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The Unique Localization of ZFP185 at Uropod of Mouse T Lymphocytes

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Abstract

Zinc finger protein 185 (ZFP185), which contains a conservative Lin-l 1, Isl-1 and Mec-3 (LIM) domain at its C-terminus, is a member of mouse LIM protein family. Although brahma-related gene 1(BRG1), the upstream regulation gene of ZFP185, is essential in the development and activation of T lymphocytes, there is no report on the involvement of ZFP185 in lymphocyte activation. The expression of ZFP185 protein was up-regulated significantly in T lymphocytes during their activation by phorbol 12-myristate 13-acetate (PMA)/ionomycin, compared with non-activated T lymphocytes, as determined by western blot assays. However, over-expression of ZFP185 in EL4 cells showed no significant effect on the cell proliferation. Importantly, expression of ZFP185-enhanced green fluorescent protein fusion protein in EL4 cells showed that ZFP185 uniquely localized in the uropod of lymphocytes as observed with a two-photon laser scanning confocal microscope. The direct interaction between ZFP185 and actin was supported by co-immunoprecipitation and immunofluorescence assays. The special distribution of ZFP185 in uropod indicates the potential involvement of ZFP185 in T-cell morphology and movement.

Introduction

LIM domain, named after Lin-l 1, Isl-1 and Mec-3 protein [1], is a conservative cysteine-rich domain characterized by two tandem zinc finger structures [2]. Proteins containing one or more LIM domains are defined as LIM proteins which play important roles in many biological processes including cytoskeleton, differentiation, signal transduction and cancerogenesis [3, 4]. Zinc finger protein 185 (ZFP185), a member of LIM protein family in mice, contains a single conservative LIM domain at its C-terminus [5]. Evidences showed that ZNF185 (homologous molecular of ZFP185 in humans) significantly down-regulated in some kinds of tumor cells, especially in metastatic cells [6]. A recent study showed that human ZNF185 co-localized with F-actin cytoskeleton in cytoplasm of prostate cancer cells and its over-expression suppressed the cell proliferation [7]. All the evidences mentioned above suggest that ZNF185 (ZFP185) may be a cytoskeleton regulatory protein involved in the regulation of cell motility, adhesion and proliferation.

In the present study, we firstly detected the expression of ZFP185 in mouse $CD4^+$ $CD25^-$ T lymphocytes during their activation, and then observed the ZFP185 sub-cellular location and its effect on cell proliferation in a T-lymphoma cell line EL4 by over-expressing ZFP185-enhanced green fluorescent protein (EGFP) fusion protein. Although over-expressing ZFP185 showed no significant effect on EL4 cell proliferation, ZFP185 protein uniquely localized in the uropod of the cells, a special protrusion structure in lymphocyte surface. These data indicate that ZFP185 may be potentially involved in T-cell morphology and movement, at least in EL4 cells.

Materials and methods

Plasmid construction and purification. The full-length of coding regions of *zfp185* was inserted in-frame into the multiple cloning sites of pEGFP-C2 (Clontech, Mountain View, CA, USA) and pCDNA3.1-His (Invitrogen, Carlsbad, CA, USA) to obtain recombinant plasmid pEGFP-C2-ZFP185 and pCNA3.1-His-ZFP185. The recombinant plasmid and control plasmid were purified by QIAGEN Plasmid Midi Kit (QIAGEN, Palo Alto, CA, USA) according to the standard protocol.

Purification and activation of $CD4^+$ $CD25^-$ T lymphocytes. Lymphocytes were harvested from lymph nodes of BALB/*c* mice. $CD4^+$ T cells were purified by negative selection with Dynal® Mouse CD4 Negative Isolation Kit (Dynal, Oslo, Norway) according to its instruction and as reported previously [8]. In brief, 200 μl Antibody/Mix was added to 1×10^8 lymph node cells followed by 20-min incubation at 4 °C. The cells were then washed and added with 2-ml prewashed magnetic beads. After 15-min incubation at 25 °C, $CD4^+$ T lymphocytes were purified as reported previously [9]. Then isolation of $CD4^+$ $CD25^-$ T lymphocyte was performed with Mouse CD25 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to its instruction. The purity of isolated $CD4^+$ $CD25^-$ T cells was always >98% as detected by flow cytometry [10].

