhere on FN-coated dishes for 20 min and photographed by phase-contrast. Quantitative comparison of cell spreading efficiency was obtained by calculating the percentage of spread cells. All bar graphs represent mean values (± the S.E.) of three different experiments (at least 150 cells were counted from randomly chosen optical fields). Total cellular α4 was shown by immunoblotting. *, p < 0.005 for FLAG-α4 cells versus Vec cells using paired Student t test. D, α4^flox^ MEFs were infected with MIGR1-GFP (Vec) or MIGR1-GFP-Cre (Cre) for 48 h. E, MEFs expressing either vector control (Vec) or FLAG-α4. Cells were allowed to spread on FN-coated coverslips for 60 (D) or 30 min (E) and were then fixed and immunostained with antibody to vinculin, which in turn was detected with a secondary rhodamine-conjugated antibody (red). GFP expression of the cells in the same field was visualized by the use of FITC filter (green) in panel D. The immunostained spikes for vinculin were counted for each cell. Bar graphs represent mean values (± the S.E.) of at least 20 cells from randomly chosen optical fields. *, p < 0.001 for Cre-infected cells versus Vec-infected cells using paired Student t test. **, p < 0.005 for FLAG-α4 cells versus Vec cells using paired Student t test. F, MEFs expressing either vector control (Vec) or FLAG-α4 were trypsinized and split into two portions; one was left in the tube in suspension (susp) and the other was replated onto FN-coated dishes (FN) for 30 min. Cells were collected and immunoprecipitation and immunoblotting were performed with antibodies as indicated. Bars, 100 μm in panels A–C and 10 μm in panels D and E.
α4 Regulates Cell Spreading and Migration

The total abundance of Rac1 was also determined by immunoblot analysis of cell lysates.

RESULTS

α 4 Modulates Cell Spreading and Focal Adhesion Formation—During experiments to characterize the PP2A regulatory subunit α4, we observed that α4-deficient MEFs spread at a lower rate than control cells when trypsinized and transferred to fresh culture plates. To quantify this defect, α4<sub>lox</sub> MEFs were infected with retroviruses encoding either green fluorescence protein (GFP) expressed from an internal ribosome entry site (IRES) alone (Vec) or both a Cre recombinase and an IRES-GFP (Cre). Infected cells were collected at 48 h and plated on FN-coated dishes. As presented in Fig. 1A, a striking difference in the appearance of cell spreading on FN was observed. At 60 min, over 95% of the vector control cells appeared well extended and had achieved a flattened morphology. In contrast, only ~22% of α4-deficient cells spread well.

To determine whether loss of α4 protein was loaded on pre-cast gels and transferred to nitrocellulose. Membranes were blocked with BLOTTO (5% nonfat dry milk and 0.1% Tween 20 in phosphate-buffered saline) and incubated with antibodies against α4 (custom antibody from BIOSOURCE), phospho-Src-Ser-416, p-paxillin (Cell Signaling), actin (Santa Cruz Biotechnology), hemagglutinin (Roche Applied Science), and Bcl-xL (19). To examine the phosphorylation of focal adhesion kinase, cells were lysed and immunoprecipitation was performed using anti-IP antibody (Santa Cruz) followed by Western blot using focal adhesion kinase antibody (Cell Signaling).

PP2A Activity Assay—The activity of cellular protein phosphatase 2A was assayed with a PP2A immunoprecipitation phosphatase assay kit (Upstate). Briefly, PP2Ac subunit was immunoprecipitated from 500 μg of total cell lysates using 2 μg of anti-PP2Ac antibody (clone 1D6; Upstate) for 2 h at 4 °C. PP2A activity was then assayed by incubating the immunoprecipitated protein with the synthetic phosphopeptide K-R-pT-I-R-R following the manufacturer’s instructions.

