Role of Rho A and F-actin for uropod formation in T lymphocytes

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Two distinct morphological features, leading edge and uropod, in mobile T lymphocyte are crucial for efficient directional movement. The uropod is a unique rear protrusion in migrating lymphocytes, in which several proteins, including CD44, ERM (ezrin/radixin/moesin), and F-actin cytoskeleton are concentrated and concerted. F-actin cytoskeleton is a basic mold for the shape maintenance. Rho A small GTPase acts as cytoskeleton organizer. So far, various pathways potentially can induce the Rho activation. PDZ domain is able to increase active Rho A form (Rho-GTP) level, reorganize F-actin cytoskeleton, disrupts the uropod structure and cell migration was diminished, suggesting that signaling pathways between Rho and F-actin cytoskeleton are related to uropod formation.

Key words – Uropod, Rho A, F-atctin, migration, PDZ

Introduction

The attraction of T lymphocytes to peripheral tissues is an essential step in the inflammatory phenomenon and the host responses to invasive infection. A requirement for a cell to initiate migration is the acquisition of a polarized morphology that allows it to turn intracellularly generated forces into net cell motility. The clear cell polarity derived from exterior stimulations such as physical contact between cell-to-cell, cell-to-microenvironment, or soluble factors like cytokines or chemokines, and migrating lymphocytes possess a distinct cellular archtecture called leading edge taken place for pulling cell body upward to chemoattractive gradient and uropod (trailing edge) which should be crucial for the rapid migration [7,13]. Recently, it has been addressed that the "backness" signal is generated by the activation of Rho by chemoattractant receptors in neutrophils [9,16]. Rho is small GTPase for acting as key regulators of the actin cytoskeleton. Rho GTPases are involved in the control of morphological change of eukaryotic cells, including lymphocyte [4,5]. During movement of the cell, actin-myosin based contraction of actin network pulls the cell to move forward. Uropod formation facilitates migration through constricted spaces in three-dimensional matrix assay [10]. However, until now, the biological roles of uropod in lymphocyte migration as well as the molecular mechanisms orchestrating this mysterious structure are largely unknown. The findings that several molecules are selectively partitionized at uropod provides some clues for clarifying these subjects. F-actin mainly accumulated at leading edges, along with a-actinin, vinculin and talin, and also is localized in uropod of migrating cells [3]. The cytoskeleton of the rear part that drive the cell uropod is poorly reported. In the present study, we focus on the connection between Rho A small GTPase and F-actin cytoskeleton, and provide potential roles of Rho A small GTPase for the uropod formation using EL4 T lymphoma, which constitutively bears a clear uropod and is good model system to understand uropod mechanisms.

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Materials and methods

Cell culture conditions

The mouse T cell line, EL4 was obtained from a parental EL4 line by limiting dilution. Cells were grown in standard RPMI 1640 medium (GibcoBRL, Paisley, UK) supplemented 10% fetal calf serum (FCS, GibcoBRL), 50 μM mercaptoethanol and 100 U/ml of both penicillin and streptomycin at 37℃ in 5% CO_2 atmosphere.

Chemical reagent and treatment of chemical reagents to living cells

The following antibody reagents were used for immunostaining, Western blot. FITC-anti-mouse CD44 were obtained from Pharmingen (California, USA), anti-γtubulin (Sigma, Saint Louis, MS). Alexa Fluor 546 phalloidin for labeling F-actin and Alexa fluor 488 goat anti-rabbit IgG were purchased from Molecular Probes (Oregon, USA).

Rabbit poylclonal anti-phoshpo-ezrin/radixin/moesin (ERM) were obtained from Cell Signaling Technology (Beverly, MA). Cytochalasin D and G418 were ordered from Nacalai Teschque (Kyoto, Japan). Recombinant murine stromal-cell derived factor-1 (SDF-1α) for chemokine assay was purchased from PeproTech (London, UK). Cells were incubated with DMSO, and 10uM cytochalasin D was treated at 37℃ for 1h.

