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Deficiency of a STE20/PAK family kinase LOK leads to the acceleration of LFA-1 clustering and cell adhesion of activated lymphocytes

Junji Endo[a,d,e], Noriko Toyama-Sorimachi[a], Choji Taya[b], Satomi Kuramochi-Miyagawa[a,1], Kisaburo Nagata[a], Keisuke Kuida[a,2], Tohru Takashi[c], Hiromichi Yonekawa[b], Yasuyuki Yoshizawa[d], Nobuyuki Miyasaka[e], Hajime Karasuyama[a,*]

[a] *Department of Immunology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan*
[b] *Department of Animal Science, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan*
[c] *Tokyo R&D Center, Daiichi Pharmaceutical Co. Ltd., 1-16-13, Kita-Kasai, Edogawa-ku, Tokyo 134-8630, Japan*
[d] *Department of Pulmonary Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[e] *First Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*

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Abstract Lymphocyte-oriented kinase (LOK) is a member of the STE20/p21-activated kinase (PAK) family and expressed predominantly in lymphoid organs. Generation of LOK-deficient mice revealed that the leukocyte-function-associated antigen (LFA-1)/intercellular adhesion molecules (ICAM)-mediated aggregation of mitogen-stimulated T cells was greatly enhanced in the absence of LOK. Though levels of total LFA-1 and ICAMs as well as the active form of LFA-1 on T cell blasts were comparable in the presence and absence of LOK, clustering of active LFA-1 detected by binding of soluble ICAM-1 was accelerated in the absence of LOK. These results suggest that LOK is potentially involved in the regulation of LFA-1-mediated lymphocyte adhesion.

Key words: Lymphocyte-oriented kinase; STE20/p21-activated kinase family; Integrin; Leukocyte-function-associated antigen-1; Clustering; Adhesion

1. Introduction

The STE20/p21-activated kinase (PAK) family is a growing family of serine/threonine kinases which have been implicated in the regulation of a number of cellular activities such as mitogen-activated protein (MAP) kinase signaling pathways, apoptosis, cell cycle and cytoskeletal dynamics [1–4]. This family can be divided into two major subfamilies based on their structure [1]. The PAK subfamily members such as yeast Ste20p and mammalian PAK have a kinase domain at the C-terminus and a conserved p21 Rac and Cdc42-binding domain (regulatory domain) at the N-terminus. On the other hand, the germinal center kinase (GCK) subfamily members lack the p21 Rac/Cdc42-binding domain and have a kinase domain at the N-terminus and a putative regulatory domain at the C-terminus. The members of the PAK subfamily were initially considered to lie at the top of the MAP kinase cascade and activate the cascade [1,2]. Recently, however, there are growing volume of studies focusing on the role of the PAK subfamily as a upstream regulator of cell morphogenesis and actin cytoskeleton [1–3]. On the other hand, the role of the GCK subfamily besides in the regulation of MAP kinase pathway remains largely unknown [4].

We previously cloned a novel protein kinase gene, *lok* (lymphocyte-oriented kinase) [5,6]. It codes for a 130-kDa serine/threonine kinase which is expressed predominantly in lymphoid organs. LOK carries a kinase domain homologous to that of the STE20/PAK family members at the N-terminus, a long coiled-coil structure at the C-terminus and a proline-rich region in between. Thus, LOK is a new member of the GCK subfamily. In contrast to many other members of the STE20/PAK family, LOK activated none of the known MAP kinase isoforms as far as analyzed in COS7 cells. These results suggested that LOK could be involved in a novel signaling pathway distinct from the known MAP kinase cascades in lymphocytes. To clarify the physiological role of LOK in vivo, we generated LOK-deficient mice by gene targeting. Our results suggest that LOK is involved in regulation of cell adhesion.

2. Materials and methods

2.1. Generation of $lok^{-/-}$ mice

A 5.5-kb *Hin*dIII–*Eco*RI fragment and a 2.5-kb *Hin*dIII–*Eco*RI fragment, upstream and downstream regions of exon 1 of the *lok* gene, respectively, were isolated from a genomic DNA library of 129/SVJ mice (Stratagene, CA, USA) and subcloned in conjunction with neomycin-resistance gene into a plasmid-carrying thymidine kinase gene [7]. Linearized construct was electroporated into W9.5 ES cells [8]. Clones resistant to both G418 and gancyclovir were selected and homologous recombination in these clones was checked by Southern blot analysis using a 1.2-kb *Eco*RI–*Bam*HI fragment of the *lok* gene as a probe (Fig. 1A) to obtain $lok^{+/-}$ ES clones. $lok^{-/-}$ mice were generated from the ES clones by the standard method [8].