Rac1 Activation Assay—Rac1 activity was measured using the Cdc42/Rac Activation Assay kit (SGT445; Chemicon) according to the manufacturer’s protocol. In brief, cells in one 100-mm dish were lysed, and 15 μl of 50% P21-activated kinase 1 P21-binding domain-agarose slurry was added to the cell lysates. Subsequently, an immunoblot was performed and PAK-precipitated GTP-Rac1 was detected by probing the membrane with a Rac1-specific monoclonal antibody, followed by horseradish peroxidase-conjugated secondary antibody, induced a complete impairment or a delay of cell spreading, we investigated cell spreading at different time points. As shown in Fig. 1B, α4-deficient cells exhibited a significant delay in cell spreading compared with control cells yet they were still able to spread and achieve a flattened morphology if enough time were given, indicating that α4 deletion delayed but did not prevent spreading. Similar results were observed when cells were plated in serum or transferred to fibronectin-coated plates (Fig. 1B). Photographs using GFP fluorescence validated over 95% of the cells shown are GFP-positive cells (data not shown). In our previous study, we identified that α4 is required to maintain cell viability (19). Here we show that deletion of α4 leads to impaired cell spreading and migration. This effect could not be accounted for by apoptotic cell death because the α4<sub>lox</sub> MEFs used here were stably transfected with Bcl-xL and cell viability was >90% at the time of assay (data not shown). In addition, α4-deleted cells were still able to spread and achieve a flattened morphology if enough time were given (Fig. 1B). Thus, these data suggest that α4 is able to regulate cell spreading independently of its effect on apoptosis.

Next, to further rule out the possibility that apoptosis may indirectly affect the cell cytoskeleton in α4-deleted cells, we investigated whether enforced expression of α4 promoted cell spreading. To address this question, several immortalized MEF cell lines expressing FLAG-tagged α4 were generated. Cells overexpressing α4 showed similar viability compared with vector control cells (data not shown). As presented in Fig. 1C, 20
\( \alpha 4 \) Regulates Cell Spreading and Migration

min after plating on FN-coated dishes, >40% of \( \alpha 4 \)-overexpressing cells attained a flattened morphology whereas only 15% of control cells achieved a flattened morphology at this time point. These data are representative of each of the stable \( \alpha 4 \)-expressing cell lines we derived. These results suggest that \( \alpha 4 \) is a limiting component in the regulation of cell spreading.

The effect of \( \alpha 4 \) on focal adhesion formation was next examined by staining the cells for vinculin. After 30 min of adhesion to FN, cells were processed for immunofluorescent labeling with anti-vinculin antibody. In vector-infected \( \alpha 4^{\text{flox}} \) MEFs, vinculin was localized in focal adhesions present in multiple cell extensions. In contrast, Cre-infected cells possessed membrane ruffles but very few extensions or focal adhesions (Fig. 1D). The total number of focal adhesion spikes in \( \alpha 4 \)-transfected cells was substantially increased in comparison with controls (Fig. 1E). In addition, similar results were obtained when staining for another focal adhesion protein, paxillin (data not shown). Despite the effects of \( \alpha 4 \) on adhesion complex formation, \( \alpha 4 \) overexpression did not affect the phosphorylation of the focal adhesion regulators Src, focal adhesion kinase, and paxillin (Fig. 1F).

\( \alpha 4 \) Regulates Cell Migration—The role of \( \alpha 4 \) in regulating cell migration was also examined. The directed migration of vector-infected and Cre-infected \( \alpha 4^{\text{flox}} \) MEFs was compared using an in vitro wound assay. As shown in Fig. 2A, when a confluent plate of cells was scratched by a sterile pipette tip, vector-infected cells extensively migrated into the wound area within 6 h. In contrast, the Cre-infected cells failed to migrate into the denuded area.

We next determined whether \( \alpha 4 \) overexpression could promote cell migration. As shown in Fig. 2B, MEFs stably expressing FLAG-\( \alpha 4 \) displayed enhanced cell migration into the wound area. Using video time-lapse microscopy, we were able to track the migration of individual cells during this experiment. By tracking over 60 cells, we confirmed that cells overexpressing \( \alpha 4 \) displayed significantly increased motility (29.0 ± 1.6 \( \mu \)m/h in \( \alpha 4 \)-expressing cells compared with 18.0 ± 1.3 \( \mu \)m/h in control cells; \( p < 0.0001 \)) (Fig. 2C). Similar results were obtained using different clones.

To further confirm the role of \( \alpha 4 \) in regulating cell motility, we used a two-chamber migration assay in which cells migrated through a membrane with 8-μm pores from serum-free medium toward a lower chamber with or without 10% serum. Consistent with the results from the wound assay, overexpression of \( \alpha 4 \) promoted cell migration either in the presence or absence of serum in the lower chamber (Fig. 3A). By taking advantage of \( \alpha 4^{\text{flox}} \) MEFs infected with IRES-GFP or IRES-GFP-Cre, cells that migrated into the lower chamber were monitored by photographing the inner membrane with GFP fluorescence. Compared with vector-infected cells, the ability of Cre-infected cells to migrate into the lower chamber containing 10% serum was significantly reduced (Fig. 3B).

