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Uncoordinated 119 Protein Controls Trafficking of Lck via the Rab11 Endosome and Is Critical for Immunological Synapse Formation¹

Magdalena M. Gorska,*† Qiaoling Liang,* Zunayet Karim,* and Rafeul Alam²*†

The activation of T cells through the TCR is essential for development of the adaptive immune response. TCR does not have any enzymatic activity and relies on the plasma membrane-associated lymphocyte-specific protein tyrosine kinase (Lck) for initiation of signaling. Here we uncover a mechanism that is responsible for plasma membrane targeting of Lck. We show that Lck is transported to the membrane via a specific endosomal compartment. The transport depends on the adaptor protein Uncoordinated 119 (Unc119), on the GTPase rat brain 11 (Rab11), and on the actin cytoskeleton. Unc119 regulates the activation of Rab11. Consequently, Unc119 orchestrates the recruitment of the actin-based motor protein, myosin 5B, and the organization of multi-protein complexes on endosomes. The Unc119-regulated pathway is essential for immunological synapse formation and T cell activation. The Journal of Immunology, 2009, 183: 1675–1684.

The Rous sarcoma oncogene homolog (Src)³ lymphocyte-specific protein tyrosine kinase (Lck) family initiates signaling from the TCR and is critical for T cell activation (1, 2). Upon binding of the Ag-MHC complex to TCR, Lck phosphorylates ITAMs on TCR-associated CD3ζ proteins (3). Phosphorylated ITAMs recruit another tyrosine kinase-ζ-associated protein, 70 kDa (ZAP70; Ref. 4). ZAP70 in concert with Lck activates critical downstream signaling pathways, which lead to cytokine production and T cell division. The fundamental role of Lck in the immune system was underscored by the finding that Lck mutations in humans and in mice cause SCID (1, 5). In resting T cells, Lck localizes primarily at the plasma membrane what is necessary for a rapid mobilization of the kinase upon TCR activation (6, 7). The mechanism by which Lck is delivered to the plasma membrane is not understood. Here we show that the transport of Lck is an active, directed process, which depends on the vesicular trafficking machinery.

We and two other groups have independently cloned a signaling molecule called Uncoordinated 119 (Unc119; Refs. 8–10). We subsequently showed that Unc119 deficiency profoundly inhibited Lck activation, T cell proliferation, and IL-2 production (11). Here we show that Unc119 activates the GTPase ras gene from rat brain 11 (Rab11) to transport Lck from perinuclear endosomes to the plasma membrane. The Rab family proteins are known to organize multimolecular complexes on endosomes and other endomembranes (12). These complexes facilitate transport of the vesicular cargo to specific cellular locations. Rab11 and its effectors are critical for recycling of integral plasma membrane proteins, e.g., receptors as well as for exocytosis of newly synthesized secretory proteins (13–15). Here we uncover a new role of Rab11 in the delivery of a cytosol-derived signaling molecule Lck to the plasma membrane. Lck is essential for formation of the immunological synapse (IS; Refs. 16 and 17). Unc119 knockdown T cells are unable to establish IS with APCs. Thus, the endosome-dependent transport of signaling molecules and receptors is critical for formation of the immunological synapse.

Materials and Methods

Antibodies

The following Abs were used: mouse monoclonal anti-Lck (clone 3A5); rabbit polyclonal anti-Rab5, anti-FAK, anti-CD4, anti-hemagglutinin (HA) tag, and goat polyclonal anti-actin (all from Santa Cruz Biotechnology); rabbit polyclonal anti-GM130 (Cell Signaling Technology); rabbit polyclonal anti-Rab11 (Invitrogen); rabbit polyclonal anti-myosin 5B (Mys5B; Antagene); mouse monoclonal anti-Rab11 (clone 47) and anti-CD4 (clone RPA-T4; BD Biosciences); and mouse monoclonal anti-CD3ζ (clone OKT3) (eBioscience). The generation of rabbit polyclonal anti-Unc119 Ab was described (8, 11). The rabbit polyclonal anti-phospho-Lck (Y394) Ab was generously provided by Dr. Andrey S. Shaw (Washington University School of Medicine, St. Louis, MO; Ref. 18).

Immunoprecipitation, polyacrylamide gel electrophoresis, and Western blotting

Immunoprecipitation, electrophoresis, Coomassie staining of gels, and Western blotting were done as described (8, 11). Coomassie-stained gels were read using the Odyssey Infrared Imaging System (LI-COR Biosciences).