Immunofluorescence microscopy

Immunofluorescent staining of both EL4 and other PDZ cells with primary and second antibodies was carried out using a slightly modified trichloroacetic acid (TCA, ICN) fixation method described previously [6]. After a brief centrifuge, cells were resuspended and fixed by 10% TCA solution for 15 min in room temperature (RT). For double staining between phospho-ERM, and F-actin, or F-actin staining, fixation by 10% TCA plus 1% paraformalhdehyde in PBS for 30min in room temperature was used. Treated samples were permeabilized with 0.2% Tween-20/PBS for 15min, and blocked for 1h at RT in PBS with 5% BSA. Primary antibodies were added for 1h at RT in blocking solution. After washing, to detect anti-phospho ERM, alexa fluor 488 goat anti-rabbit IgG (1/500) in blocking solution was added for 1h at RT. Nucleus was visualized with DAPI. Cells were washed, transferred onto slide glasses, dried and mounted using Permafluor aqueous mounting medium (Immunotech, France). Samples were observed using a laser scanning confocal microscope (MRC-1024; Bio-Rad, Osaka, Japan) equipped with 63×/1.4Plan-Apochromat oil immersion objectives. The digital images captured were analyzed with Adobe Photoshop software (Adobe Systems, San Jose, CA) [8].

Plasmid constructs and stable transfection

cDNA fragment encoding human EBP50 PDZ fragment (1-97aa) [11] was amplified from a full length human EBP50 cDNA with PCR. The PCR products were sub-cloned into expression vectors (pcDNA3.1/His A, invitrogen). For stable transfection, all cells were transfected by electroporation (BioRad Gene Pulser at 380 V and 960 μF). After 24h, G418 was supplemented with 400μg/ml to select for stable transfectants. Selection was maintained for 3 weeks, during which time the medium was replaced every three days. G418-resitance cells were enriched by Western blotting and sub-cloned by two rounds of limiting-dilution culture. Five stable clonal empty vector, PDZ-expressing cell lines were obtained, as confirmed by Western blotting.

Rho A pull down assay

More than 3×108cells were lysed with 2 ml of RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0). The amount of Rho-GTP in the reaction solution was measured by a pull-down method based on a specific binding to Rhotekin-RBD followed by Western blotting using anti-Rho antibody (Rho activation assay biochem kit, BK306, Cytoskeleton).

Chemokine assay

Chemotaxis assay of EL4 cells, vector, and PDZ cell line was performed in transwell cell culture chambers with polycarbonate filters, 6.5 mm diameter, 10 μm thickness and 5um pore size (Costar). A total of 2×10^5 cells suspended in 100ul RPMI 1640 were added to the upper chamber and 600 μl of the same medium were added to the lower well. When indicated, 10 nM and 100 nM SDF-1α were added to the lower well. Cells were allowed to migrate for 4 h at 37℃ and 5% CO_2 atmosphere. Counting of migrated cells was carried out using a FACScalibura flow cytometer using Cell Quester software (BD Biosciences). The assay was carried out at least in duplicates and the results were expressed as the average.

Results

Confirmation of uropod in EL4 T-lymphoma

In general, the uropod is induced in chemoattractant- or adhesion-dependent fashion [13,14], thus it seems relatively unstable. We took an advantage of a mouse T lymphoma cell line, EL4, which constitutively equipped the uropod with high frequency (over 80%) when grown on non-adhesive culture dishes (Fig. 1a, arrows), hence this cell line is suitable for uropod study. EL4 T lymphoma cells exhibited a hand mirror-shaped morphology with a narrow-neck, which segregates the cell body and the spheric uropod protrusion (Fig. 1). To confirm uropod in EL4, one of the membrane bound markers, CD44 and cytosol marker, p-ERM as hallmarkers of uropod were used. Confocal microscopic analysis clearly demonstrated that transmembrane proteins, CD44 and membrane cross linker, p-ERM selectively localized at the protruding part (Fig. 1b). In contrast, F-actin enriched in opposite side to the

