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Zap70 controls the interaction of talin with integrin to regulate the chemotactic directionality of T-cell migration

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ABSTRACT

Aberrant lymphocyte infiltration is crucial for many disorders such as tumor immune escape and autoimmunity. In this study, we have investigated T-cell migration in a three-dimensional collagen matrix containing tumor spheroids and by using μ-Slide chemotaxis and found that Zap70 regulates directionality during cell chemotaxis. Jurkat cells actively migrated toward SDF-1, nutrition, and spheroids of MCF-7 breast carcinoma cells embedded in collagen matrix. Inhibition of Zap70 activity impaired transmigration and μ-Slide chemotaxis but not the random movement of T cells in the collagen/fibronectin matrix. P116 cells, a Zap70 deficient variant of Jurkat, showed active random movement but failed to migrate against chemoattractants. P116 cells exhibited a reduced polarization of cell morphology, showing less lamellipodia formation accompanied with a fast pseudopod turnover rate. Instead of direct interacting with F-actin, Zap70 formed a complex with talin which is an integrin scaffold for F-actin. SDF-1 enhanced Zap70 phosphorylation and also stimulated binding of talin and β1 integrin activation. P116 cells showed reduced complex of talin and β1 integrin in parallel with impaired integrin activation. Collectively, Zap70 modulates integrin activation by interacting with talin, which contributes to directionality of T-cell migration, severing as a potential target for anti-inflammation therapy.

1. Introduction

Understanding lymphocyte migration, by which appropriate immune responses are initiated, is essential for elucidating the etiology of many inflammation related disorders. Cell migration is well controlled by a complex network of signaling pathways, which are activated by chemokines and different sets of integrin receptors. Among those migratory signals for T cells, activation of ERK and AKT are important (Boehme et al., 1999; Vlahakis et al., 2002). Although a dominant-negative Vav1 mutant, but not ERK, blocks SDF-1-induced T-cell migration (Ticchioni et al., 2002), integration of the signals of chemokine stimuli and T-cell receptors (TCR) is crucial to warrant a successful antigen-specific infiltration. Zap70 is a kinase binding to the intracellular domain of TCR and is quickly phosphorylated upon TCR engagement with antigen loaded on the major histocompatibility complex (Chan et al., 1992; Hatada et al., 1995). A defect in Zap70 causes aberrant thymic selection resulting in severe immunodeficiency and autoimmune arthritis (Chan et al., 1994; Sakaguchi et al., 2003). Evidence indicates that Zap70 plays also a role in T-cell migration upon chemokine stimulation. For instance, blockage of Zap70 signal reduces LFA-1-dependent transmigration induced by SDF-1 (Soede et al., 1998; Ticchioni et al., 2002). How Zap70 controls T-cell migration is not fully understood.

Considering that tumor cells rarely express only one attractant, the degree of lymphocyte infiltration is the result of many factors produced by tumor cells. Besides, it is known that chemokines may exert opposite effects on target cells depending on the local concentration they reach. A low concentration of SDF-1 is an attractant for T cells, while a high concentration is a repellant (Poznansky et al., 2000). Therefore, the contribution of chemotactic signals for T-cell infiltration in a tumor site can be underestimated if they are measured separately. To observe the T-cell migration in the vicinity of tumor cell mass, we have applied a 3D coculture system using collagen as the supporting matrix to simultaneously observe the mobility and directionality during T-cell chemotaxis. Tumor spheroids, which show biological properties similar to those of tumor nodules grown *in vivo* (Sutherland, 1988) and have been used to study cellular motility (Raeber et al., 2005; Schor et al., 1983), were chosen as the source of chemotactic factors.

Abbreviations: Zap70, zeta-associated protein-70; SDF-1, stromal cell-derived factor-1; 3D culture, three-dimensional culture.

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The movement of T cells toward tumor spheroids in the collagen matrix, expected to approximate an *in vivo* environment, was recorded using time-lapse videomicroscopy. Furthermore, we used two methods, Boeden-chamber-based transmigration assay (one-dimensional) and μ-Slide chemotaxis (two-dimensional), to verify the function of Zap70 for T-cell migration in a defined environment. The former method measures the net migration of cells toward a chemotactic source, while the latter method simultaneously records active mobility and directionality. Combining pharmacological inhibitors and Zap70 deficient T cells, we demonstrate for the first time that Zap70 plays an important role in cell directionality during T-cell chemotaxis.

2. Materials and methods

2.1. Cell culture, spheroids and transfection

Human T-cell leukemia cell line Jurkat (E6-1), breast carcinoma cell line MCF-7, and glioblastoma cell line U-118MG, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Jurkat derivatives P116 and CAM1.6, originally from ATCC, were kindly provided by Dr. PL Shu, College of Medicine, National Taiwan University. Jurkat cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% FBS, and 1% antibiotic–antimycotic. MCF-7 and U-118MG were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and 1% antibiotic–antimycotic. Cells were grown at 37 °C in an air 5% CO_2 atmosphere at constant humidity. MCF-7 and U-118MG spheroids were formed by culturing 1×10^4 cells 5 days in a 96-well plate base-coated with 0.5% Noble agar prepared in DMEM. Plasmid encoding Zap70 was purchased from Gendiscovery (Taiwan). For transfection, those plasmids were delivered into p116 cells using a Microporator (Digital Bio Technology, Korea) according to the manufacturer's instructions.