RNA interference, plasmids, and retroviral infection of T cells

Small interfering RNA (siRNA) for human Unc119 was from the Predesigned siGenome Collection from Dharmacon. siRNA against firefly luciferase (non-targeting siRNA 2; Dharmacon) was used as a control. Electroporation of Jurkat T cells with siRNA oligonucleotides was performed according to the Amaxa protocol. Small hairpinRNAs (shRNA) for murine Unc119 and luciferase (control) were cloned into the retroviral vector Ban-shee-GFP as described (19). The Unc119- and luciferase-targeting shRNA
sequences were: GAGAGGCCTAATTCGGA and AACGTACGGC-GAATACTT, respectively. Constructs encoding three other Unc119 hairpins were tested, but their silencing capacity was less potent (data not shown). cDNAs of Unc119 and Rab11 were cloned into the bicistronic retroviral vector GFP-RV (20). In GFP-RV constructs, the cDNA for the molecule of interest and the CDNA for GFP are separated by the internal ribosome entry site sequence. The HA-tag was included at the N terminus of Rab11. Rab11S25N and Rab11Q70L mutants were generated using the QuickChange (Stratagene) mutagenesis approach. Production of retroviruses, T cell infection, and sorting were done as described (11, 21). For infection, CD4 T cells were isolated from C57BL/6 splenocyte suspensions by negative selection using the murine CD4 T cell Isolation Kit (Miltenyi Biotec). Procedures on mice were approved by the Institutional Animal Care and Use Committee at National Jewish Health.

**T cell-APC conjugate formation assay**

The assay was performed as described (17, 22).

**Staining for fluorescent microscopy, image acquisition and analysis**

The uptake of Alexa 488-conjugated cholera toxin subunit B (Invitrogen) and cell staining for fluorescent microscopy were performed as described (23–25). Experiments were conducted on a Nikon TE 2000 inverted microscope using a ×100 objective. The microscope is equipped with a mercury lamp (Chiu Technical), Z-motor (Prior Scientific), excitation and emission filter wheels, and a CoolSnap HQ camera (Roper Scientific-Photometrics). Data acquisition and analysis were performed with Metamorph version 7.0 (Molecular Devices). Z-stack images were acquired at 1-μm increments. Images of experimental groups to be compared were acquired using the same software setting (e.g., same exposure time). The three-dimensional deconvolution of Z-stacks was done using the measured point spread function of the Metamorph program. All planes in the deconvolved Z-stack were examined. A representative plane of the Z-stack is shown in each figure. For quantification, three planes of the deconvolved Z-stack were analyzed. The distance between the first and the second plane and between the second and the third plane was 3 μm. Results from three planes were averaged. Images were thresholded to eliminate the input from the background fluorescence. The same threshold was applied to all images in comparison studies. The Region tool of the software was used to select regions corresponding to the entire T cell plasma membrane, the contact site with the APC, the vesicle-enriched perinuclear area, or the whole T cell area. Thresholded areas within the selected regions were used for integrated fluorescence intensity measurement. The integrated intensity is defined as a sum of intensities of all selected pixels.

**Flow cytometry**

Staining of cell surface CD3ε and CD4 was done as described (11). Stained cells were analyzed using the CyAn ADP cytometer (Beckman-Coulter).

**GTPase assay**

The GTPase assay was done as described with modifications (26). Jurkat cell lysates were immunoprecipitated with an anti-Rab11 Ab or control Ig. Immunoprecipitates were washed twice in the modified radioimmunoprecipitation buffer (11) and twice in buffer A (20 mM Tris (pH 8.0), 2 mM EDTA, 1 mM DTT). Next, immunoprecipitates were incubated with 2 μCi (0.074 mCi/mmol) of [35S]GTPyS in 40 μl of buffer B (20 mM Tris (pH 8.0), 5 mM MgCl₂, 1 mM DTT) at 37°C for the time indicated in the figures. Reactions were stopped by addition of 40 μl of buffer C (0.2% SDS, 5 mM EDTA, 5 mM GDP, 5 mM GTP) and by heating at 65°C for 2 min. Two microliters of each eluate were separated on TLC plates using 0.75 M KH₂PO₄, pH 3.5, as a running buffer. Plates were read, and the GDP and GTP radioactivity was quantified using the Typhoon PhosphorImager (GE Healthcare-Amersham) and the Image Quant software, respectively.

**Statistical analysis**

Microscopy experiments were performed 3–5 times, and 300 cells (or cell conjugates) were visually examined per cell type and per experiment. A presented staining pattern was seen in 75–90% of cells. For fluorescent intensity measurements, deconvolved Z-stacks of 15 representative cells (or cell conjugates) from 3 experiments were studied. Statistical analyses were done with the two-tailed Student’s t test. Results are shown as mean ± SD. A value of p < 0.05 was considered statistically significant.