2.2. Re-aggregation assay of mitogen-stimulated lymphoblasts

Lymph node cells (2×10^6 cells/ml) were stimulated in vitro with 5 μg/ml concanavalin a (Con A) for 2 days as described [9]. Con A blasts were resuspended (2×10^6 cells/ml) in complete medium with

*Corresponding author. Fax: (81)-3-56856608.
E-mail: h-karas@rinshoken.or.jp

[1] Present address: Department of Molecular Cell Biology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan.
[2] Present address: Vertex, 130 Waverly Street, Cambridge, MA 02139-4242, USA.

Abbreviations: LOK, lymphocyte-oriented kinase; PAK, p21-activated kinase; GCK, germinal center kinase; MAP, mitogen-activated protein; LFA, leukocyte-function-associated antigen; ICAM, intercellular adhesion molecule; Con A, concanavalin A

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15 mM α-methyl mannoside to prevent Con A-mediated cell adhesion, in the absence or the presence of 50 μg/ml of an anti-mouse leukocyte-function-associated antigen (LFA-1) antibody, KBA. Each cell suspension (200 μl) was added to a well of 96-well flat bottom-plate and shaken at 200 rpm in a CO_2 incubator at 37°C for 30 min. For quantification of cell aggregation, samples were applied to Coulter Multisizer Counter (Coulter Scientific Instruments, Hialeah, FL, USA) as described [10]. The percentage of cells involved in aggregation = 100×[1−(number of non-aggregated cells/number of input cells)].

2.3. Antibodies and flow cytometric analysis

Cells were incubated with antibodies specific to LFA-1 (KBA), intercellular adhesion molecule (ICAM)-1 (KAT-1), ICAM-2 (PharMingen, CA, USA), washed and then stained with FITC-goat F(ab′)$_2$ anti-rat IgG (Tago Immunologicals, CA, USA). Stained cells were analyzed by FACSCalibur (Becton Dickinson, San Jose, CA, USA).

2.4. ICAM-1 binding assay and confocal microscopic analysis

Experiments were performed according to the method of Stewart et al. [11]. Briefly, Con A blasts were suspended (1×10^5 cells/50 μl) in staining buffer (PBS/5 mM Mg^{2+}/1 mM EGTA/0.2% BSA) and incubated with 20 μg/ml of soluble ICAM-1 chimeric with the Fc portion of human IgG1 (D1D2-IgG) [12]. After a 30 min incubation either at 37 or 4°C, cells were reacted with 15 μg/ml of FITC-goat anti-human IgG Fc specific antibody (Jackson Immuno Research Laboratories, PA, USA) for 20 min on ice and applied to FACSCalibur. Stained cells were also examined with confocal laser scanning microscopy (LSM510, Carl Zeiss, Germany) after fixation with 3% formalin/PBS for 30 min at RT. Fixation of the cells before incubation with the FITC-labeled second antibody gave comparable results even though the background of fluorescence became a little higher (data not shown). In each sample from $lok^{-/-}$ or $lok^{+/+}$ mice, 100 cells were analyzed for the presence of cap formation by using a 3D projection image analyzer (Carl Zeiss). Cap formation was classified in three types, 1/4 cap, 1/2 cap and patch. Cells showing fluorescence on approximately 25 and 50% of the membrane periphery were considered as having 1/4 and 1/2 cap, respectively. Cells showing fluorescence in small, irregular clusters disseminated throughout the cell surface without obvious polar distribution were considered as having patch.