The isolated $CD4^+$ $CD25^-$ T lymphocytes were stimulated with PMA/ionomycin (final concentration: PMA 0.5 ng/ml, ionomycin 0.5 μM) in a full RPMI1640 medium as reported previously [11].

Cell culture and gene transfection. $CD4^+$ $CD25^-$ T lymphocytes and EL4 (T-lymphoma cell line) were cultured in RPMI-1640 medium (Invitrogen Life Technologies) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA) in a humidified 5% CO_2 atmosphere at 37 °C. Gene transfection was performed by means of electroporation. In brief, EL4 cells were washed with RPMI-1640 medium without serum for three times and then adjusted to 2×10^7/ml. Electroporation was performed at 130 V for 20 ms with ECM 630 Electroporation System (Harvard Apparatus, Inc., Holliston, MA, USA). The cells were then cultured with complete RPMI-1640 medium (10% foetal bovine serum).

Co-immune-precipitation and Western blot. The cell lysates of those $CD4^+$ $CD25^-$ T lymphocytes stimulated with PMA/ionomycin at different time were prepared with radioimmunoprecipitation assay (RIPA) lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail). Western blot was then performed with anti-ZFP185 and anti-actin antibodies.

The EL4 cells were transfected with pCDNA3.1-His vector, pCDNA3.1-His-zfp185 plasmid or no plasmid respectively by means of electroporation. Twenty-four hours post-transfection, the cell lysates were prepared as described above. The cell lysates were incubated with an anti-His-tag antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The immune-complexes were precipitated with protein A–Agarose (Santa Cruz). After washing with the lysis buffer, the co-precipitated proteins were detected by Western blot with anti-ZNF185 and anti-actin antibodies [7]. Tubulin was detected as control of 1/10 input of each cell lysate. The resulting images were analysed by UTHSCSA ImageTool (Version 3.0) software (University of Texas Health Science Center, San Antonio, TX, USA) and the ratio of ZFP185 to correspondent actin was calculated.

Cell staining and fluorescence microscopy observation. For visualizing the localization of GFP-fused ZFP185 in lymphocytes, pEGFP-C2-ZFP185 recombinant plasmid and pEGFP-C2 control plasmid were transfected into EL4 cells by means of electroporation respectively. Twenty-four hours after transfection, cells were stained by adding Hoechst 33342 into culture medium to final concentration 1 μg/ml and additional culture in 37 °C for 30 min. Then, the living cells were observed directly by a two-photon laser confocal scanning microscope (Zeiss LSM 510 META).

For the immunofluorescence staining of ZFP185 and CD44, the EL4 cells were spinned to slides and then fixed with 2% (w/v) paraformaldehyde in PBS (pH7.4) for 15 min at room temperature. After washing twice, the cells were permeabilized with 0.2% Triton X-100 for 10 min. After another two washes with PBS, the cells were blocked with 2% BSA dissolved in PBS. The cells were then stained with anti-ZFP185 polyclonal antibody for 90 min at 37 °C [7]. After three washes, the cells were stained with fluorescein isothiocyanate-labelled goat-anti-rabbit IgG antibody (Zhongshan, Beijing, China) and anti-CD44-PE (BD Pharmingen, San Diego, CA, USA) or tetra-methyl-rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. The slides were washed for three times and mounted with antifade regent (Zhongshan, Beijing). The cells were then observed under a LSM510 laser confocal microscope with 63 × oil objective.

Cell proliferation assay. For proliferation assay, cells transfected with pEGFP-C2-ZFP185 recombinant plasmid, pEGFP-C2 control plasmid or none were cultured at 37 °C in a 5% CO_2 incubator in complete RPMI 1640 medium in 96-well plates (5×10^4 cells/200 μl/well). Twenty-four hours after transfection, cells were pulsed with 0.5 μCi of ^{3}H-labelled thymidine (radioactivity, 185 GBq/mmol; Atomic Energy Research Establishment, China) per well, after 18 h further incubation, were harvested onto glass fibre filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a liquid scintillation analyser (Beckman Instruments, Fullerton, CA, USA) as reported previously [12].