**Results**

**Unc119 controls the exit of Lck from Rab11-positive endosomes and Lck targeting to the plasma membrane**

We examined the effect of Unc119 knockdown on Lck localization in resting T cells. In our experiments we used the human CD4⁺ T cell line Jurkat as well as murine primary CD4 T cells. Unc119 knockdown was induced by electroporation with siRNA (Jurkat T...
Unc119 or by retroviral infection with shRNA (primary T cells; Fig. 2). As expected, in control Unc119-sufficient CD4+ T cells, Lck localized primarily at the plasma membrane (Fig. 1, B and C, and Fig. 2, B and C). A small portion of Lck was located in punctate, vesicle-like structures in the cytoplasm (6). In the majority of T cells, these structures clustered in the perinuclear region. Unc119 knockdown resulted in a profound reduction in plasma membrane targeting of Lck. Lck was almost exclusively...
localized to perinuclear vesicle-like structures (Fig. 1, B and C). We set out to determine the identity of these Lck-positive endomembranes. Perinuclear Lck was reported to colocalize with endocytosed transferrin (27, 28). After endocytosis, transferrin enters the early endosomal compartment, which is regulated by the GTPase Rab5 (29). Later it moves to the recycling endosome/trans-Golgi network compartment, which is controlled by the GTPase Rab11. T cells were costained with an anti-Lck Ab and an Ab against Rab5 or Rab11 (Figs. 1B and 2B). Vesicular Lck colocalized with Rab11 in both control and Unc119 knockdown T cells. The colocalization with Rab5 was minimal. We also studied if Lck-positive vesicles overlap with the Golgi apparatus, which is another perinuclear organelle (Fig. 1B). The colocalization of Lck with the Golgi marker protein GM130 was minimal. Jurkat cells stained with control IgGs or the Lck-deficient Jurkat clone J.CaM1.6 treated with the anti-Lck Ab did not show any fluorescence (Figs. 1B and 2D). We have previously shown that Unc119 activates another Src family kinase, Fyn (11). In normal T cells, Fyn is primarily localized in the perinuclear region (Fig. 2E; Ref. 6). The subcellular distribution of Fyn was not affected by Unc119 knockdown. The localization of the non-Src tyrosine kinase FAK was not changed as well (Fig. 2F).

Mislocalization of a protein may influence its degradation kinetics. The total level of Lck was not affected by Unc119 knockdown (Figs. 1A and 2A). Protein overexpression and deficiency usually give opposite phenotypes. Indeed, Lck was absent from the perinuclear compartment of Unc119-overexpressing Jurkat T cells (Fig. 2, G and H). It localized solely at the plasma membrane. Thus, on the way to the plasma membrane, Lck traffics through the Rab11-positive endosomal compartment. The egress of Lck from Rab11-positive endosomes is regulated by Unc119.

**FIGURE 3.** Unc119 is essential for Lck accumulation at the IS and the IS formation. A and B, Fluorescent images of T cell-B cell conjugates. Jurkat T cells transfected as in Fig. 1A were copelleted with CMAC (blue dye)-stained, SED-loaded Raji B cells to induce the IS formation and incubated at 37°C for 1, 5, or 10 min. Cell conjugates were immunostained for Lck (green) and Rab11 (red). A, T cell-B cell conjugates at the 10-min time point. Experiment was performed 3 times, and 300 cell conjugates were visually examined each time. Presented staining pattern was seen in 90% of the cell conjugates. Z-stacks of green, red, and blue fluorescence were acquired and deconvoluted as in Fig. 1B. Digital image correlation images of the same cell conjugates are shown to the extreme left. Deconvolved Z-stacks of Lck fluorescence were quantified. B, mean ± SD values of integrated fluorescent intensity (IFI) of Lck at the T cell-APC contact site per micrometer of the contact site. Images of 15 conjugates at each time point from 3 different experiments were quantified. *, p < 0.05. C, Cell conjugates were prepared as in A and stained with an anti-CD4 Ab. C, Conjugates at the 10-min time point. The experiment was done three times. Each time 300 conjugates were examined. Deconvolution of Z-stacks was done as in A. D, Mean percent ± SD of synapse-forming T cells. Percentage of synapse-forming T cells in individual experiments (n = 3) was calculated using the formula ([synapse-forming T cells/all T cells] × 100) at the 10 min time point. Synapses were identified by immunostaining with anti-CD4 Ab as in C. *, p < 0.05.
At the plasma membrane, a fraction of Lck resides in lipid rafts. Lipid rafts are known to cycle between the plasma membrane and endomembrane compartments (23, 24, 30). We studied the trafficking of lipid rafts via the Rab11<sup>+</sup> endosomes. The plasma membrane lipid rafts were labeled by incubation of Jurkat cells on ice with the Alexa 488-conjugated subunit B of cholera toxin (CTB). Next, cells were switched to 37°C and allowed to internalize CTB for 10, 30, and 60 min. Subsequent immunostaining of fixed cells revealed moderate colocalization of CTB with Rab11 at 10 min (supplemental Fig. 1).<sup>4</sup> The colocalization was diminished at 30 and 60 min. There was no apparent difference in overall subcellular distribution of CTB in control and Unc119 knockdown cells. Thus, rafts are transported via the Rab11<sup>+</sup> compartment, and this transport does not seem to depend on Unc119. In the later part of the article, we show that Unc119 regulates Rab11. Previous study has demonstrated that the transport of rafts from Rab11<sup>+</sup> endosomes to the plasma membrane does not depend on Rab11 but relies on another GTPase Arf6 (30).