2.2. Preparation of collagen gel

Type I collagen was prepared from pork skin according to the established procedure described previously (Speranza and Valentini, 1986). Stock solution was made of 0.2% collagen dissolved in 0.025 N acetic acid. For 3D collagen gel culture, collagen stock (3 volume) was mixed with 5.7× DMEM (1 volume), 2.5% $NaHCO_3$ (0.5 volume), 0.1 M HEPES (1 volume), 0.17 M $CaCl_2$ (0.1 volume), 1 N NaOH (0.1 volume), and 1× serum free culture medium (RPMI; 4.3 volume) containing cells and spheroids. The mixture was dispensed in a culture dish and allowed to gelatinize by incubation in humidified air with 5%CO_2, at 37 °C.

2.3. Analysis of lymphocyte migration in collagen gel

Time-lapse videomicroscopy was used to measure the migration of lymphocytes embedded in 3D collagen gel according to a previously described method (Allen et al., 1998). The positions of Jurkat cells relative to their starting places and migration distances were graphed. On these graphs, the distance from the origin to a point reflects the travel distance; the absolute *X* values represent lateral deviation along the direction toward the tumor spheroid. Jurkat cells that moved at least 20 μm over the observed time period and had positive *X* coordinates were considered as migrating toward the tumor spheroid. Alternatively, transmigration assay of T cells was done in 24-well chemotaxis chambers (6.5-mm diameter; Costar, Cambridge, MA). Polycarbonate transwell culture inserts with a 5-μm pore size were used. RPMI 1640 medium or tumor-conditioned media was added to the lower chamber, and the lymphocyte suspension in RPMI 1640 medium was added to the upper well. The number of lymphocytes that migrated into the lower well in 4 h was counted.

2.4. Chemotaxis assay using μ-Slide chemotaxis

The μ-Slide chemotaxis was done according to the manufacturer's instructions (Integrated BioDiagnostics, Germany). To enhance T-cell attachment, the observable area of a μ-Slide rack was coated with a solution containing fibronectin (50 μg/ml) for 30 min and the chamber allowed to dry overnight. A cell suspension of 10^6 cells/ml was loaded in the chamber. Images of cell movement toward SDF-1 or nutrition were evaluated by ImageJ plug-in (free software of Chemotaxis and Migration Tool: http://www.ibid.de/applications/ap-chemo.html) for plotting and analyzing the tracked data.

2.5. Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from MCF-7 and U-118MG cell lines using TRIzol reagent (Life Technologies, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription of 2 μg total RNA was performed at 37 °C for 60 min in a 20 μl solution including 500 ng of oligo-dT primers, 4 μl of 5× RT buffer (250 mM Tris–HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$; 50 mM DTT), 0.5 μl of 40 mM dNTPs, 0.5 μl of 200 units/μl RNase inhibitor (Promega, Madison, WI), 3.5 μl of diethyl pyrocarbonate-treated water, and 1 μl of 200 units/μl of M-MLV reverse transcriptase (Promega, Madison, WI). A polymerase chain reaction of 1 μl cDNA was carried out in a 50 μl reaction including 10 mM Tris/HCl, pH9.0, 200 μM dNTPs, 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, and 10 pmol of oligonucleotide primers (Table 1). Human β-actin served as a quantitative control in PCR. PCR products were fractionated by agarose, stained with ethidium bromide, and visualized under UV light.

2.6. Western blot analysis

Cells were lysed in whole cell extraction buffer (250 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors cocktail containing 500 μM AEBSF, 1 μg/ml aprotinin, 1 μM E-64, 500 μM EDTA, and 1 μM leupeptin). About 50–100 μg of the proteins were separated in 10–12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked with 5% skim milk and then probed with antibodies specific for β1 integrin (BD PharMingen, San Diego, CA), Zap70, phospho-Zap70 (Tyr-493 in the kinase domain, New England Biolabs, Beverly, MA; final dilution:1/1,000), and GAPDH (Chemicon, Temecula, CA; final dilution:1/5000). The activation of β1 integrin was determined by using anti-LIBS antibody (Chemicon, Temecula, CA) to recognize an epitopes that is exposed only after activation (Shimaoka et al., 2002). After being probed with appropriate secondary antibodies, immunodetected proteins were visualized by an ECL system according to the manufacturer's instruction (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.7. Immunoprecipitation assay

Total protein extract of Jurkat cells and P116 cells pre-cleared with protein G-agarose beads, was incubated with a monoclonal antibody specific for Talin (Millipore, MA) at 4 °C for 3 h. Talin antibody complex was pulled down with protein G-agarose beads. Immunoprecipitates were collected and washed with a lysis buffer. Talin, Zap70, and β1 integrin in the immunoprecipitates were detected using Western blot analysis with appropriate antibodies.

Table 1
Oligonucleotide primers used for RT-PCR.

	Sense (5′ → 3′)	Antisense (5′ → 3′)	Expected size of product
SDF-1	TGAACGCCAAGGTCGTGGTC	CCTCAGGCGTCTGACCCTC	301 bp
RENTES	TCATTGCTACTGCCCTCTGCG′	TGGCACACACTTGGCGGTTC	189 bp
β-Actin	GATGAGATTGGCATGGCTTT	CACCTTCACCGTTCCAGTTT	139 bp

2.8. Morphological observation

Human fibronectin (Santa Cruz Biotechnology, CA) was dissolved in PBS to a working concentration of 20 μg/ml. To prepare fibronectin-coated dish, 300 μl fibronectin solutions were added to a 40 mm-dish and incubated for 1 h. The dish was then washed twice with PBS to remove unbounded fibronectin. Freshly prepared fibronectin-coated dishes were used in the experiments.