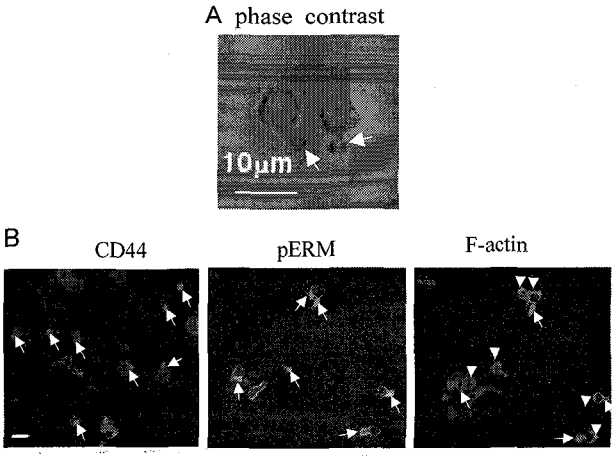

Fig. 1. EL4.G8 cells show polarized morphology with a clear uropod. Fixed, permeabilized cells were stained with fluorescence-labeled probes for cell surface CD44 and intracellular p-ERM, or F-actin. And the images were obtained through phase-contrast or confocal microscope. (A) Phase-contrast images showing the hand mirror-shaped morphology of EL4 T lymphoma cells with clear uropods (arrows). (B) Selective localization of the uropod markers (CD44) and p-ERM in the EL4 T cell protrusion and accumulation of F-actin in leading edge (head arrow) and uropod (arrow), but enrich in leading edge of F-actin. Scale bars, 10 μm.

protrusive structure, thus this part is likely the leading edge, and a signal for actin filaments was also detected at uropod (Fig. 1b).

Uropod requires actin cytoskeleton

When EL4 cells were treated with actin polymerization inhibitor, cytochalasin D, uropod was also disrupted to bring about uniform p-ERM and CD44 distributions in EL4 cells (Fig. 2). Therefore, actin cytoskeleton are required for the maintenance of uropod in EL4 T lymphocytes.

PDZ domain increases active Rho A (Rho-GTP) level

Uropod contains membrane bound-factors, cross-linkers, and cytoskeleton (espically F-actin, Fig 1) in EL4 T lymphoma. For the maintenance of uropod architecture, actin cytoskeleton as a basic mold is required in Fig 2. The Rho GTPases-Rho acts as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state, to regulate the actin cytoskeleton, and is associated with a variety of fundamental biological processes including symmetric and asymmetric morphological change [1,2]. So far, several domains are able to increase activated

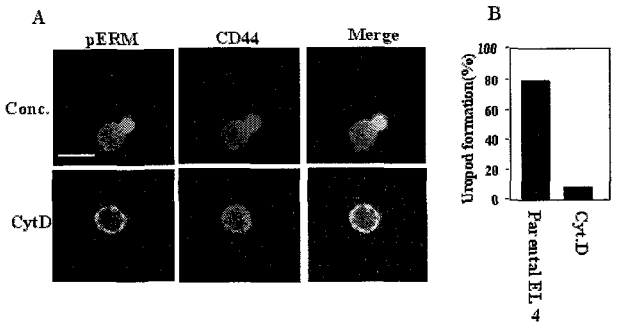

Fig. 2. Uropod requires actin cytoskeleton. Cells were incubated with 10 μM cytochalasin D in the medium for 1h at 37℃. Samples were observed using a laser scanning confocal microscope (MRC-1024; Bio-Rad, Osaka, Japan) equipped with 63×/1.4Plan-Apochromat oil immersion objectives. (A) Cytochalasin D disrupts uropod structure. Cell were treated with DMSO (cont.) or cytochalasin D (cyt.D) and stained for p-ERM and CD44. (B) Percentage of cells exhibiting uropod structure in A (n>150). Scale bars, 10 μm.

Rho A level. PDZ domain is one of the candidates for activation of Rho A. In order to underdstand the role of endogenous activated Rho for uropod, stable EL4 T lymphoma transfectants with plasmid bearing PDZ domain were established. We performed rhotekin-RBD pull down assay for the detection of activated Rho (Rho-GTP form) from stable EL4 T lymphoma cells. Active Rho A level was increased to about 4 fold level compared to control level (Fig 3).