Unc119 interacts with the CD3 complex and CD4 (11). Thus, we investigated whether Unc119 affected the subcellular distribution of these receptors. In normal T cells, the CD3ε subunit was localized at the plasma membrane as well as in the belt-like area, which encircled the nucleus (Fig. 2). The cytoplasmic staining likely reflects the accumulation of freshly-synthesized CD3ε in the endoplasmic reticulum or another compartment of the biosynthetic pathway (28). CD4 was localized exclusively at the plasma membrane (Fig. 2K). Rab11 showed very little overlap with the aforementioned receptors (Fig. 2, I and K). The subcellular distribution (microscopy; Fig. 2, I and K) and the surface expression (flow cytometry; Fig. 2, J and L) of CD3ε and CD4 were not influenced by Unc119 knockdown.

Our results showed that Lck and CD4 were localized in different compartments in Unc119 knockdown T cells. We wanted to confirm the fluorescent microscopy data with a biochemical approach. Coimmunoprecipitation experiments demonstrated that the association of Lck with CD4 is significantly reduced in Unc119 knockdown T cells (Fig. 2M).

**Unc119 regulates the Lck translocation to the immunological synapse and the synapse formation**

Because experiments with Jurkat and primary T cells gave identical results, for simplicity, we used the former cell type for further investigations. We examined the effect of Unc119 knockdown on recruitment of Lck to the IS (Fig. 3, A and B). Superantigen (staphylococcal enterotoxin D; SED)-pulsed Raji B cells were used as APCs (22, 28). In control T cells, Lck translocated to the IS within first minute of the contact with the APC. Lck accumulation at the IS was also visible at 5 and 10 min after the initiation of the T cell-APC contact. In Unc119 knockdown T cells, the concentration of Lck at the IS was reduced at all examined time points. Unc119 knockdown did not affect the translocation of CD4 to the IS (Fig. 3C).

The results suggest that Unc119 is critical for accumulation of Lck at the IS. Because Lck is critical for formation of stable IS, we examined the effect of Unc119 knockdown on IS development. We studied T cells that had been exposed to superantigen-loaded APCs for 10 min. The percentage of synapse-forming Unc119 knockdown Jurkat T cells was reduced 2.9-fold (Fig. 3D). Thus, Unc119 is important for IS formation.

**Unc119 is present on Rab11-positive endosomes**

We set out to determine the mechanism by which Unc119 affected the transport of Lck. We analyzed the subcellular distribution of Rab11 by immunostaining (Fig. 4A). Unc119 showed a punctate cytoplasmic localization with a significant enrichment in the perinuclear region. Perinuclear Unc119 strongly colocalized with Rab11 (Fig. 4B). Anti-Unc119 immunostaining of Unc119 knockdown cells did not produce fluorescence (Fig. 4A). We previously showed that Unc119 was critical for Lck kinase activity (11).