For confocal microscopic imaging, Jurkat cells were seeded on a fibronectin-coated coverslip at 1×10^5 cells/ml and incubated at 37 °C for 1 h followed by SDF-1 stimulation (50 ng/ml) for 5 min. Cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 1% BSA. Immunostaining was done by hybridization with antibodies (Millipore, MA) against talin, Zap70 or phospho-Zap70 for 3 h, followed by incubation with secondary antibodies conjugated with Alex 594 or Alex 488. Imaging was done using a Leica TCS SPII confocal microscope (Nussloch, Germany) with excitation set at 488 nm.

2.9. Statistical analysis

Data are expressed as mean ± SEM. The differences between groups were analyzed using Student's *t*-test. One-way ANOVA and Tukey's honestly significant difference method were used to make pairwise comparisons between the treatments in most experiments. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Zap70 is required for Jurkat cells to migrate toward tumor spheroids in 3D collagen gel culture

To correlate the attraction of T cells, we measured the expressions of known T cells attractants, SDF-1 and RENTES, in tumor cells. In agreement with a previous report (Zhou et al., 2002), transcripts of SDF-1 and RANTES were detected in MCF-7 cells, but not in U-118MG cells (Fig. 1A). Accordingly, MCF-7-conditioned medium attracted Jurkat cells migrating across the insert membrane in a transwell experiment, but U-118MG-conditioned medium did not (Fig. 1B). Tumor spheroids, secreting chemoattractants, create a chemotaxis gradient in collagen gel and attract lymphocytes to migrate toward them. Using time-lapse videomicroscopy, we established a convenient 3D collagen gel culture to simultaneously evaluate directionality and motility. MCF-7 cells formed typical tumor spheroids on Nobel agar in 3 days (Fig. 1C). There was no obvious cell detachment from MCF-7 spheroids cultured in 0.067% collagen type I gel in 12 h (data not shown). The migration behavior of Jurkat cells in collagen gel toward MCF-7 spheroids was recorded by time-lapse videomicroscopy. The directionality and overall moving distance of the migrating Jurkat cells were determined by plotting the final positions of individual cells from their original position versus the place of the tumor spheroid (Fig. 1D). In collagen gel without any attractant, Jurkat cells barely moved; less than 20 μm of centroid movement over a 12 h observation period. About 40% of Jurkat cells near the MCF-7 spheroids were motile. Among motile Jurkat cells, 80% cells migrated toward MCF-7 spheroids, which was comparable to the transmigration result deduced from a transwell experiment in which a conditioned medium of MCF-7 cells attracted Jurkat cells (Fig. 1B). Surprisingly, Jurkat cells moved actively in a random fashion after Zap70 was blocked by piceatannol. A recent report demonstrated that TCR signals downstream kinase LCK and its substrate Zap70 regulates T-cell chemotaxis induced by SDF-1 (Dar and Knechtle, 2007). In addition, Jurkat cells lacking Zap70 exhibit lower level of migration toward a SDF-1 source than wild-type Jurkat cells do (Ottoson et al., 2001). We thus measured the migration of CAM1.6 and P116 cells, which are Jurkat derivatives with defects in LCK and Zap70, respectively. Similar to piceatannol treatment, P116 cells showed an elevated random movement when observed alone in collagen gel. Furthermore, P116 cells near MCF-7 spheroids in collagen gel also underwent random migration. CAM1.6 cells, defective in LCK, were not motile in 3D collagen gel with or without MCF-7 spheroids. To better understand the migratory behavior of T cells, we used U0126 to inhibit ERK which is important in regulating cell motility. Compared with the control groups, inhibiting ERK significantly reduced the overall migration of Jurkat cells to the extent that they showed very short centroid movement (Fig. 1D). Consistent with these findings, U0126 also inhibited the transmigration of Jurkat cells toward nutrition gradient in a conventional transwell experiment (data not shown).

3.2. Deficiency in Zap70 causes loss of directional movement along a chemotactic gradient resulting in low transmigration rate

In previous studies using a transwell assay system, Jurkat cells defective in LCK and Zap70 exhibited lower levels of migration toward a SDF-1 source than did wild-type Jurkat cells (Dar and Knechtle, 2007; Ottoson et al., 2001). Consistent with these observations, the addition of piceatannol to block Zap70 severely impaired the transmigration of Jurkat cells (Fig. 2A). P116 and CAM1.6 cells also showed a low transmigration rate toward MCF-7-conditioned medium in a transwell experiment (Fig. 2B). We further used μ-Slide chemotaxis assay to differentiate the action of Zap70 on cell motility (Fig. 3). Jurkat cells migrated actively toward SDF-1 on fibronectin-coated plate. In contrast, P116 cells moved in much random manner in a SDF-1 gradient (Fig. 3). In the absence of defined chemoattractant, P116 cells attached well on fibronectin-coated plate and showed random movement similar to that of wild type Jurkat cells.

3.3. Zap70 affects focal adhesion turnover

Videomicroscopic images showed a dynamic change of cell morphology during migration. When Jurkat cells were plated on a fibronectin-coated dish, three types of cell morphology appeared: round, rough, and ameba-like (Fig. 4A). Rough Jurkat cells had short podia-like protrusions which retracted frequently. Ameba-like cells had a flat, fibroblast-like shape with morphological polarization, and migrated randomly on a fibronectin matrix. By counting cells with distinct shapes, we found that the morphological changes induced by fibronectin were cell-type dependent (Fig. 4B). Jurkat cells stably adhered to fibronectin and about 40% of them became ameba-like in 1 h; 30% were round and 30% rough. Among P116 cells on fibronectin, 37% were round, 37% rough, and 25% ameba-like. We further measured the dynamic changes of pseudopod (Fig. 4C and D). Jurkat cells with an ameba-like shape had an about 115 s