\( \alpha 4 \) Functions through Modification of PP2A Activity—Several lines of evidence suggest that PP2A activity is required for cell migration and adhesion (24, 25). Using the in vitro phosphatase assay, we observed that deletion of \( \alpha 4 \) resulted in reduced PP2A activity and overexpression of \( \alpha 4 \) led to increased PP2A activity (Fig. 4, A and B), suggesting PP2A activity is involved in \( \alpha 4 \)-regulated cell migration. Inhibition of PP2A by either okadaic acid or forsterlein completely blocked \( \alpha 4 \)-promoted cell migration (Fig. 4C).

\( \alpha 4 \) Expression Levels Correlate with the Cellular Level of GTP-bound Rac1—Rac1 is known to be a critical regulator in cell spreading and motility and is required for focal adhesion formation (3, 26). We observed that \( \alpha 4 \) localized in the cell periphery, especially at protruding edges, during spreading in a pattern similar to that of the active form of Rac1 (Fig. 5A). Using an assay that specifically recognizes the active GTP-bound form of Rac1 in both \( \alpha 4 \)-deficient and -overexpressing cells, the deletion of \( \alpha 4 \) by Cre infection was found to reduce the activation of Rac1 (Fig. 5B). Moreover, the level of GTP-bound Rac1 was enhanced by \( \alpha 4 \) expression (Fig. 5C).
α4 Regulates Cell Spreading and Migration

**FIGURE 4. α4 functions through modification of PP2A activity.** A, α4ffloxMEFs were infected with MIGR1-GFP-Cre (Cre) for the times indicated. **B**, MEFs stably expressing retroviral vector (Vec) or FLAG-α4. PP2A activity was measured as described under "Experimental Procedures" (left panel). Results are expressed as the fold of control and are representative of three independent experiments. Immunoblotting shows total cellular Rac1 and GTP-bound Rac1 levels as described under "Experimental Procedures." *Bar, 10 μm.* C, monolayer of MEF clones expressing retroviral vector (Vec) or FLAG-α4 were wounded as described under "Experimental Methods," and the same spots were photographed at 0 and 6 h after scratch in the presence or absence of PP2A inhibitors. Migration was quantified by counting cells that migrated into a defined wound area after 6 h. Cells in six defined areas/group/experiment were quantified. **Bar graphs** are representative of three independent experiments and are shown ± the S.E. *, p < 0.001 compared with control FLAG-α4 cells using paired Student t test.

**Rac1 Is a Mediator of α4-regulated Cell Migration**—The above data suggest that α4 regulation of cell spreading and migration is epistatic to Rac1. If this is the case, the cytoskeletal defect induced by α4 deletion should be rescued by constitutively active Rac1. To test this, we first generated cell lines expressing a tamoxifen-inducible Cre-ER fusion protein in α4ffloxMEFs. Based on this, we further generated stable cell lines expressing constitutively active hemagglutinin-Rac1V12 in α4ffloxCre-ER MEFs (Fig. 6A). Consistent with the previous results, α4-deleted cells spread much less than control cells whereas expression of constitutively active Rac1 almost completely overcame this defect (Fig. 6B). Moreover, the two-chamber transwell assay demonstrated that Rac1V12 rescued the defect of cell migration caused by α4 deletion (Fig. 6C). To further show a functional correlation between α4 and Rac1, we tested whether inhibition of Rac1 would block α4-promoted cell migration. As shown in Fig. 6D, α4-promoted cell migration was almost completely abolished by a specific Rac1 inhibitor, 553502.

**Transgenic α4 Promotes Primary T Cell Motility**—It has been reported that α4 is expressed in both T and B lymphocytes (27). To test whether α4 regulation of cell motility is specific for fibroblasts or is also present in other cells, we generated T cell-specific α4 transgenic mice using a T cell-specific CD2 promoter/enhancer. T cells were isolated and purified to >99% purity as indicated by staining with the T cell marker Thy 1.2. Characterization of α4-transgenic T cells showed that an increased level of α4 has no observed effect on T cell development, proliferation, and viability (Fig. 7A and data not shown). To examine whether α4 regulates T cell motility, purified T cells from either wild-type or transgenic mice were analyzed using the two-chamber transwell assay in the absence or the presence of the chemokine SDF. Similar to our findings in fibroblasts, α4 transgenic T cells displayed enhanced migration both in the absence or presence of SDF (Fig. 7B). In agreement with the fibroblast results, increased GTP-bound Rac1 was found in α4 transgenic T cells (Fig. 7C). Using this in vivo transgenic system, we demonstrate that the ubiquitously expressed protein α4 plays a similar role in promoting cell migration both in fibroblasts and T cells.