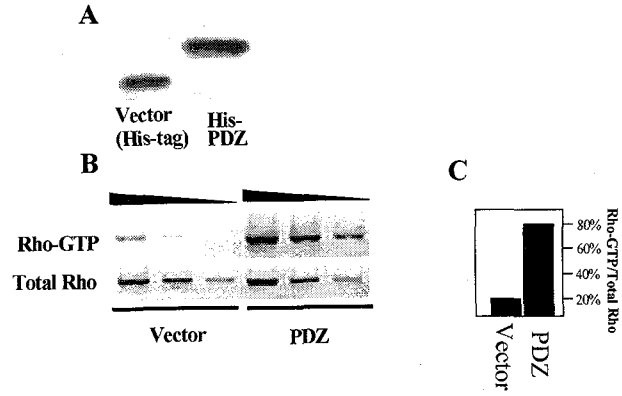

Fig. 3. PDZ domain increases the active Rho A (Rho-GTP form) level. (A) PDZ domain is expressed in stable EL4 T lymphoma transfectants. (B) Rho-GTP in the reaction was pull-downed and detected by Western blotting (upper). Total Rho was also confirmed by Western blotting (lower). Five-fold serial dilutions of each protein were carried out. (C) Relative amount of active Rho compared to the control from B was calculated by measuring the band density of Rho and normalized by total Rho density (histograms).

Endogenous active-Rho guanine nucleotide (Rho-GTP) is involved in uropod formation

EL4 T lymphoma was stained to detect localization of F-actin, p-ERM, one of the uropod marker, and morphological change. Increased endogeneous active Rho level was affected to disrupt uropod structure in EL4 T lymphoma (Fig 4, arrow, head-arrow). Surprisingly, F-actin or p-ERM accumulated at the rear part of the EL 4 T lymphoma (Fig. 4, head-arrow).

Chemotaxis in PDZ stable transfectants

We next tested the chemotaxis assay using transfectants containing PDZ domain. Among the transfectants, parental EL4 and vector transfectants represented higher chemotactic response toward SDF-1 (100ng), while PDZ transfectants showed decreased chemotatic response to the control cells (Fig. 5). Almost similar results were also obtained using other PDZ clones. Collectively, in cell free environments, these results support the notion that uropod controls effective lymphocyte migration.

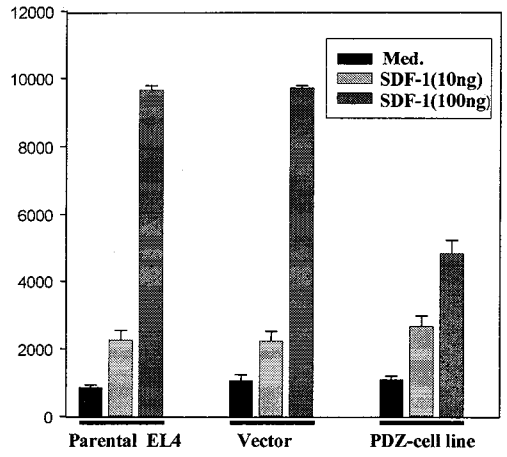

Fig. 5. Chemotatic migration of parental EL4 cells, vector and PDZ cells in response to SDF-1. Chemotaxis assay of EL4, vector, and PDZ cells was performed in Transwell cell culture chambers with polycarbonate filters, 6.5 mm diameter, 10 μm thickness and 5 μm pore size (Costar). EL4 cells exhibit typical chemotaxis toward SDF-1(100 ng/ml), which is markedly inhibited in PDZ EL4 stable cell line. Chemotatic activity is determined by a constant period counting using a flowcytometer and shown as means ± SD. A typical result of duplicate.