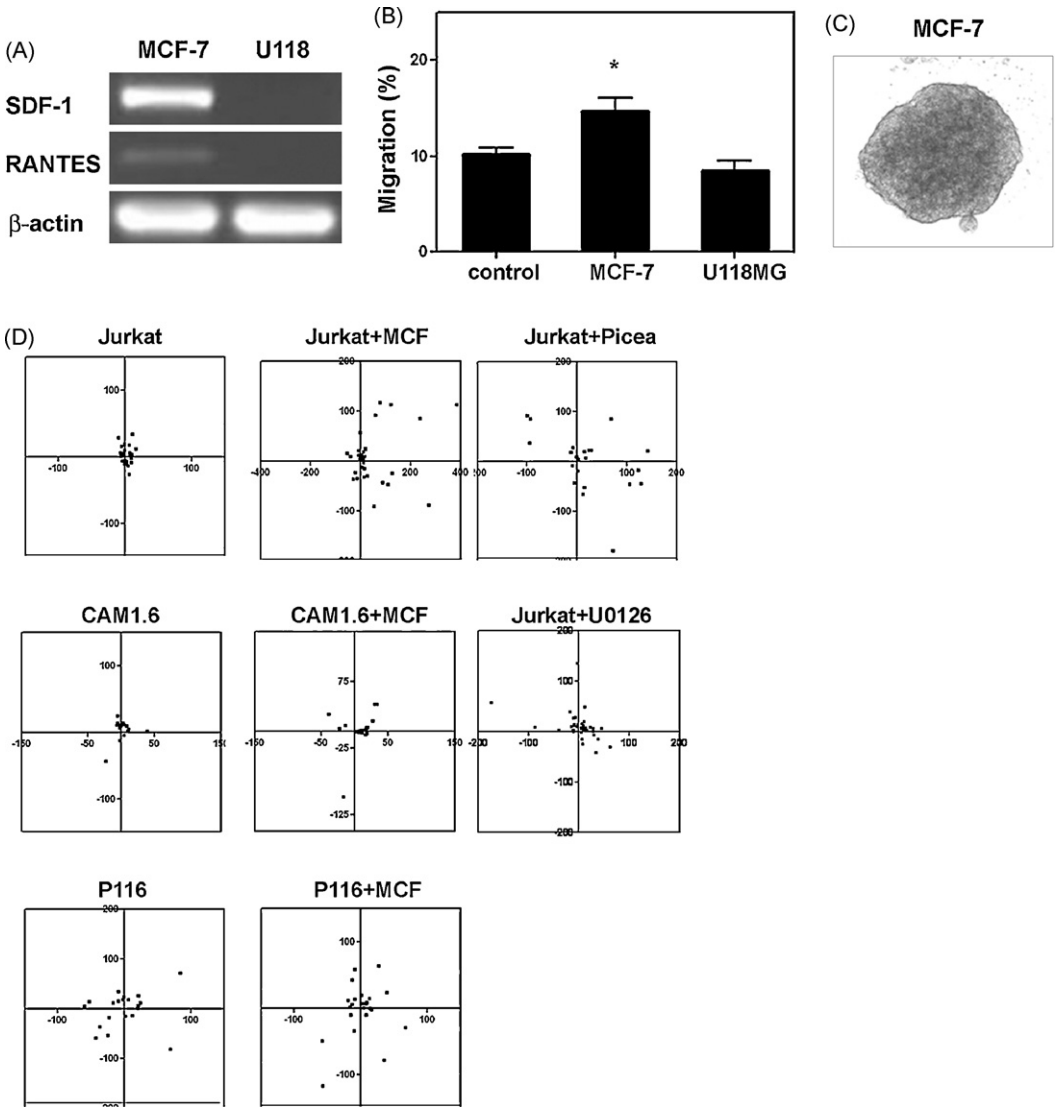

Fig. 1. Migration of Jurkat cells toward MCF-7 tumor spheroids. (A) Transcripts of SDF-1 and RENTES in MCF-7 and U-118MG were determined by RT-PCR. (B) Transmigration of Jurkat cells toward conditioned media of MCF-7 or U-118MG cells was determined in a transwell unit. Conditioned media of tumor cells were added to the lower chamber to serve as attractants. Jurkat cells (5×10^5/well) were added to the upper chamber. Jurkat cells that transmigrated across the insert membrane of the transwell unit were counted at 4 h. (C) MCF-7 spheroids were formed by culturing 1×10^5 cells 5 days in a 96-well plate base-coated with 0.5% Noble agar prepared in DMEM. (D) Jurkat cells, CAM1.6 or P116 cells were embedded alone or with the MCF-7 spheroids in collagen type I gel. To block Zap70 activity, cells were pretreated with piceatannol (25 μM) for 30 min before coculture with tumor spheroids. ERK activity was inhibited by U0126 (25 μM). Time-lapse videomicroscopy was used to record the position of cells at 5-min intervals for 12 h. The final positions of Jurkat cells were plotted in relation to their original positions versus the place of the tumor spheroid. The distance in μm and direction to spheroid are indicated. Migration toward the tumor spheroid is defined as *x*-positive.