3. Results

3.1. Generation of $lok^{-/-}$ mice

For generation of LOK-deficient mice, a targeting vector shown in Fig. 1A was used to replace the exon 1 of the *lok* gene with a neomycin-resistance gene. Southern blot analysis of mouse tail DNA showed the correct targeting of the *lok* locus and identified the genotype of progeny (Fig. 1B). The absence of LOK expression in homozygous mice was confirmed by Western blot (Fig. 1C). An intercross of heterozygotes produced wild-type ($lok^{+/+}$), heterozygous ($lok^{+/-}$) and homozygous ($lok^{-/-}$) offspring at the expected Mendelian ratio. $lok^{-/-}$ mice did not exhibit any obvious gross histopathologic abnormalities. Flow cytometric analysis showed a normal distribution of B cells, T cells and their precursors in the bone marrow, thymus, spleen and lymph nodes of $lok^{-/-}$ mice (data not shown), indicating that their lymphocytes developed normally.

3.2. Enhanced cell adhesion in mitogen-stimulated T cells from $lok^{-/-}$ mice

To examine possible functional alteration of $lok^{-/-}$ lymphocytes, lymphocytes obtained from various lymphoid organs were stimulated in vitro with mitogens such as LPS and Con A, antibodies specific to IgM, CD40, CD3 and CD28 or IL-2. Proliferation of B and T cells from $lok^{-/-}$ mice was comparable to that of cells from $lok^{+/+}$ mice as judged by thymidine uptake and cell counts (data not shown). Interestingly, however, we noticed that Con A-stimulated T cell blasts from $lok^{-/-}$ mice formed much larger and tighter aggregates than those from wild-type littermates did (Fig. 2A). No remarkable difference was observed in the aggregation of Con A blasts derived from $lok^{+/+}$ and $lok^{+/-}$ mice (data not shown). To quantitate the extent of cell aggregation, the re-aggregation assay was performed under shaking conditions. Many of the $lok^{-/-}$ cells formed aggregates again (Fig. 2B, top, right), whereas most of the $lok^{+/+}$ cells remained as single cells and only some cells formed aggregates but smaller than those formed by the $lok^{-/-}$ cells (Fig. 2B, top, left). Quantitative analysis with the multisizer counter revealed that about 50% of $lok^{-/-}$ cells were involved in cellular aggregation, while only 15% of the $lok^{+/+}$ cells were involved (Fig. 2C). These results indicated that cell adhesion of Con A blasts was enhanced in the absence of LOK.

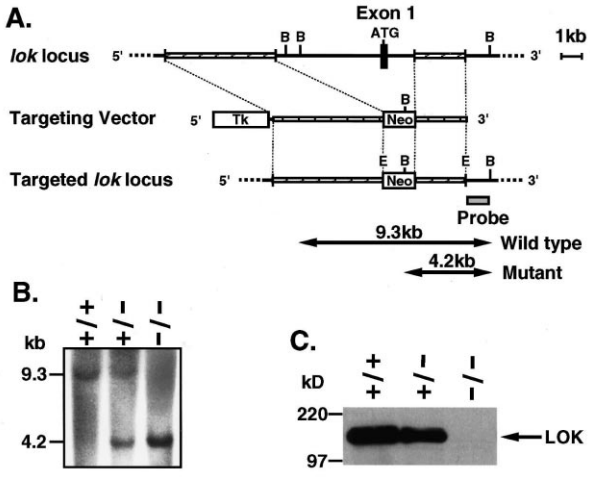

Fig. 1. Generation of $lok^{-/-}$ mice by gene targeting. A: Partial restriction maps are shown for the wild-type *lok* locus (top), the targeting vector (middle) and the targeted *lok* locus (bottom). Neo, neomycin resistance gene; Tk, *Herpes simplex* virus-thymidine kinase. Restriction sites; B, *Bam*HI; E, *Eco*RI. B: Southern blot analysis of offspring from intercrosses of $lok^{+/-}$ mice. Tail DNA was digested with *Bam*HI and hybridized with the 1.2 kb *Eco*RI–*Bam*HI fragment shown in A. Bands corresponding to the wild-type allele (9.3 kb) and those to the mutant allele (4.2 kb) were indicated. C: Western blot analysis of LOK protein. The expression of LOK protein in splenocytes from $lok^{+/+}$, $lok^{+/-}$ and $lok^{-/-}$ mice was examined by Western blotting with anti-LOK antibody [5].