**DISCUSSION**

Reversible protein phosphorylation controlled by protein kinases and phosphatases is a major mechanism for regulating a variety of cellular processes. Unlike kinases, the activity and specificity of serine/threonine phosphatases is governed largely by their associated proteins (11). The PP2A phosphatase complex contains two distinct forms of evolutionarily conserved regulatory subunits that do not appear to be functionally redun-
\(\alpha 4\) Regulates Cell Spreading and Migration

**A**

\(\alpha 4^{\text{Cre-ER}}\) MEFs expressing Cre-ER fusion protein (\(\alpha 4^{\text{Cre-ER}}\)) were stably transfected with LPC vector (Vec) or LPC-hemagglutinin-Rac1V12 (Rac1V12). Cells were treated with or without 200 nM 4-hydroxytamoxifen (4HT) for 72 h. A, cells were collected, and immunoblotting was performed with antibodies indicated. B, cells were trypsinized and replated onto dishes coated with FN (10 \(\mu g/ml\)) for 60 min and then photographed using phase-contrast microscopy. Quantitative comparison of cell spreading efficiency was obtained by calculating the percentage of spread cells from three different experiments (mean ± the S.E.), \(\ast p < 0.005\) for Rac1V12 cells versus Vec cells in the presence of 4HT using paired Student \(t\) test. Bar, 100 \(\mu m\). C, cell migration was analyzed by the two-chamber assay in the presence of 10% serum in the lower chamber. After 6 h, migrated cells were stained and photographed as described under “Experimental Procedures.” Bar graphs show mean value ± the S.E. from three independent experiments, \(\ast p < 0.0001\) for Rac1V12 cells versus Vec cells in the presence of 4HT using paired Student \(t\) test. D, monolayer of MEF clones expressing retroviral vector (Vec) or FLAG-\(\alpha 4\) were wounded as described under “Experimental Procedures,” and the same spots were photographed at 0 and 6 h after scratch in the presence or absence of Rac1 inhibitor (50 \(\mu M\)). Migration was quantified by counting cells that migrated into a defined wound area after 6 h. Cells in six defined areas/group/experiment were quantified. Bar graphs are representative of three independent experiments and are shown ± the S.E., \(\ast p < 0.005\) compared with control FLAG-\(\alpha 4\) cells using paired Student \(t\) test.

The \(\alpha 4\) homologue in yeast, Tap42, has been reported to influence actin organization via a Rho GTPase-dependent mechanism (21, 28). The present findings suggest that mammalian \(\alpha 4\) retains this ability. Although in yeast PP2A activity might play a negative role in controlling the actin cytoskeleton (21), our data suggest that PP2A activity is required for \(\alpha 4\) regulation of cell migration.

The results demonstrate that Rac1 is a downstream effector for \(\alpha 4\)-mediated cell spreading and migration. Deletion of \(\alpha 4\) resulted in impaired activation of Rac1 and overexpressing \(\alpha 4\) led to an increase in GTP-bound Rac1. Expression of an active mutant of Rac1 in \(\alpha 4\)-deficient cells rescued \(\alpha 4\) deletion-induced defects in cell spreading and migration, suggesting \(\alpha 4\) functions upstream of Rac1 activation. These observations are consistent with the previous studies that Rac1 is a critical regulator of both cell spreading and migration (29–31). Despite the fact that \(\alpha 4\) overexpression led to increased focal adhesion spikes and deletion resulted in impaired focal adhesion formation, the phosphorylation status of several critical focal adhesion regulators did not change, including focal adhesion kinase, Src, and paxillin. Other studies have shown that Rac1 does not affect phosphorylation of Src or focal adhesion kinase yet it is required for Src, focal adhesion kinase, and vinculin to form a stable molecular complex, important for cell focal adhesion (26). Our results suggest that \(\alpha 4\) may regulate the focal adhesion complex via Rac1.