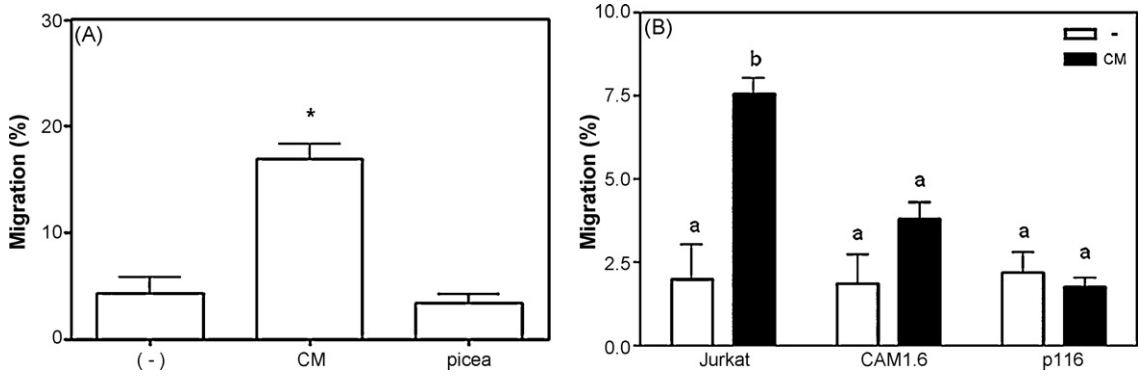

Fig. 2. Involvement of Zap70 and LCK activities in transmigration of Jurkat cells. Transmigration of cells was determined in a transwell unit. (A) Conditioned medium of MCF-7 cells were added to the lower chamber to serve as attractants as described in Fig. 1. To block Zap70, cells were pretreated with piceatannol (25 μM) for 30 min. (B) Transmigration of Jurkat, CAM1.6, and P116 cells were measured at 4 h. Data presented are averages ± standard deviation obtained from three independent experiments. The differences between treatments marked with different letters are statistically significant ($P < 0.05$).

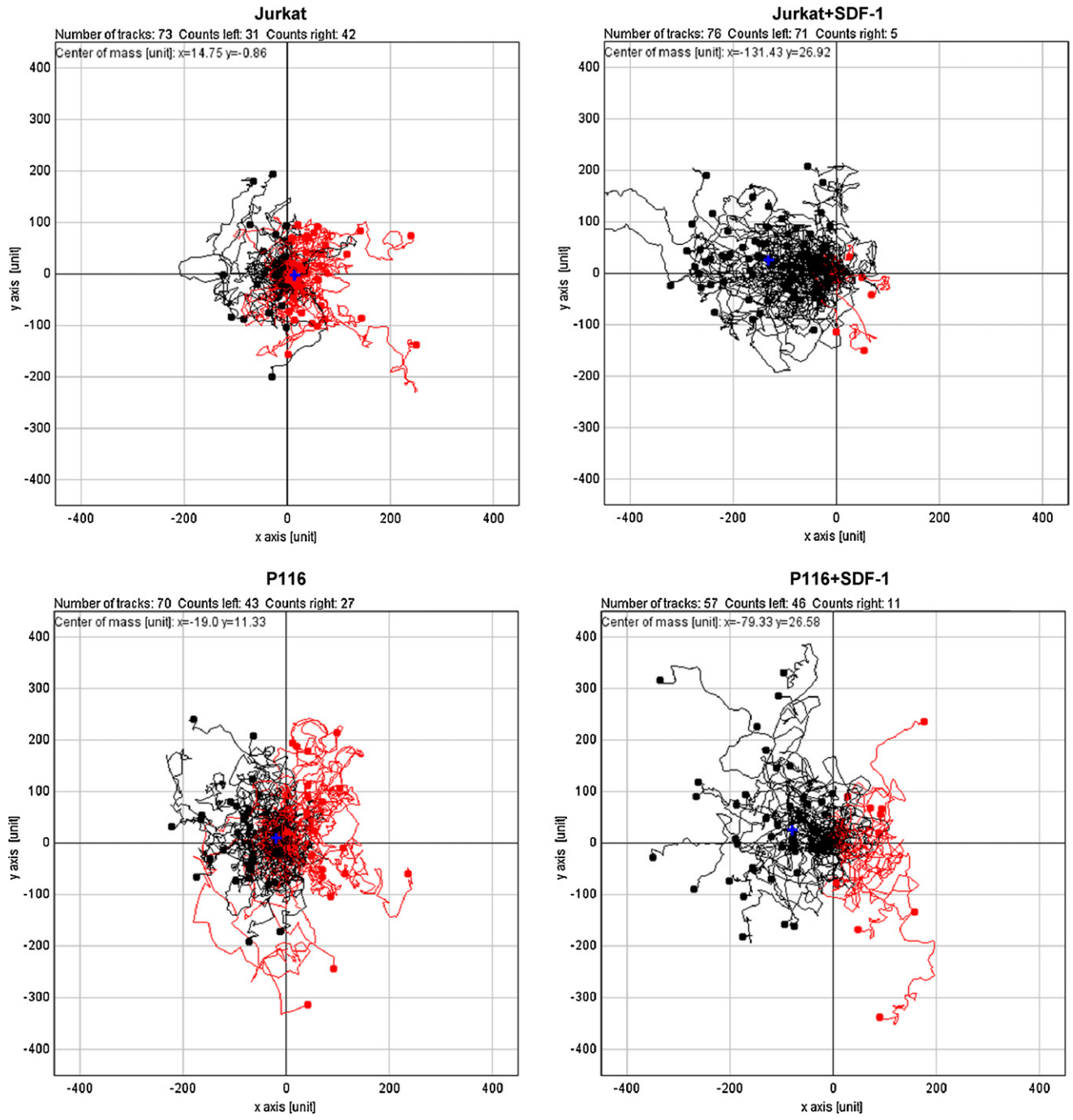

Fig. 3. Directional migration of cell toward SDF-1. Track plots of control Jurkat cells and P116 cells of representative μ-Slide chemotaxis experiments. Starting point of each single cell is placed in the center of the diagram. Tracks of cells moving toward SDF-1 are black; tracks of cells without attraction to SDF-1 are red.

turnover rate of a pseudopod, from appearance to retraction. The pseudopod turnover rate of Jurkat cells with a rough shape was about 50 s. The pseudopod turnover of P116 cells was very dynamic, averaging about 50 s and 35 s for cells with ameba-like and rough shapes, respectively.