3.3. Enhanced clustering of active LFA-1 on $lok^{-/-}$ Con A blasts

When anti-LFA-1 antibody was added in culture during the re-aggregation assay, the aggregation of $lok^{-/-}$ Con A blasts was almost completely blocked as in the case of $lok^{+/+}$ Con A blasts (Fig. 2B lower panels and Fig. 2C). Therefore, the enhanced cellular aggregation observed in $lok^{-/-}$ Con A blasts was thought to be mediated mainly by the LFA-1/ICAMs adhesion pathway. However, flow cytometric analysis showed no significant difference between $lok^{-/-}$ and $lok^{+/+}$ unstimulated or Con A-stimulated cells in the expression levels of LFA-1, ICAM-1 and ICAM-2 (Fig. 3A).

We next investigated the surface expression level of the active form of LFA-1, which is competent to bind ICAM-1, by using soluble ICAM-1/Fc chimeric protein (D1D2-IgG). As reported previously [11], unstimulated $lok^{+/+}$ cells bound

little if any D1D2-IgG while $lok^{+/+}$ Con A blasts bound substantial amounts of D1D2-IgG when incubated at 37°C (Fig. 3B). To our surprise, the amount of D1D2-IgG bound to $lok^{-/-}$ Con A blasts was comparable to that observed in $lok^{+/+}$ Con A blasts as judged by fluorescence intensity (Fig. 3B). The incubation at 4°C instead of 37°C abolished this binding, indicating that the observed binding of D1D2-IgG to Con A blasts was temperature-dependent and not non-specific.

