Phosphorylation of Rab5a Protein by Protein Kinase Cε Is Crucial for T-cell Migration

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Background: Rab5a GTPase plays important roles in intracellular transport and cell signaling. Rab5a GTPases control membrane traffic and receptor-mediated endocytosis. Within this context, Rab5a plays an important role in the spatial regulation of intracellular transport and signal transduction processes. Here, we report a previously uncharacterized role for Rab5a in the regulation of T-cell motility. We show that Rab5a physically associates with protein kinase Cε (PKCε) in migrating T-cells. After stimulation of T-cells through the integrin LFA-1 or the chemokine receptor CXCR4, Rab5a is phosphorylated on an N-terminal Thr-7 site by PKCε. Both Rab5a and PKCε dynamically interact at the centrosomal region of migrating cells, and PKCε-mediated phosphorylation on Thr-7 regulates Rab5a trafficking to the cell leading edge. Furthermore, we demonstrate that Rab5a Thr-7 phosphorylation is functionally necessary for Rac1 activation, actin rearrangement, and T-cell motility. We present a novel mechanism by which a PKCε-Rab5a-Rac1 axis regulates cytoskeleton remodeling and T-cell migration, both of which are central for the adaptive immune response.

Significance: The study provides novel insights into the role of Rab5a in the adaptive immune response.

Rab GTPases control membrane traffic and receptor-mediated endocytosis. Within this context, Rab5a plays an important role in the spatial regulation of intracellular transport and signal transduction processes. Here, we report a previously uncharacterized role for Rab5a in the regulation of T-cell motility. We show that Rab5a physically associates with protein kinase Cε (PKCε) in migrating T-cells. After stimulation of T-cells through the integrin LFA-1 or the chemokine receptor CXCR4, Rab5a is phosphorylated on an N-terminal Thr-7 site by PKCε. Both Rab5a and PKCε dynamically interact at the centrosomal region of migrating cells, and PKCε-mediated phosphorylation on Thr-7 regulates Rab5a trafficking to the cell leading edge. Furthermore, we demonstrate that Rab5a Thr-7 phosphorylation is functionally necessary for Rac1 activation, actin rearrangement, and T-cell motility. We present a novel mechanism by which a PKCε-Rab5a-Rac1 axis regulates cytoskeleton remodeling and T-cell migration, both of which are central for the adaptive immune response.

Leukocyte trafficking is a key element of an immune response that is critically dependent on a multifunctional molecular array of integrin-mediated adhesions and chemokine signals. Interaction of the αLβ2 integrin leukocyte function associated-antigen-1 (LFA-1), expressed on T-cells, with its ligand intercellular adhesion molecule-1 (ICAM-1), expressed on the surface of high endothelial venules and other cell types, promotes firm adhesion of T-cells to blood vessel walls and their migration through endothelial junctions into secondary lymphoid organs and sites of inflammation (1–3). In addition, chemokines (for example the stromal cell-derived factor-1α (SDF-1α)) and their interactions with specific receptors on T-cells (such as C-X-C chemokine receptor type 4 (CXCR4)) direct T-cells to arrest on post-capillary venules at sites of infection/injury and secondary lymphoid organs (4). This multistep process of T-cell migration is precisely regulated by a plethora of signaling molecules including kinases, adaptors, and motor proteins that ultimately result in the reorganization of cytoskeletal systems and provide mechanical force to propel the cell forward. Emerging evidence suggests an important role for endocytic transport and vesicular traffic in the process of cytoskeletal remodeling and integrin-mediated cell motility (5–8).

Small GTPases of the Rab family are known to function as molecular switches that regulate a variety of cellular processes including proliferation, differentiation, signal transduction, and cytoskeletal reorganization (9–11). In particular, they control intracellular vesicle transport, such as receptor-mediated endocytosis, exocytosis, and receptor recycling (12–14), all of which are important for cell migration. Rab GTPases cycle between a GDP-bound inactive state and a GTP-bound active state. Inactive GDP-bound Rab proteins typically reside in the cytosol, whereas GTP-bound active Rab proteins localize on intracellular membranes where effector targets reside. Thus, the Rab family of proteins typically require turnover of GTP/GDP to function. This reversible control of Rab GTPase activity via GDP/GTP exchange is crucial to their function as regulators of vesicular trafficking (15). One member of the Rab GTPase family, Rab5a, is known to regulate endocytosis, endosomal dynamics, tethering, and fusion of early endosomes. Rab5a con-
tributes to cytoskeletal remodeling in part by regulating the endocytic trafficking of Rac, a member of the Rho GTPase subfamily and an important component of the actin cytoskeletal system (16–18).

The protein kinase C (PKC) family comprises a group of highly related multifunctional serine/threonine kinases that phosphorylate a wide variety of protein targets and play important roles in cellular signaling pathways including those involved in cell migration (19). More than 10 isoforms of PKC have been identified in mammalian tissues, each of which plays a distinct role in the processing and modulation of a variety of physiologic and pathologic responses to external signals. There is substantial evidence that members of the PKC family regulate integrin-mediated cell spreading and migration (20–23). For example, we have established that signaling through the integrin LFA-1 in T-cells results in the association of PKCβ with the centrosome and microtubule cytoskeleton, a process that is essential for cell migration (21). In addition, another PKC family member PKCe has been identified as a regulator of β1 integrin-dependent cell migration (23–25).