3.4. *Zap70 forms a complex with talin and activates integrin*

P116 cells showed rapid focal adhesion turnover suggesting an impairment of the β1 integrin activation. In agreement with unstable focal adhesion, P116 cells showed less activated β1 integrin than Jurkat cells (Fig. 5A). To address whether Zap70 is important for β1 integrin activation, Zap70 gene was introduced into p116 cells (Fig. 5A). Ectopic expression of Zap70 in P116 cell enhanced the active form of β1 integrin (compare Fig. 5A lane p116/Zap with lane p116/CV). The cytoskeletal protein talin couples integrins to F-actin and controls focal adhesion turnover (Albiges-Rizo et al., 1995; Calderwood et al., 1999). We thus predicted that P116 cells are defective in talin-integrin interaction. The pull-down and immunoprecipitation data confirmed that the binding of talin and β1 integrin was greatly reduced in P116 cells (Fig. 5B). In addition, we also succeeded to pull down Zap70 and talin together by immunoprecipitation with anti-talin (Fig. 5C). Some chemotaxis-controlling molecules are spatially regulated and asymmetrically

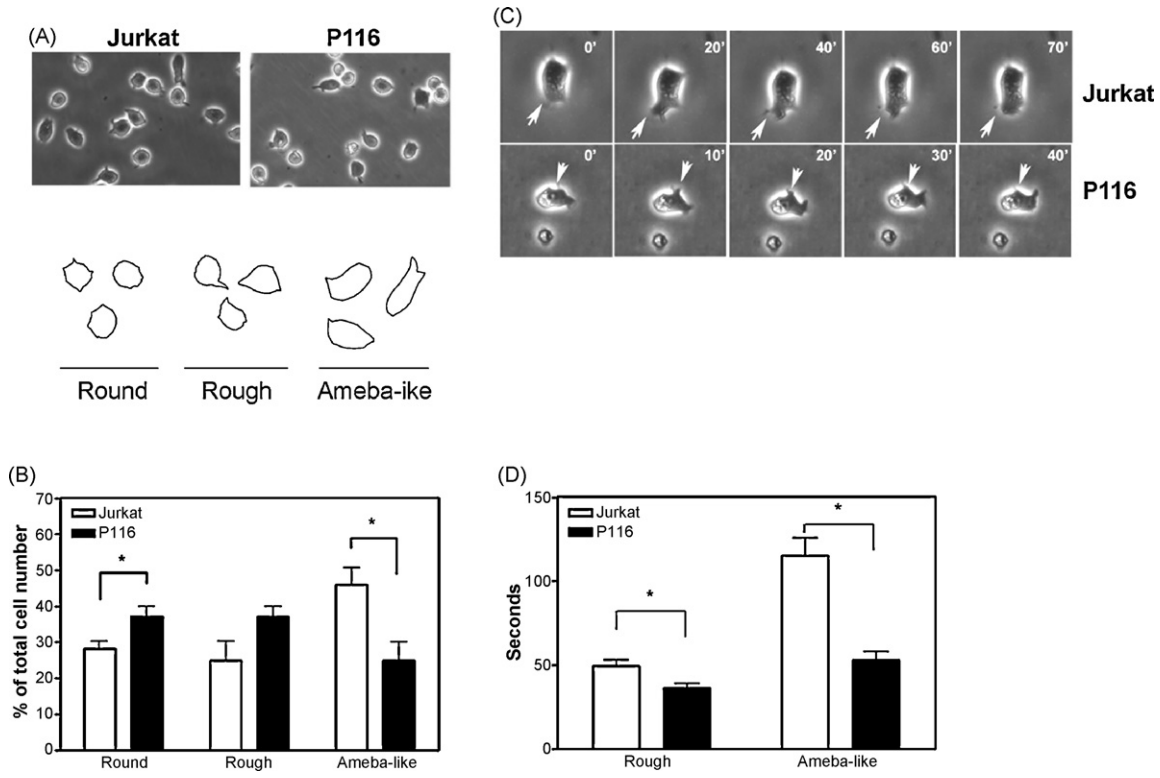

Fig. 4. Morphology of migrating cells. (A) Cells were incubated on a fibronectin-coated plate for 1 h. Morphological changes of the cells were evaluated in ten randomly selected observations with microscope (×200) using time-lapse microscopy. Shown are representative images of Jurkat and P116 cells. Based on the shape, cells were classified into round, rough, and ameba-like shapes. Percentages of each cell type were calculated. (B). Changes in morphology of Jurkat and P116 cells on fibronectin-coated plates. Data presented are averages ± standard deviation obtained from three independent experiments. (C) Representative images of the morphological dynamics. Arrowheads indicate the formation and retraction of a pseudopod of an ameba-like cell. (D) The duration of pseudopod retraction was calculated. Values shown are averages of 20 cells. Asterisk indicates $P < 0.05$ compared with other groups.

distributed in polarized migrating cells (Servant et al., 2000). In addition, colocalization of Zap70 and talin was observed by confocal imaging. Many Phospho-Zap70 were also colocalized with talin at the protrusive structures in ameba-like cells (Fig. 5D). Although P116 cells are Zap70 deficient, they attached normally to fibronectin and exhibited normal F-actin staining at the leading front suggesting that Zap70 is apparently not required for F-actin polymerization (data not shown). As shown earlier, Jurkat cells migrated actively toward SDF-1 (Fig. 3). When expose to SDF-1, Zap70 of Jurkat cells was phosphorylated on tyrosine 493 in 5 min (Fig. 6A). SDF-1 stimulated the talin/β1 integrin complex formation in Jurkat cells and in parallel enhanced β1 integrin activation (Fig. 6A and B). We also observed a slight increase in the β1 integrin activation in P116 cells upon SDF-1 treatment (Fig. 6A) suggesting a Zap70-independent way for integrin activation.