Disscusion

In general, about 80% EL4 cells show the morphology bearing uropod but 20% cells still display the round shape. We thought that presence of these rounded cells might some correlation with their cell cycle. During the cell division, EL4 cells turned into a round shape with the temporary disappearance of uropod and CD44 localization. This reflects that the cells abrogate not only the polarized morphology but also plasma membrane polarity upon entering the mitosis. On the contrary, it was frequently observed that the uropod-like tail structure, in which CD44 was concentrated with the remains of polar microtubles, was reconstructed at the cleavage furrow during cytokinesis (Fig. 6A, arrow). ERM protein is cross-linker between membrane-bound protein and F-actin cytoskeleton in uropod area. Using p-ERM of EL4 cells, we next tracked the activated ERM localization in each mitotic phase (Fig. 6B). During prophase or metaphase, p-ERM localization as well as CD44 polarization was never observed. These findings indicate that asymmetrical p-ERM localization on the plasma membrane is cell-state-dependent. As consistent to the cytochalasin D treatment (Fig. 2), it seems to require an extrinsic mechanism other than p-ERM itself, e.g. actin cytoskeleton or other interactive partner. As sister chromatids were segregated toward two poles in anaphase and cleavage furrow was gradually formed in telophase, CD44 and p-ERM stared to accumulate around the furrow especially at a site corresponding to the contractile ring (Fig. 6B, arrow). The contractile ring finally cleaved the two daughter cells that migrated to opposite direction each other. Throughout the mitotic phases, CD44 well co-localized with p-ERM. It is also worthy of noting that the tails of daughter cells, which have been the parts at the cleavage

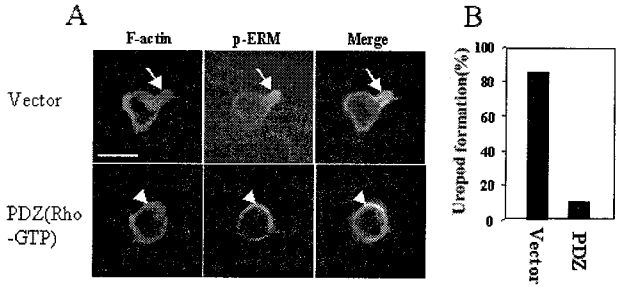

Fig. 4. Endogenous active-Rho guanine nucleotide (Rho-GTP) is involved in uropod formation. (A) EL4 T lymphoma was stained to detect localization of F-actin, p-ERM and morphological change. Increased endogenous active Rho level was affected to disrupt uropod structure in EL4 T lymphoma (arrow, head-arrow). F-actin or p-ERM was accumulated at the rear part of the EL 4 T lymphoma(head-arrow). (B) Percentage of cells exhibiting the uropod in B (n > 203). Scale bars, 10 μm.