**FIGURE 4.** Unc119 associates with Rab11. A, Fluorescent images of Jurkat T cells transfected as in 1A and costained with an anti-Unc119 Ab (green) and 4′,6′-diamidino-2-phenylindole (blue). Z-stacks of green and blue fluorescence were collected and deconvolved as in Fig. 2D. A representative plane is shown. B, Fluorescent images of normal Jurkat T cells costained with an anti-Unc119 (green) and anti-Rab11 (red) Abs. Z-stacks of green and red fluorescence were collected and deconvolved as in Fig. 1B. A representative plane is shown. C, Fluorescent images of Jurkat T cells transfected as in Fig. 1A and costained with anti-phospho-Lck (pLck) (Y394) (green) and anti-Lck (red) Abs and a nuclear dye, 4′,6′-diamidino-2-phenylindole (blue). Z-stacks of green, red, and blue fluorescence were collected and deconvolved as in Fig. 1B. A representative plane is shown. Experiments in A–C were performed 3–5 times with 300 cells visually inspected per condition and per experiment. D, Deconvolved Z-stacks of pLck fluorescence from C of 15 representative cells from 3 experiments were quantified. Integrated fluorescent intensity (IFI) of pLck in the entire cell area was measured. Values are the mean ± SD IFI of pLck per square micrometer of the cell area. *, p < 0.05. E, Western blot (WB) analysis of immunoprecipitates (IP) generated by incubation of Jurkat T cell lysates with anti-Unc119 Ab or with the control Ig (clgG). Immunoprecipitates were sequentally blotted with anti-Rab11 and anti-Unc119 Abs. n = 3; IgH, Ig H chain.

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<sup>4</sup>The online version of this article contains supplemental material.
Thus, we hypothesized that Unc119 controlled the vesicular transport of Lck via stimulation of its kinase activity on endosomes. Therefore, we examined the activation of Lck on endosomes by staining with an Ab against phosphorylated Tyr$^{394}$ (pY394) of Lck (Fig. 4C; Ref. 18). Tyr$^{394}$ is located in the activation loop of Lck. Phosphorylation of Tyr$^{394}$ results in enzymatic activation. Phospho-Lck was located exclusively at the plasma membrane in both control and Unc119 knockdown Jurkat T cells. In agreement with our previous data, Unc119 knockdown Jurkat T cells demonstrated a significant reduction of Lck phosphorylation (Fig. 4D). Thus, it is unlikely that Unc119 activated Lck on endosomes. We asked whether there were any connections between Unc119 and the Rab11-controlled vesicular trafficking molecules. Coimmunoprecipitation experiments revealed the association of Unc119 with Rab11 (Fig. 4E). The colocalization and coimmunoprecipitation results collectively suggest that Unc119 and Rab11 coexist in the same protein complex on perinuclear endosomes.

**Unc119 controls the ability of Rab11 to form complexes with other endosomal proteins**

The Rab family GTPases are activated on membranes through the exchange of GDP for GTP (12). Activated Rabs bind a wide range of downstream effectors such as motor proteins (e.g., myosins or kinesins), enzymes involved in lipid metabolism (e.g., PI3Ks) or vesicle-tethering factors (e.g., components of exocyst complex; Refs. 12 and 31). These effectors act cooperatively in vesicle movement, tethering, and fusion with destination membranes. Because the plasma membrane targeting of the Rab11-associated vesicular cargo was impaired in Unc119 knockdown cells, we set out to determine whether this effect was due to diminished Rab11 interactions with other proteins. The Rab11-associated proteins were immunoprecipitated from the control and Unc119 knockdown T cells using an anti-Rab11 Ab. Complexes were run on a polyacrylamide gel and stained with the Coomassie stain (Fig. 5A). Unc119 knockdown profoundly reduced the interaction of Rab11 with Rab5, Actin, and Myo5B (Fig. 5B). Unc119 knockdown substantially reduced the association of myosin 5B with Rab11 (Fig. 5F). Unc119 knockdown profoundly reduced the interaction of Rab11 with other proteins. Control experiments showed that Unc119 knockdown had no effect on the interaction of Rab5 with its binding partners as assessed by Coomassie staining of the gel (Fig. 5B).

The motor protein myosin 5B is one of the key effectors recruited by activated Rab11 to endosomes (31). It has the ability to move along actin filaments and drive the movement of endosomes. Unc119 knockdown substantially reduced the association of myosin 5B with Rab11 (Fig. 5F). On the contrary, Unc119 overexpression resulted in the significant increase of Rab11-bound myosin 5B (Fig. 5G). Importantly, similarly to Rab11, the total level of myosin 5B was not influenced by Unc119 (Fig. 5, C and D). These observations suggest that Unc119 is essential for Rab11 to
establish interactions with other proteins and thus for its biological activity.

**Depolymerization of actin filaments inhibits Lck exit from the perinuclear compartment**

The substantial effect of Unc119 on the association of Rab11 with the actin-based motor protein prompted us to investigate the role of actin cytoskeleton in the transport of Lck-positive vesicles. Latrunculin A changes the structure of actin monomers and thereby precludes their participation in polymerization. Because the polymerization and the depolymerization of actin dynamically balance each other all the time, inhibition of one process will result in the acceleration of the other. Latrunculin A treatment caused depolymerization of actin filaments (Fig. 6A) and led to the accumulation of Lck in the Rab11-positive compartment (Fig. 6B). Overall, the distribution of Lck in latrunculin A-treated T cells resembled that seen in Unc119 knockdown T cells. Thus, an intact actin cytoskeleton is critical for the transit of Lck from the Rab11 compartment to the plasma membrane.