4. Discussion

Previous studies using a Boeden-chamber-based transwell system reported that Zap70 is required for SDF-1-mediated T-cell migration (Ottoson et al., 2001; Ticchioni et al., 2002). We verified this observation with P116 cells, which are Zap70 deficient. The transmigration of P116 cells in the transwell system was impaired toward nutrition gradients and conditioned medium of MCF-7. However, P116 cells migrated in random directions toward MCF-7 spheroids in 3D gel and in μ-Slide chemotaxis for SDF-1. In addition, inhibiting Zap70 activity by piceatannol did not block the random migration of Jurkat cells in collagen gel, but blunted their directional movement toward the MCF-7 spheroid. Therefore, impaired migration observed in transwell experiments for Zap70 deficient cells is not due to inability of movement but rather to loss of directionality.

It is of interest to know how Zap70 affects the directionality of moving cells. ERK is an essential kinase regulating cell motility and has been found as a downstream substrate of Zap70 in TCR activated or SDF-1 stimulated T cells (Ticchioni et al., 2002; Williams et al., 1998; Zhang and Samelson, 2000). However, cells become immotile by suppression of ERK. A previous study showed that Zap70 did not directly colocalize with the cortical actin cytoskeleton (Huby et al., 1997) which is the primary structure determining cell shape and motility (Oelz et al., 2008). In agreement with these reports, P116 cells exhibit normal actin polymerization and basal motility, indicating that Zap70 may be not a constitutive part of the locomotion engine.

When we seeded Jurkat cells on fibronectin, a portion of them rapidly attached to the matrix then started to move around. The moving Jurkat cells showed filopodia and lamellipodia locating along the edge of the moving direction. We measured the turnover rate of pseudopods of moving cells and found that defect in Zap70 resulted in frequent retraction of the pseudopod indicating unstable focal adhesion. Formation of filopodia and lamellipodia is controlled by several actin-binding proteins affecting actin polymerization that consequently determines the move direction toward a chemoattractant (Pollard and Borisy, 2003). Failure of stable focal adhesion formation or frequent readjustment of focal adhesion to a new direction may result in random cell movement (Vicente-Manzanares et al., 2009). In agreement with this, activation of Zap70 by SDF-1 strongly enhanced β1 integrin activation. P116 cells, defect in Zap70, showed impairment of integrin activation. Accordingly, re-expression of Zap70 in P116 cell significantly