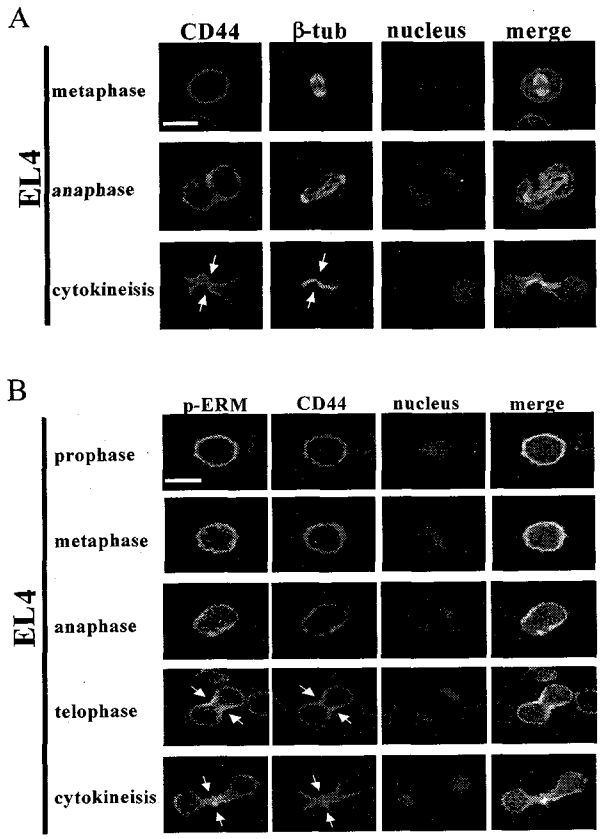

Fig. 6. Uropod relocalization of p-ERM and CD44 during parental EL4 T lymphoma cell division. Abrogation and reconstruction of the plasma membrane polarity during the mitotic phases of EL4 cells. (A) Cells were stained for CD44 and β-tubulin and cells in the indicated mitotic phase are shown. Uropod-like tail structure was frequently observed at the cleavage furrow during cytokinesis (arrow). (B) Changes in the localization of p-ERM and CD44 during cell division. p-ERM dynamically redistributes during the mitotic phases. p-ERM transfectants were stained for CD44. In later phases of mitosis, p-ERM was accumulated at the cleavage furrow especially in contractile ring (arrows). Scale bars, 10 μm.

furrow, seemed to become the uropods. Thus p-ERM seems to construct the plasma membrane polarity even in the mitosis in proper site according to newly formed cytoplasmic polarity axis of the cell. In this research, chiefly using EL4 T lymphoma cells, we showed the preferential localization of p-ERM protein, CD44, faint F-actin in the uropod. So far, various pathways potentially can induce the Rho activation [2,12]. Recently, it has mentioned that the backness signal is occurred by the activation of Rho by means of chemoattractant receptor in neutrophils [9,15]. PDZ stable transfectants of EL4 T lymphoma were established. PDZ domain displays increased endogenous active Rho (Rho A-GTP) level and various cellular phenomena, including the reorganization of F-actin in the uropod, abrogation of uropod, decrement of chemotaxis. Cytochalasin D, actin depolymer, treated cells showed the disruption of uropod structure. These evidences support the notion that connection factors between Rho A small GTPase and F-actin cytoskeleton are the crucial components for lymphocyte morphology and motility. Uropod protrusion seems to depend on Rho A small GTPase in EL4 T lymphoma cells. Thus what coordinates the Rho A small GTPase and the downstream component system remains undetermined at this time. Triggering lymphocyte migration via various attractants include complicated signaling of which the whole picture remains poorly understood. The environmental directional cues are supposed to trigger the sequential events. Our observations suggest that this process can be connected with the Rho small GTP ase mediated uropod protrusion. In the future researches, it will be important to identify the down- stream components of Rho A small GTPase together with linking the upstream signaling during the lymphocyte migration.

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초록 : T 세포의 Uropod 형성에 있어 Rho A와 F-actin의 역할

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외부 병원체 침입으로 이동하고 있는 T 세포는 두가지 뚜렷한 형태적인 변화, 즉, leading edge와 uropod를 형성하여 효과적으로 T세포 이동에 영향을 미친다. Uropod 구조물은 이동하는 림프구들의 뒤쪽에서 관찰 할 수 있는 아주 독특한 구조로 CD44, ERM, F-actin과 같은 단백질들이 서로 영향을 미치며 모인다. F-actin cytoskeleton은 세포의 형태를 유지하는 기본적인 틀을 제공한다. Rho A small GTPase는 이러한 cytoskeleton을 재구성하는 organizer로 역할을 한다고 보고되어 왔다. 지금까지, 다양한 경로를 통하여 Rho A가 활성화 되어 진다고 보고 되었다. 본 실험에서 PDZ 도메인이 세포 내부 Rho A에 GDP가 결합된 불활성화 형태의 Rho A를 GTP가 결합된 활성화 형태로 전환한다는 것을 알았고, F-actin cytoskeleton을 재구성 하며, PDZ 도메인을 함유한 세포는 uorpod 구조물이 없어졌으며 세포 이동 속도도 감소하는 것을 알았다. 따라서 Rho A와 F-actin cytoskeleton 사이의 신호 전달 과정이 uropod 형성에 아주 중요한 기능을 할 것이라는 것을 알았다.