Results

Up-regulation of ZFP185 expression in activated $CD4^+$ $CD25^-$ T lymphocytes

The essential role of BRG1, the upstream regulatory gene of ZFP185, in immune system development and lymphocyte activation promoted us to characterize the potential involvement of ZFP185 in mouse $CD4^+$ $CD25^-$ T

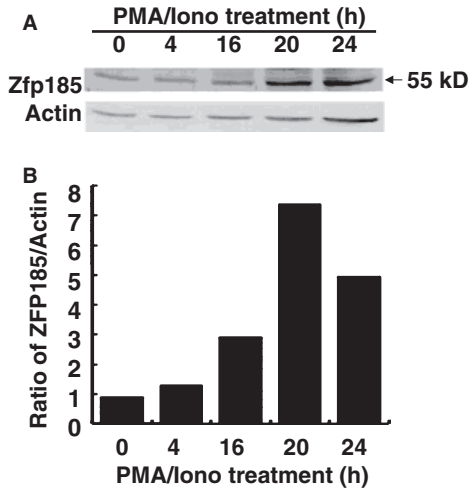

Figure 1 Zinc finger protein 185 (ZFP185) expression was up-regulated during $CD4^+$ $CD25^-$ T-lymphocyte activation. (A) Western blot detected the expression level of ZFP185 in different time post-stimulation by phorbol 12-myristate 13-acetate (PMA)/ionomycin *in vitro*. (B) Ratio of the area between ZFP185 and actin in Fig. A.

lymphocytes. As shown in Fig. 1, ZFP185 expression in $CD4^+$ $CD25^-$ T lymphocytes was significantly up-regulated during activation and reached peak at approximately 20 h after activation. This suggests that ZFP185 may function at the early stage of T-lymphocyte activation.

Over-expression of ZFP185 had no significant effect on cell proliferation in EL4 cells

Accumulated evidences suggested that actin cytoskeleton regulatory proteins might have essential function in cell apoptosis and proliferation [13, 14]. To determine the potential function of ZFP185 on cell proliferation, we detected the proliferation of EL4 cells transfected with or without ZFP185. But no significant alteration could be detected in the three kinds of cells as determined by thymidine incorporation assays (Fig. 2), so did the results from cell cycle detection (data not shown). The results suggest that ZFP185 may have no effects on cell proliferation, at least in EL4 cells.

Localization of ZFP185 in the uropod structure of T-lymphoma cells, EL4

We over-expressed ZFP185 in a mouse T-lymphoma cell line EL4, a well-known cell line that is often used for investigating T-cell function [15]. Electroporation was performed to transfected EGFP-ZFP185 fusion protein gene into EL4 cells. Twenty-four hours after transfection, the living cells were stained with Hoechst 33342, a fluorescent dye to label nucleus of living cell, and then observed by two-photon laser confocal scanning microscope. Layer-by-layer scanning of the EGFP-positive cells showed that

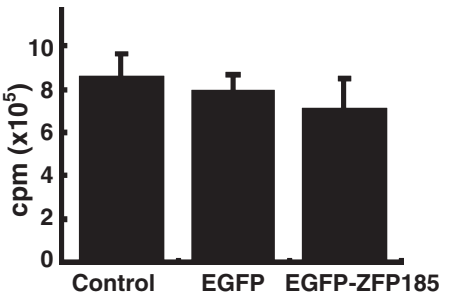

Figure 2 Overexpression of zinc finger protein 185 (ZFP185) did not change EL4 cell proliferation. Control, the cells performed electroporation but without plasmid; enhanced green fluorescent protein (EGFP), the cells performed electroporation with pEGFP-C2 plasmid; EGFP-ZFP185, the cells performed electroporation with pEGFP-C2-ZFP185 plasmid. Cell proliferation was detected by thymidine incorporation assay.

ZFP185 localized specially at the uropod structure of EL4 cells (Fig. 3A). In contrast to ZFP185-EGFP fusion protein, EGFP itself localized diffusively in EL4 cells transfected with EGFP alone (Fig. 3B). CD44 is a well-known uropod marker in T cells. To confirm the localization of ZFP185 in uropod, we performed the co-immunofluorescence staining with anti-ZFP185 antibody and anti-CD44 antibody (Fig. 3). As shown in Fig. 3C, CD44 and ZFP185 were co-located in the uropod.