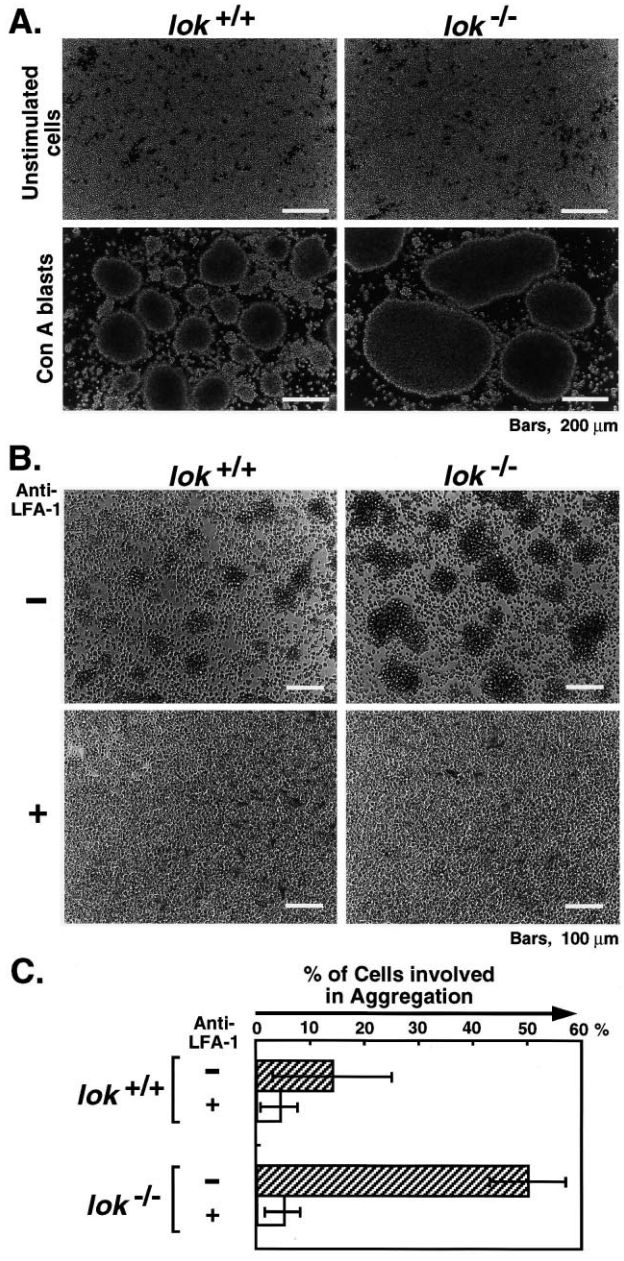

Fig. 2. Enhanced cell adhesion through LFA-1/ICAMs interaction in Con A blasts from $lok^{-/-}$ mice. A: Lymph node cells from $lok^{+/+}$ and $lok^{-/-}$ mice were cultured with Con A at 37°C for 2 days. Photomicrographs were taken before (upper panels) and after the culture (lower panels). B: Con A blasts from $lok^{+/+}$ and $lok^{-/-}$ mice were resuspended in the absence (upper panels) or the presence of 50 μg/ml of anti-LFA-1 antibody (lower panels) and 200 μl of each cell suspension was shaken at 200 rpm at 37°C for 30 min. Photomicrographs taken just after the 30 min-shaking were shown. C: The extent of cell re-aggregation shown in (B) was quantified with a multisizer counter. Data are shown derived from three independent experiments, triplicate in each.

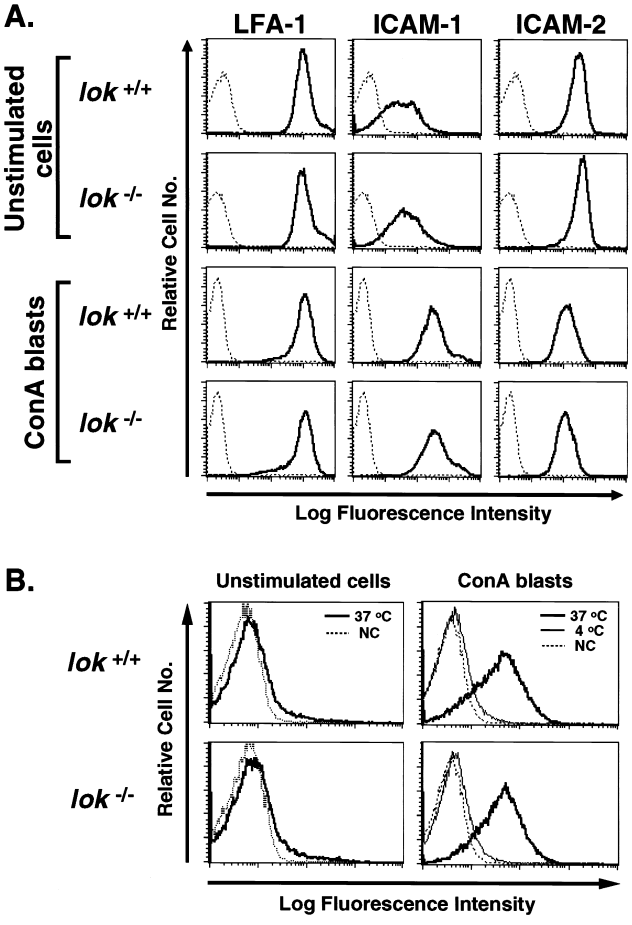

Fig. 3. lok$^{+/+}$ and $lok^{-/-}$ Con A blasts express comparable levels of LFA-1, ICAM-1/2 and active form of LFA-1 on the surface. A: Unstimulated cells and Con A blasts from $lok^{+/+}$ and $lok^{-/-}$ mice were stained with antibodies specific to LFA-1, ICAM-1 or ICAM-2. The histograms are shown as overlaid with those of negative controls (dotted lines). B: Unstimulated cells and Con A blasts from $lok^{+/+}$ and $lok^{-/-}$ mice were reacted with soluble ICAM-1/Fc chimeric protein either at 37°C (bold lines) or 4°C (thin lines). One representative experiment of six is shown as histograms overlaid with those of negative controls (NC, dotted lines).

Intriguingly, the analysis with confocal microscopy revealed that the cell surface distribution of active LFA-1 molecules reactive to D1D2-IgG differed in $lok^{-/-}$ and $lok^{+/+}$ Con A blasts. LFA-1 molecules binding to D1D2-IgG were localized in large clusters on $lok^{-/-}$ Con A blasts while they were rather dispersed on $lok^{+/+}$ Con A blasts (Fig. 4A, lower panels). Analysis with a 3D projection image analyzer revealed that approximately 60% of the $lok^{-/-}$ cells formed caps of LFA-1 while only 20% of the $lok^{+/+}$ cells formed such caps (Fig. 4B). The difference was especially prominent when highly capped cells were compared. About 25% of the $lok^{-/-}$ cells formed '1/4 cap' while only 2% of the $lok^{+/+}$ cells did. These results indicate that the clustering of active LFA-1 was greatly enhanced in the absence of LOK.