What is upstream of \(\alpha 4\) in controlling Rac1? It has been shown that the rapamycin-insensitive TOR complex TORC2 is involved in regulating cytoskeleton both in yeast and mammalian cells (32–34). Like yeast TORC2, mammalian TORC2 also functions upstream of the Rho GTPase Rac1 (34). Our data suggest that \(\alpha 4\) might act as a mediator of mammalian TORC2 in regulating Rac1 and cytoskeletal organization. The possible factors downstream of \(\alpha 4\)-phosphatase complex in regulating cell cytoskeleton are unknown. Using co-immunoprecipitation, we did not detect a direct interaction between \(\alpha 4\) and Rac1, indicating that \(\alpha 4\) may contribute to regulating Rac1 via a non-direct mechanism. Given the fact that TOR proteins regulate Rho GTPase activity via a GTP exchange factor, ROM2, in...
α4 Regulates Cell Spreading and Migration

Given the fact that overexpression of α4 up-regulates the GTP-bound state of Rac1, it is not surprising that α4 promotes migration in transgenic T cells. Moreover, Rac1-mediated migration of T cells generally is integrin-dependent. Our results demonstrate that inhibition of integrin signaling using the integrin β1 antibody completely abolished the ability of α4 to promote T cell migration. These data further confirmed the cellular functional correlates between PP2A-α4 and Rac-1 signaling in regulating cell migration. Our previous work had demonstrated that α4-PP2A is involved in T cell receptor signaling, because the cytoplasmic domains of both CTLA-4 and CD28 can associate with PP2A and α4 (13). Recently, another group showed that CTLA-4 increases T cell motility and over-rides the T cell receptor-induced stop signal, yet how CTLA-4 regulates T cell motility remains unknown (36). Our data suggest that α4 might play a critical role in regulating T cell motility mediated by CTLA-4.

Acknowledgments—We thank Drs. Margaret M Chou and Eric Brown for providing the Rac1 constructs and lentivirus supernatant expressing Cre-ER fusion protein, respectively. We thank members of the Thompson laboratory for their inspiration and discussion.

REFERENCES

1. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) Science 302, 1704–1709
2. Webb, D. J., Parson, J. T., and Horwitz, A. F. (2002) Nat. Cell Biol. 4, E97–E100
3. Hall, A. (2005) Biochem. Soc. Trans. 33, 891–895
4. Étienne-Manneville, S., and Hall, A. (2002) Nature 420, 629–635
5. Rossman, K. L., Der, C. J., and Sondek, J. (2005) Nat. Rev. Mol. Cell Biol. 6, 167–180
6. Alahrí, S. K., Reddig, P. J., and Juliano, R. L. (2002) Int. Rev. Cytol. 220, 145–184
7. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. M. (1998) Science 280, 1614–1617
8. Yu, D. H., Qu, C. K., Henegariu, O., Lu, X., and Fing, G. S. (1998) J. Biol. Chem. 273, 21125–21131
9. Zeng, L., Si, X., Yu, W. P., Le, H. T., Ng, K. P., Teng, R. M., Ryan, K., Wang, D. Z., Ponniah, S., and Allen, C. J. (2003) J. Cell Biol. 160, 137–146
10. Ito, A., Kataoka, T. R., Watanabe, M., Nishiyama, K., Mazaki, Y., Sabe, H., Kitamura, Y., and Nojima, H. (2000) EMBO J. 19, 562–571
11. Janssens, V., and Goris, J. (2001) Biochem. J. 353, 417–439
12. Inui, S., Kuwahara, K., Mizutani, J., Maeda, K., Kawai, T., Nakayasu, H., and Sakaguchi, N. (1995) J. Immunol. 154, 2714–2723
13. Chuang, E., Fischer, T. S., Morgan, R. W., Robbins, M. D., Duer, J. M., Vander Heiden, M. G., Gardner, J. P., Hambor, J. E., Neveu, M. J., and Thompson, C. B. (2000) Immunity 13, 313–322
14. Goldberg, Y. (1999) Biochem. Pharmacol. 57, 321–328
15. Murata, K., Wui, J., and Brautigan, D. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10624–10629
16. Nanahoshi, M., Tsujihata, Y., Tokunaga, C., Inui, S., Sakaguchi, N., Hara, K., and Yonezawa, K. (1999) FEBS Lett. 446, 108–112
17. Pickett, T. D., and Brautigan, D. L. (2006) J. Biol. Chem. 281, 30503–30511
18. Di Como, C. J., and Arndt, K. T. (1996) Genes Dev. 10, 1904–1916
19. Kong, M., Fox, C. J., Mou, J., Solt, L., Xu, A., Cinalli, R. M., Birnbaum, M. J., Lindsten, T., and Thompson, C. B. (2004) Science 306, 695–698
20. Inui, S., Sanjo, H., Maeda, K., Yamamoto, H., Miyamoto, E., and Sakaguchi, N. (1998) Blood 92, 539–546
21. Wang, H., and Jiang, Y. (2003) Mol. Cell. Biol. 23, 3116–3125