**Unc119 controls Lck localization via regulation of the Rab11 activation**

The binding of effector proteins to GTPase is dependent on its association with GTP. The hydrolysis of GTP to GDP causes the dissociation of effectors from the GTPase and thus renders it biologically inactive. We measured the GTPase activity of Rab11, which had been isolated by immunoprecipitation from control, Unc119 knockdown, and Unc119-overexpressing T cells. The Unc119 knockdown cell-derived Rab11 hydrolyzed GTP to GDP faster than the control cell-derived Rab11 (Fig. 7, A and B). In contrast, Rab11 from Unc119-overexpressing cells demonstrated a substantially reduced ability to break down GTP (Fig. 7, C and D). Immunoprecipitates generated using control IgG did not hydrolyze GTP (Fig. 7A).

Our experiments collectively show that Unc119 decelerates the hydrolysis of the Rab11-bound GTP and thus increases the amount of activated Rab11. Next, we wanted to determine whether the effect of Unc119 on Rab11 determined the subcellular distribution...
of Lck. We took a stepwise approach and first examined whether an inactivation of Rab11 in normal, Unc119-sufficient T cells have an effect on Lck trafficking. Normal Jurkat T cells were infected with the retrovirus encoding the GTP binding-defective, dominant negative variant of Rab11, Rab11S25N (13). Dominant negative variants of GTPases are believed to block endogenous enzymes by sequestrating guanine nucleotide exchange factors. Control cells were infected with the retrovirus encoding the wild-type Rab11 or empty retrovirus. Cells were sorted on the basis of the selection marker (GFP) expression and the expression of HA-tagged Rab11 variants was verified by Western blotting (Fig. 8A). T cells expressing Rab11S25N, but not control cells, showed loss of Lck from the plasma membrane and the retention of the kinase on perinuclear endosomes (Fig. 8, B and C).

Overexpression of a GTPase often results in its hyperactivation. We examined whether the overexpression of normal Rab11 or the introduction of GTP-locked Rab11 could correct the localization defect of Lck in Unc119 knockdown cells. Jurkat T cells were infected with the retroviruses encoding the wild-type Rab11 or the GTPase deficient variant of Rab11, Rab11Q70L (13). Because Rab11Q70L does not hydrolyze GTP, it is locked in the GTP-bound state and is constitutively active. Control cells were infected with the retrovirus encoding Rab11S25N or the empty retrovirus. Next, the GFP-positive cells were sorted and electroporated with Unc119 siRNA oligonucleotides. A separate sample of empty retrovirus-infected cells was electroporated with control siRNA oligonucleotides. Neither the infection with the empty virus nor the infection with Rab11S25N-encoding virus impacted on the Lck localization of Unc119 knockdown cells (Fig. 8D, E and F). We examined 300 cell conjugates per T cell type and per experiment. Presented staining pattern was detected in 90% of cells. n = 3 experiments. IFI, Integrated fluorescent intensity.

FIGURE 8. Unc119 regulates the Lck localization via Rab11 and is important for immunological synapse formation. A, Western blot (WB) analysis of Jurkat T cells sorted on the basis of the GFP expression after infection with a retrovirus encoding GFP alone (vector) or GFP and HA-tagged Rab11 variants: wild-type (WT) Rab11 (Rab11WT); Rab11S25N; and Rab11Q70L. Western blot membrane was probed with anti-HA and anti-actin Abs. n = 3. B, Fluorescent images of Jurkat T cells infected and sorted as in A and costained with an anti-Lck Ab (red) and 4',6'-diamidino-2-phenylindole (blue). For B, D, and F, Z-stacks of green (GFP), red and blue fluorescence were obtained. Z-stacks of red fluorescence were deconvolved as in Fig. 1B. A representative plane is shown. C, Deconvolved Z-stacks of images from B were quantified as for Fig. 1C. Images of 15 cells from 3 experiments were quantified. *, p < 0.05. D, Fluorescent images of Jurkat T cells transfected and sorted as in A, next transfected as in Fig. 1A, and costained as in B, E. Deconvolved Z-stacks of images from D were quantified as in Fig. 1C. Fifteen cells from three independent experiments were analyzed. *, p < 0.05 (comparison with cells transfected with siUnc119 plus vector); #, p < 0.05 (comparison with cells transfected with control siRNA (siC) plus vector). F, Fluorescent images of T cell-B cell conjugates. Jurkat cells subjected to the double transfection as in D were copelleted with CMAC-stained, SED-loaded Raji B cells as in Fig. 4A and incubated at 37°C for 10 min. Cell conjugates were immunostained for Lck (red). We examined 300 cell conjugates per T cell type and per experiment. Presented staining pattern was detected in 90% of cells. n = 3 experiments. IFI, Integrated fluorescent intensity.
localization in Unc119-knockdown T cells (Fig. 8, D and E). On the contrary, the infection with the Rab11Q70L- or wild-type Rab11-encoding viruses restored the plasma membrane targeting of Lck. The restoration of Lck at the plasma membrane was accompanied by the shrinkage of the endosomal pool of the kinase. In fact, in the majority of Rab11Q70L-overexpressing cells, the endosomal pool of Lck was no longer visible.