4. Discussion

LFA-1 is a leukocyte-specific adhesion receptor that be-

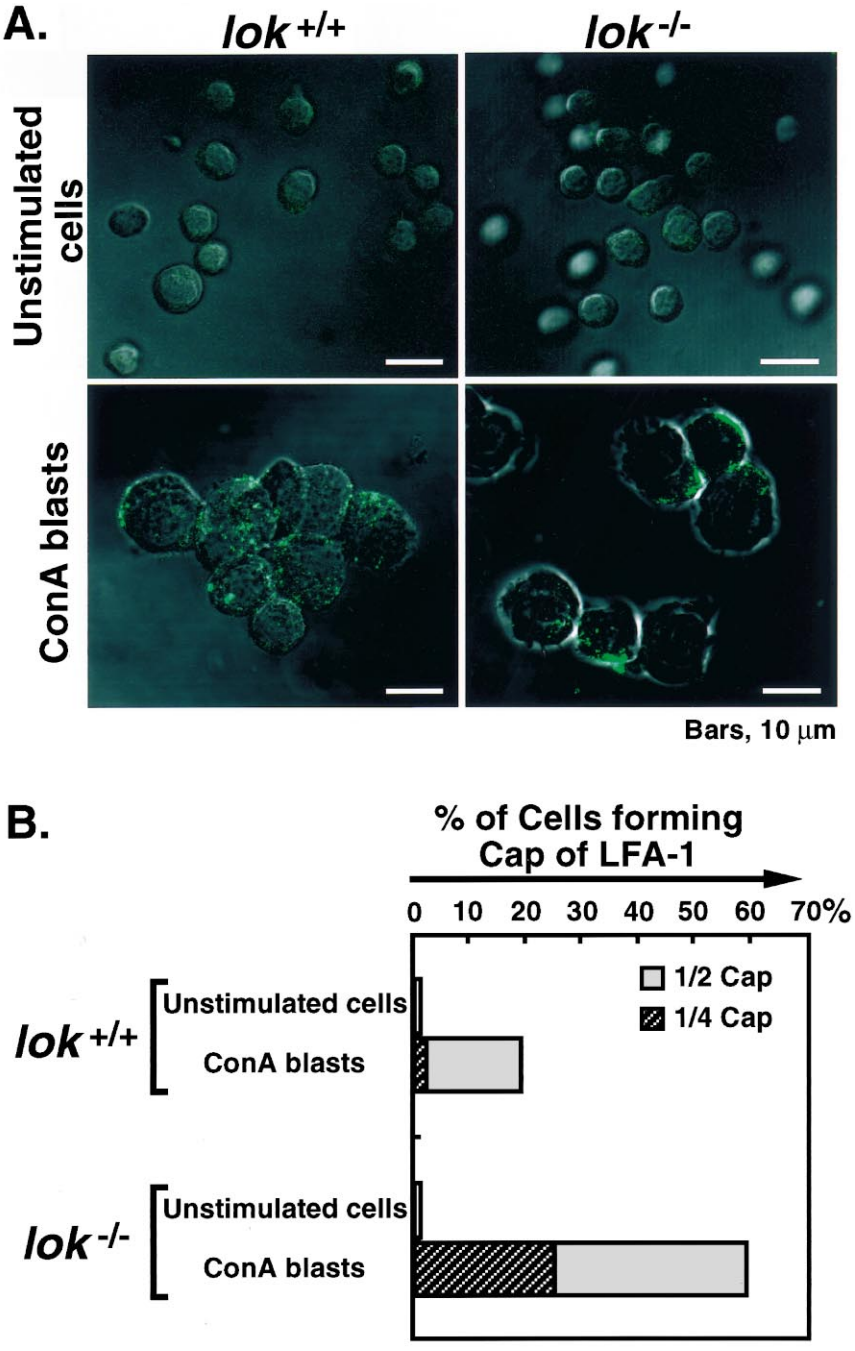

Fig. 4. Enhanced clustering of active LFA-1 on $lok^{-/-}$ Con A blasts. A: Unstimulated cells and Con A blasts from $lok^{+/+}$ and $lok^{-/-}$ mice were stained with soluble ICAM-1/Fc chimeric protein as in Fig. 3B and examined with confocal laser scanning microscopy. B: In each sample from $lok^{+/+}$ or $lok^{-/-}$ mice, 100 cells were analyzed for the presence of cap formation of LFA-1 by using 3D projection image analyzer. Cap formation was classified in three types: 1/4 cap, 1/2 cap and patch. Percentages of cells forming cap of LFA-1 are shown.