<FIGURE 7. Transgenic α4 promotes T cell migration.> Purified T cells from either wild-type (WT) or α4 transgenic (α4-Tg) animals were cultured overnight without stimulation. A, overexpression of α4 had no effect on cell viability. T cell viability was determined by propidium iodide (PI) staining followed by flow cytometry at the time indicated. B, α4 promoted T cell migration. T cell migration was determined in a two-chamber transwell assay with or without 10 ng/ml of SDF in the bottom chamber. Cells that migrated into the bottom chamber were counted. The bar graph represents results from three independent experiments (mean ± the S.E.) * p < 0.01 for WT versus α4-Tg in the absence of SDF. ** p < 0.005 for WT versus α4-Tg in the presence of SDF. C, α4 up-regulated Rac1 activity in T cells. T cells were collected from either wild-type (WT) or α4 transgenic (α4-Tg) animals. Rac1 activity was measured as described under “Experimental Procedures.” Total cellular Rac1 and α4 were shown by immunoblotting.

yeast, it is possible that α4-phosphatase complex signals to the cell cytoskeleton through modification of GTP/GDP exchange factor activity (32).

Using T cell-specific transgenic mice, we are able to demonstrate that α4 also promotes T cell migration. It has been shown by many groups that Rac1 is a critical regulator in T cell migra-
α4 Regulates Cell Spreading and Migration

22. Cinalli, R. M., Herman, C. E., Lew, B. O., Wieman, H. L., Thompson, C. B., and Rathmell, J. C. (2005) Eur. J. Immunol. 35, 786–795
23. Kong, M., Mournier, C., Dumas, V., and Posner, B. I. (2003) J. Biol. Chem. 278, 5837–5844
24. Kiely, P. A., O’Gorman, D., Luong, K., Ron, D., and O’Connor, R. (2006) Mol. Cell. Biol. 26, 4041–4051
25. Takahashi, K., Nakajima, E., and Suzuki, K. (2006) J. Cell Physiol. 206, 814–820
26. Guo, F., Debidda, M., Yang, L., Williams, D. A., and Zheng, Y. (2006) J. Biol. Chem. 281, 18652–18659
27. Onda, M., Inui, S., Maeda, K., Suzuki, M., Takahashi, E., and Sakaguchi, N. (1997) Genomics 46, 373–378
28. Torres, J., Di Como, C. J., Herrero, E., and De La Torre-Ruiz, M. A. (2002) J. Biol. Chem. 277, 43495–43504
29. van Leeuwen, F. N., van Delft, S., Kain, H. E., van der Kammen, R. A., and Collard, J. G. (1999) Nat. Cell Biol. 1, 242–248
30. Hamelers, I. H., Olivo, C., Mertens, A. E., Pegtel, D. M., van der Kammen, R. A., Sonnenberg, A., and Collard, J. G. (2005) J. Cell Biol. 171, 871–881
31. Hall, A. (1998) Science 279, 509–514
32. Schmidt, A., Bickle, M., Beck, T., and Hall, M. N. (1997) Cell 88, 531–542
33. Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004) Curr. Biol. 14, 1296–1302
34. Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A., and Hall, M. N. (2004) Nat. Cell Biol. 6, 1122–1128
35. Fukui, Y., Hashimoto, O., Sanui, T., Oono, T., Koga, H., Abe, M., Inayoshi, A., Noda, M., Oike, M., Shirai, T., and Sasazuki, T. (2001) Nature 412, 826–831
36. Schneider, H., Downey, J., Smith, A., Zinselmeyer, B. H., Rush, C., Brewer, J. M., Wei, B., Hogg, N., Garside, P., and Rudd, C. E. (2006) Science 313, 1972–1975