Association between ZFP185 and actin

Uropod is recognized as an actin-rich region in cells. The potential interaction between ZFP185 and actin was investigated by using a co-immunoprecipitation (IP) assay. The proteins pull-down by anti-His mAb in ZFP-185-transfected but not control vector-transfected EL4 cells showed both positive for ZFP185 and actin as determined by western blot, indicating that ZFP185 may be associated with actin directly (Fig. 4A). This speculation was further supported by the observation that anti-ZFP185 antibody and TRITC-phalloidin were co-stained in EL4 cells as detected by a two-photon laser confocal scanning microscope (Fig. 4B).

Discussion

Encode gene of zfp185 localizes in the Xq28 site of mouse X chromatin, a special pseudoautosomal region. The corresponding region of Xq28 on human X chromatin was determined to associate with a great deal of hereditary diseases, such as haemophilia A [16], X linked mental retardation [17], neuronal intestinal pseudo-obstruction [18] and Happle syndrome [19]. The C-terminus of ZFP185 is a cysteine/histidine-rich LIM domain named after three proteins Lin-l 1, Isl-1 and Mec-3 containing the domains and its sequence is highly conservative—homology between the LIM domain of human

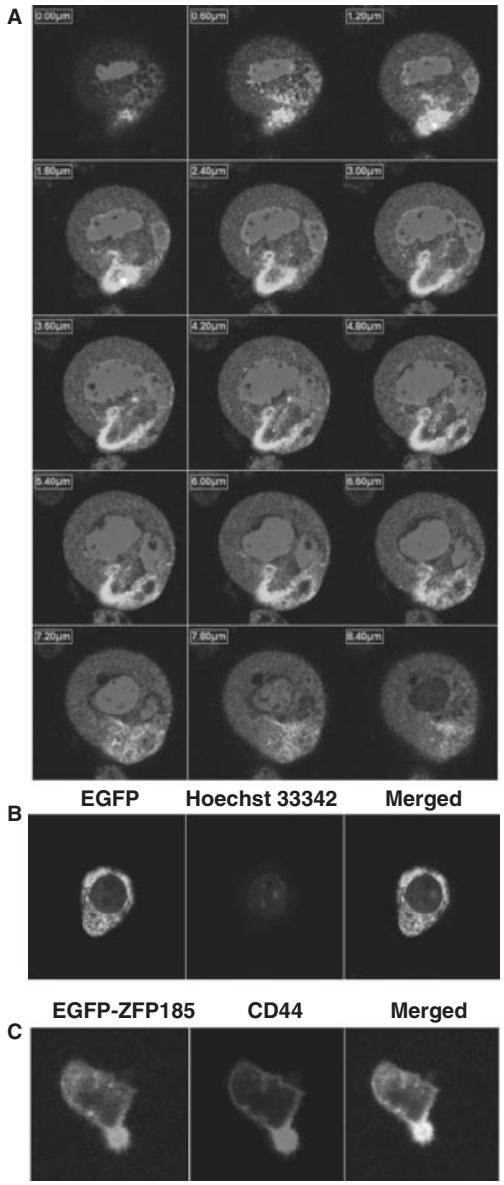

Figure 3 Zinc finger protein 185 (ZFP185) localized in the uropod of EL4 cells. (A) EL4 cells were transfected with enhanced green fluorescent protein (EGFP-ZFP185) fusion protein gene by means of electroporation and cultured for 24 h. After stained with Hoechst 33342, cells were observed in multiple layers by a two-photon laser confocal scanning microscope. EGFP was excited at 488 nm and observed at 520 nm. Hoechst was excited by two-photon laser at 750 nm and observed at 430 nm. (B) El4 cells were transfected with EGFP vector as a control. (C) EL4 cells were stained with anti-ZFP185 antibody and anti-CD44 antibody.