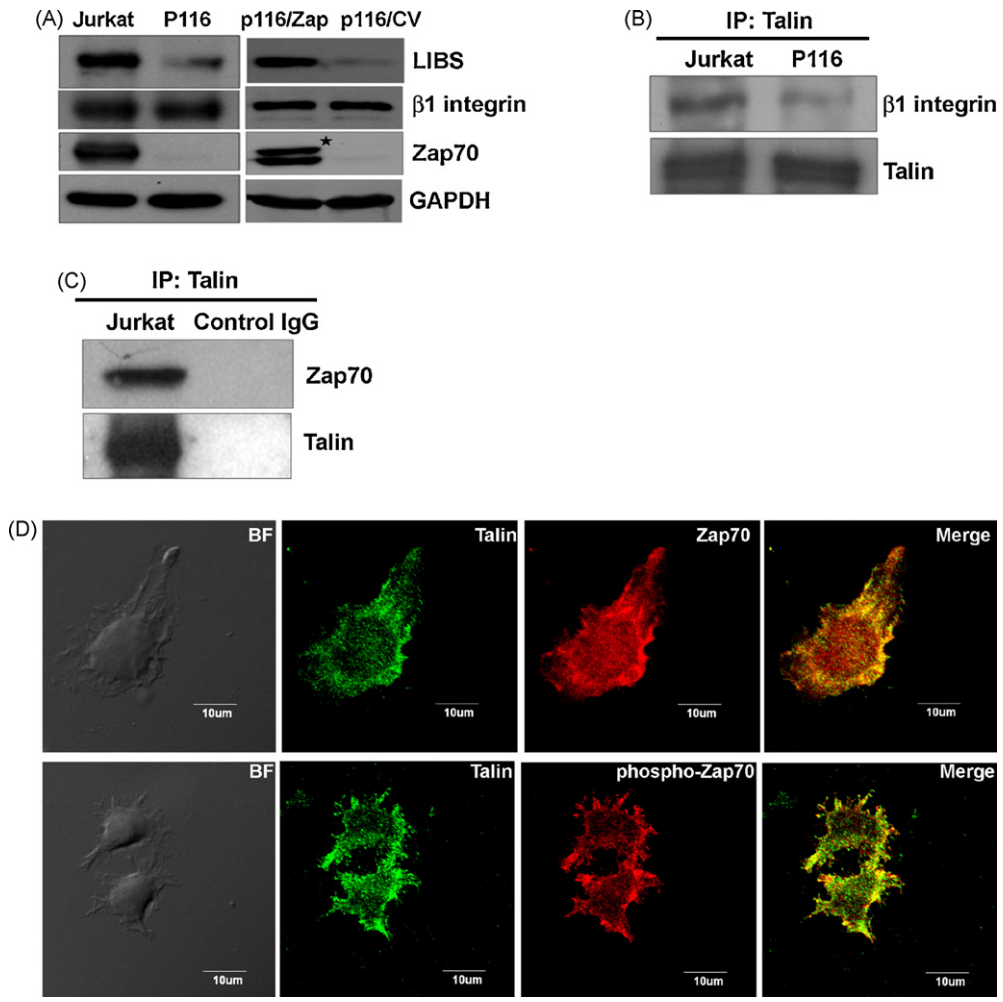

Fig. 5. β1 integrin activation and complex of talin and Zap70. Jurkat cells and P116 cells were culture for 48 h. P116 cells were transfected either with Zap70 or control vector (CV). Cells were harvested 48 h posttransfection. Protein lysates were prepared from these cells. Active β1 integrin [ligand-induced binding sites (LIBS)], total β1 integrin, Zap70 and GAPDH were detected by western blot analysis (A). The asterisk marks an immunoreactive band with molecular weight higher than Zap70 that is probably generated by post-translational modification. For immunoprecipitation assay, talin was pulled down by antibodies (B and C). Talin, β1 integrin and Zap70 in immune complex were detected by western blot analysis. (D) To do confocal imaging, Jurkat cells were cultured on a fibronectin-coated coverslip in the presence of SDF-1 (50 ng/ml). Cells were fixed, permeabilized and stained with antibodies against Zap70 (red), phospho-Zap70 (red) or talin (green). Images were taken using a confocal microscope. BF: bright field.

increased the activation of integrin. A damped induction of β1 integrin activation in P116 cells by SDF-1 treatment may be due to activation of other kinases, such as PI3K and protein kinase C-θ (PKC-θ) (Shahabi et al., 2008).

Although Zap70 did not directly interact with the cortical actin cytoskeleton (Huby et al., 1997), we found that Zap70 and talin were colocalized at the leading front of lamellipodia by confocal microscopic imaging. Physical interaction of Zap70 and talin was also demonstrable by immunoprecipitation assay. Talin is a major cytoskeletal protein that binds to integrin β cytoplasmic domains leading to the conformational rearrangements of integrin extracellular domains that increase their affinity (Critchley, 2005; Horwitz et al., 1986; Tadokoro et al., 2003). The talin rod domain is composed of a series of helical bundles that contain multiple binding sites for integrins. We have tried but failed to detect phosphorylation of talin indicating that talin may not be a substrate of Zap70 (data not shown). The F3 subdomain of talin resembles a phosphotyrosine binding domain that poten-

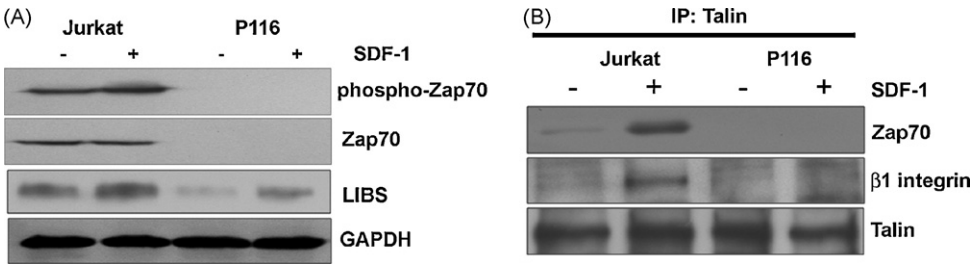

Fig. 6. Stimulation of β1 integrin/Talin and Zap70/Talin interactions by SDF-1. Jurkat cells and P116 cells were treated with or without SDF-1 50 ng/ml. After 5 min SDF-1 treatment, cells were harvested for total proteins extraction. (A) Phospho-Zap70, Zap70 and active β1 integrin (LIBS) were detected by western blot analysis. GAPDH served as a protein loading control. (B) After pulled down by talin-specific antibodies. Zap70, talin and β1 integrin in immune complex were detected.

tially binds phosphotyrosine-containing proteins (Ratnikov et al., 2005). Recently, it is shown that SDF-1 stimulates phosphorylation of Vav-1 that resulted in the dissociation of Vav-1/talin complex, subsequently enhanced α4β1 integrin activation, and that affect cell adhesion (Garcia-Bernal et al., 2009). Besides, phosphorylation of talin head controls the turnover of talin, adhesion stability and cell migration (Huang et al., 2009). In agreement with those reports, we found that SDF-1 stimulated phosphorylation of Zap70 and subsequently enhanced the binding Zap70 to talin and integrin activation. In addition, many Phospho-Zap70 were colocalized with talin at the protrusive structures in ameba-like cells, it is thus plausible that talin binds to phosphotyrosine residues of activated Zap70 and subsequently stimulates integrin activation. Collectively, our findings suggest Zap70 modulates integrin activation indirectly through talin binding in response to intracellular cues to enhance the focal adhesion along the chemotactic gradient.

Our data have important biological implications for the understanding of the role of Zap70 in inflammation. Elucidation of the mechanism underlying Zap70-associated move directionality to prevent cell accumulation in the inflamed tissue is of considerable interest that should lead to new insights into the mechanism of chronic inflammation. Together with the action of Zap70 in antigen-specific T-cell activation, we propose that targeting Zap70 kinase with inhibitors may be considered as an attractive therapeutic strategy, which not only prevents T-cell activation by autoantigens but also reduces other T effector cells in inflamed tissue.

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