We also studied whether the overexpression of wild-type or constitutively active Rab11 in Unc119-knockdown T cells was sufficient to restore the Lck translocation to the contact site with the APC. Overexpression of Rab11Q70L or wild-type Rab11 reconstituted the Lck recruitment to this adhesion site (Fig. 8F). The infection with the empty virus or the expression of Rab11S25N had no effect. Taken together, our results provided strong evidence that Unc119 controlled the Lck transport to the plasma membrane through the activation of Rab11.

Discussion

Our work reveals the mechanism of plasma membrane targeting of the critical T cell tyrosine kinase Lck. Lck is transported to the plasma membrane via an endosomal route. The trafficking of Lck depends on the activation of the endosome-associated proteins Unc119 and Rab11 and on the actin cytoskeleton. We have also demonstrated that the same mechanism is used to deliver Lck to the immunological synapse. Interference with Unc119 impairs the interaction of Rab11 with the motor protein myosin 5B. As a consequence, Lck transport to the membrane is stalled and the formation of the immunological synapse is severely disturbed.

The mechanism of Lck targeting to the plasma membrane has not been well understood. Recent studies suggested that vesicular trafficking might play a role. In one report, the association of Lck with the cell surface CD4 was shown to be reduced by the exocytosis inhibitor, brefeldin A (32). Accordingly, certain infections were shown to cause the redistribution of Lck from the plasma membrane to intracellular vesicles. In T cells infected with HIV, Lck accumulated on transferrin receptor-positive endosomes (28, 33). The distribution of Lck in HIV-infected T cell closely resembled that of Unc119-deficient T cell. Likewise, HIV-infected T cells had reduced ability to form synapses with APCs. In T cells infected with HSV, Lck was targeted to lysosomes, where it underwent degradation (34). As a result, the amount of Lck at the plasma membrane was substantially reduced. The altered targeting of Lck in infected T cells has been attributed to the action of the HIV protein Nef and the HSV protein Tip. The endogenous molecule(s) responsible for Lck transport is largely unknown. One recent report showed the importance of the integral membrane protein MAL (35). We describe an essential role for the GTPase Rab11 and the adapter protein Unc119 in this process.

Rab11 function is relatively well studied in epithelial cells and neurons. Rab11 localizes to the perinuclear recycling endosomes, trans-Golgi network and post-Golgi vesicles (13, 15). The GTPase regulates recycling of various integral plasma membrane proteins such as enzymes (e.g., H⁻/⁻-K⁺ ATPase), adhesion molecules (e.g., integrins), or neurotransmitter receptors (e.g., α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors; Refs. 36–38). Some reports indicate that Rab11 and the recycling endosome are also involved in the transport of newly-synthesized proteins from the Golgi apparatus. In Drosophila photoreceptors, Rab11 controls the transport of rhodopsin from the Golgi to the photosensitive apical plasma membrane (15). Unc119 plays an important role in photoreceptor function. The photoreceptor-specific expression of mutant Unc119 in transgenic mice causes retinal degeneration (39). The recycling endosome, and, presumably Rab11, participates in the transport of newly-synthesized vesicular stomatitis virus glycoprotein G to the plasma membrane in epithelial cells (40). Finally, Rab11 has been implicated in the regulation of TNF-α secretion by macrophages (14). The role of Rab11 in T cell biology is poorly characterized. A single but exciting finding has been made in the field of CTLs. Rab11-positive endosomes deliver Rab27a and hMunc13-4 to the cytotoxic T cell synapse (41). Rab27a and hMunc13-4 are essential for the exocytosis of granzyme and perforin-containing granules. Mutations in Rab27a and hMunc13-4 in humans underlie the pathogenesis of two diseases associated with the altered CD8 T cell function, the Griscelli syndrome and familial hemophagocytic lymphohistiocytosis, respectively (42, 43). We are the first to characterize the function of Rab11 in CD4 T cells.