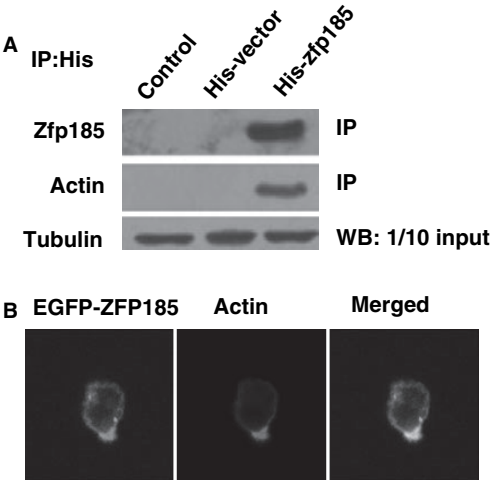

Figure 4 Association between zinc finger protein 185 (ZFP185) and β-actin was detected by immunoprecipitation and immunofluorescence assay. (A) EL4 cells were transfected with pCDNA3.1-His or pCDNA3.1-His-zfp185 plasmid by means of electroporation respectively. Immunoprecipitation was performed with anti-His antibody, the pull-down protein was assayed by Western blot with either anti-actin or anti-ZFP185 antibody. Bottom panel was the cell total proteins (1/20 input). (B) EL4 cells were stained with anti-ZFP185 antibody and TRITC-conjugated phalloidin.

ZNF185 and mouse ZFP185 is up to 93%. Moreover, N-terminus of ZFP185 is also a conservative region. The N-terminus region of human ZNF185 has been identified as an actin-binding region and can regulate the proliferation of prostate cancer cells [7].

It is reported that BRG1 (also named as SMARCA4), a member of chromatin remodelling complex, is an upstream regulatory gene of ZNF185 (ZFP185) and studies in lung cancer cell line showed the close correlation between the expression of BRG1 and ZNF185 (ZFP185). BRG1 can directly bind to the promoter region of zfp185 to regulate the expression of ZFP185 [20]. In addition, the research in *brg*-deficient mice showed that BRG1 was essential in T-lymphocyte function [21]. Although $CD4^+$ T lymphocytes can be detected in the *brg*-deficient mice, their proliferative response to a number of stimuli including T-cell receptor cross-linking using anti-CD3 or anti-CD3 plus anti-CD28 mAbs, Con A, and PMA/ionomycin reduced significantly [21]. Our present results showed that the expression of ZFP185 significantly up-regulated during the activation procedure of mouse $CD4^+$ $CD25^-$ T lymphocytes. However, over-expression of ZFP185 in EL4 cells did not significantly alter cell proliferation, indicating ZFP185 may not play a critical role in T-cell proliferation. Of course, the possibility that ZFP185 may function differently in tumour cells and primary T cells should be excluded in the future.

Most strikingly, the EGFP-ZFP185 fusion protein was mainly localized at the uropod region. Uropod, a special structure at the rear edge of polarized T lymphocytes, is an actin-rich, large protrusion at the cell surface generated by actin polymerization after stimulation [15, 22, 23]. The size of uropod is large enough to distinguish itself from other structure on cell surface such as filipodia, dendrites and lamellipodia. Though its particular function remains controversial, uropod may function in T-lymphocyte

migration, connection and apoptosis [24–26]. Inhibiting actin polymerization and uropod generation leaded to a T-lymphocyte anergic state [27]. The actin-binding capacity of ZFP185 characterized in this study suggested that ZFP185 localized at the uropod structure by means of its binding to actin. However, actin not only polymerized at uropod but also at the leading edge which also accumulated at the opposite side of uropod in polarized EL4 cells [15]. In our study, we have not observed the aggregation of ZFP185 at the leading edge of polarized EL4 cells, indicating that some additional molecules may be involved in ZFP185 distribution in cells. To determine the exact mechanism for ZFP185 in uropod, more binding partners should be characterized.

In summary, our present studies show that mouse ZFP185 expression is up-regulated during T-lymphocyte activation. Moreover, ZFP185 can specially localize at the uropod structure of cells but had no significant effect on cell proliferation, at least in EL4 cells. Immunoprecipitation and immunofluorescence assays suggest that ZFP185 may be associated with F-actin which is rich in uropod structure. The function of ZFP185 in lymphocytes need to be further determined.

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