longs to the β2 family of integrins and coordinates distinct adhesive and signaling interaction in the immune system [13–15]. LFA-1 expressed by leukocytes is generally not functional and must be activated through intracellular signaling to bind its ligand ICAMs. Though the precise mechanism by which the adhesive function of LFA-1 is regulated remains to be determined, it has been shown that the qualitative rather than quantitative change of LFA-1 is crucial in the regulation [13–15]. At least two mechanisms are thought to be involved in the qualitative change. One is the conformational alteration of LFA-1 that could affect the intrinsic affinity of LFA-1 [15]. The other is the cell surface distribution (clustered/dispersed) of LFA-1 that could affect the avidity for ligands [16,17].

In the present study, we demonstrated that homotypic cell aggregation was greatly enhanced in mitogen-stimulated T lymphoblasts derived from mice deficient for LOK. Their aggregation was completely blocked by function-blocking anti-LFA-1 antibody. Therefore, the enhancement of cell adhesion in the absence of LOK appears to be attributed to the accelerated LFA-1/ICAMs interaction rather than the induction or activation of other adhesion pathways. The expression levels of LFA-1 and ICAMs on the cell surface was found not to be

altered in the absence of LOK, indicating that loss of LOK might affect the qualitative change of these adhesion molecules. Indeed, the analysis with soluble ICAM-1 revealed that clustering of active LFA-1 on mitogen-stimulated T lymphoblasts was accelerated in the absence of LOK. The amount of active LFA-1 detected by soluble ICAM-1 was comparable in the presence and absence of LOK. These results strongly suggest that LOK is involved in the qualitative regulation of LFA-1 through control of the cell surface distribution of active LFA-1.

It remains unclear at present how LOK contributes to the regulation of LFA-1 distribution. Previous studies have indicated that the actin cytoskeleton plays an important role in the regulation of LFA-1 clustering [16–18]. LFA-1 is co-localized with F-actin [18] and concentrated in the area of contact between interacting cells [19]. Disruption of the F-actin cytoskeleton by cytochalasin D results in the disappearance of LFA-1 clusters [18]. Therefore, one may assume that an intracellular serine/threonine kinase LOK could be involved in the regulation of LFA-1 clustering through actin reorganization. In this sense, it is intriguing to note that PAK1, a member of the PAK subfamily, has been shown to dissolve focal adhesion and stress fibers when overexpressed in fibroblasts [20,21]. Moreover, it was recently reported that overexpression of a member of the GCK subfamily TNIK in adherent cells resulted in the disruption of the F-actin structure and the inhibition of cell spreading [22]. Taken together, it is plausible that LOK could participate in modulation of the cytoskeleton to control the cell surface distribution of LFA-1.

Even though we observed the enhanced cellular aggregation of $lok^{-/-}$ Con A blasts in vitro, no remarkable alteration was detected in immune responses of $lok^{-/-}$ mice as far as we examined antibody production, formation of germinal center and cytotoxic activity of activated killer cells (data not shown). Physiological stimuli to induce immune responses in vivo might be much weaker than the extraordinary stimulation with Con A in vitro. Under such weaker stimulation, the loss of LOK function could be compensated by SLK, another member of the GCK subfamily, which is ubiquitously expressed and shows very high similarity to LOK in both kinase and regulatory domains [23,24]. Establishment of LOK/SLK double deficient mice would be needed to explore this possibility.

Evidence provided in the present study gives an important insight into the possible relationship between the GCK subfamily kinases and the regulation of cell adhesion. In order to clarify a pathway connecting LOK, the cytoskeleton and adhesion molecules, we are currently identifying a substrate(s) of LOK and a molecule(s) associated with LOK in lymphocytes.

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