We have shown that Unc119 blocks the hydrolysis of Rab11-associated GTP. The exact molecular mechanism of this inhibition is unknown at this moment. The intrinsic rate of GTP hydrolysis by Rab proteins is very low. We speculate that Unc119 interferes with the action of a GTPase-activating protein(s). The knowledge about the biochemical mechanisms that control Rab11 is very limited. There is one report that shows that the oncogene Evi5 enhances GTPase activity of Rab11 in vitro (44). However, two other reports disprove it (45, 46). In another report, a neuronal adaptor protein protrudin has been demonstrated to associate with the GDP-bound Rab11 and to block its biological function (47). The mechanism of this action is not clear.

In our previous paper, we characterized the role of Unc119 in the regulation of the Lck enzymatic activity (11). Unc119 binds to CD3 and CD4 molecules. Upon TCR stimulation, Unc119 transmits the activation signal from the receptor to Lck. The signal transmission is dependent on a direct interaction between Unc119 and Lck. Through mutational analyses and various binding experiments, we have shown that an interaction occurs between the Src homology domain of Lck and one of two PXXP motifs of Unc119. The Src kinase crystal structure predicts that the binding of the ligand to the Src homology domain of the kinase would disrupt the intramolecular interactions and cause the relaxation of the conformation and kinase activation. Thus, the TCR-associated Unc119 serves as an Lck activator. In this paper, we describe the role of endosome-associated Unc119 as an Lck-transporting molecule. The latter is accomplished through the activation of Rab11. We are unable to detect phosphorylated Lck in the endosomal compartment. We believe that Lck activation by Unc119 at the cell membrane and Lck trafficking by Unc119 from the endosomal compartment are two independent processes. Unc119 is also an activator of Fyn, which is another Src family kinase expressed in T cells (11). However, Unc119 does not have any effect on Fyn localization. A previous study showed that Fyn and Lck localize to nonoverlapping cellular compartments (6). Perhaps this explains the lack of effect of Unc119 on Fyn localization.

Our results show that Unc119 is essential for Lck recruitment to the IS. This is likely due to the overall reduced level of Lck at the plasma membrane. In normal T cells, the translocation of Lck is primarily driven by CD4 and its interaction with MHC molecules on the APC (7, 18). The accumulation of Lck at the immunological synapse is necessary for the signal amplification (7).

Lck also plays a critical role in the formation of the immunological synapse (17). Lck-deficient T cells have impaired ability to form synapses with APCs. Synapse formation cannot be restored by the expression of another TCR-associated Src family kinase-Fyn. Failure to form synapses with APCs is also not due to the lack of ZAP70 activation. ZAP70-deficient T cells conjugate normally. Lck induces T cell-APC conjugate through the activation of the integrin-LFA-1 and remodeling of the actin cytoskeleton (17, 48). Thus, the impaired synapse formation by Unc119 knockdown T cells is likely due to lack of Lck recruitment. In summary, we have
identified a vesicular trafficking pathway for Lck, which is essential for CD4 T cell activation.

Disclosures
The authors have no financial conflict of interest.

References
**Supplemental Data**

**Manuscript title:** Unc119 controls trafficking of Lck via the Rab11 endosome and is critical for immunological synapse formation

**Authors:** Magdalena M. Gorska, Qiaoling Liang, Zunayet Karim, Rafeul Alam

**Supplemental Figure 1**

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Supplemental Figure 1
Legend to the Supplemental Figure 1: Cholera toxin, a lipid raft marker, is transported via the Rab11 compartment in Unc119-independent manner.

Jurkat T cells were transfected as in 1A and incubated on ice with Alexa 488-conjugated subunit B of cholera toxin (CTB; shown in green). Labeled cells were washed, placed in $37^0\text{C}$ for 10, 30 and 60 min to permit internalization and intracellular trafficking of CTB. Next, cells were fixed and stained with anti-Rab11 antibody (red) and nuclear dye-DAPI (blue) and examined by fluorescent microscopy. Supplemental Fig.1 shows cells at 10 and 30 min time points. Z-stacks of green, red and blue fluorescence were collected. Z-stacks of green and red fluorescence were subjected to 3D deconvolution as in 1B. A representative plane is shown. The boxed area in the overlay image is magnified and displayed to the right of the image. Three independent experiments were performed. Each time 300 cells were visually inspected. The presented intracellular distribution pattern of CTB and Rab11 was detected in 85% cells.