Although extensive progress has been made to understand T-cell signal transduction pathways, the potential involvement of Rab5α in T-cell migration has not been explored to the similar extent. Moreover, the precise contribution of the PKC isoforms in LFA-1-mediated downstream signaling has remained unclear. In the current study we demonstrate that T-cell stimulation through the integrin LFA-1 or the chemokine receptor CXCR4 triggers PKCe-dependent phosphorylation of Rab5α at threonine 7 (Thr-7), which is crucial for Rac1 activation, actin cytoskeleton remodeling, and T-cell migration.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Primary human peripheral blood lymphocyte (PBL) T-cells were isolated from healthy volunteers by standard methods and cultured with phytohemagglutinin and IL-2 as described previously (26). The human T-cell line HuT-78 was obtained from the American Type Culture Collection (ATCC) and cultured as described (27–30). Motility-inducing anti-LFA-1 (clone SPV-L7) was from Monosan (CamiBiotech Biosciences). Human recombinant ICAM-1-Fc was producing anti-LFA-1 (clone SPV-L7) was from Monosan (Cambridge, MA). HuT-78 was obtained from the American Type Culture collection (ATCC) and cultured as described (27–30). Motility-inducing anti-LFA-1 (clone SPV-L7) was from Monosan (Cambridge, MA).

**Induction of LFA-1-mediated T-cell Migration**—We used our well characterized migration-triggering model system where T-cells are stimulated through the LFA-1 receptor via cross-linking with an immobilized anti-LFA-1 antibody or with the physiological ligand ICAM-1 (21, 26–29). These migrating T-cells display dynamic protrusions of the leading edge, flexible oscillatory shape changes of the cell body, and detachment of the trailing edge concomitant with polarized or localized distribution of signaling molecules and cytoskeletal rearrangements. Briefly, 96-well tissue culture plates (flat bottom, Nunc™), 8-well LabTek chamber slides (Nalge Nunc Inc.), or round coverslips, depending on the particular assay type, were coated overnight at 4 °C with 5 μg/ml goat anti-mouse IgG in sterile phosphate-buffered saline (PBS). After washing with PBS, the surface was then incubated with a cross-linking anti-LFA-1 antibody (SPV-L7) for 2 h at 37 °C. ICAM-1-coated surfaces were prepared by adding 5 μg/ml anti-Fc-specific goat anti-human IgG to the culture plates/chamber slides and incubating overnight at 4 °C followed by 1 μg/ml ricAM-1-Fc for 2 h at 37 °C. After washing the surfaces with PBS, HuT-78 or PBL T-cells were loaded into the coated wells and incubated in 5% CO₂ at 37 °C for various time points as indicated in “Results” and figure legends of the particular experiment. Migration assays on ICAM-1 also contained 5 mM MgCl₂ and 1.5 mM EGTA in the cell culture medium to induce the high affinity form of the LFA-1 receptor on T cells (11).

**Plasmid Constructs and Electroporation**—Plasmid vector encoding Rab5α fused to green fluorescent protein (Rab5α-GFP), Rab5αQ79L-GFP, and Rab5αS34N-GFP were generous gifts from M. Zerial, Max Planck Institute of Molecular Cell Biology and Genetics. PKCe-GFP and kinase-dead (KD) PKCe-GFP were kind gifts from D. Romberger, Department of Internal Medicine, University of Nebraska. LifeAct-Red (31) was a generous gift from R. Wedlich-Soldner, Max Planck Institute of Biochemistry. Site-directed mutagenesis of Thr-7, Ser-84, and Ser-123 residues of Rab5α to alanine or glutamic acid was performed by Bio S&T Inc., Montreal, Quebec. Plasmid vectors expressing wild-type or Thr-7 mutant Rab5α as a photoactivable GFP fusion (Rab5α-paGFP or Rab5αT7A-paGFP constructs, respectively) were made by replacing the eGFP cDNA with paGFP cDNA (32), a generous gift from J. Lippincott-Schwartz, Cell Biology and Metabolism Branch, National Institutes of Health. The eGFP cDNA of Rab5αT7A-GFP and PKCe-GFP constructs were replaced with the mCherry cDNA (a kind gift from R. Tsien, University of California, San Diego) to generate the corresponding cherry-tagged proteins. HuT-78 T-cells were nucleofected with plasmid constructs using the Amaxa Nucleofector™ system according to the manufacturer’s recommendations.

**RNA Interference**—To deplete PKCe expression in primary PBL T-cells, a mixture of four siRNA duplexes (Dharmacon ON-TARGETplus SMARTpool® siRNA Reagents, Thermo Fisher Scientific) targeted against PKCe or nonspecific control siRNAs was used. Cells (5 × 10⁶) were nucleofected with 1000 nM siRNA using the Amaxa Nucleofector™ system according to the manufacturer’s instructions and then harvested after 72 h.

**Production of Phospho-Thr-7-specific Rab5α Antibody**—A polyclonal antibody against Thr-7-phosphorylated Rab5α was generated by immunizing two rabbits with a synthetic peptide comprising amino acids 2–12 (ASRGAPTRPNGP, where pT is phosphorylated Thr) of human Rab5α (NeoMPS PolyPeptide). The total serum was then purified by affinity chromatography.
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on phosphopeptide-coupled columns (Amino-link, Pierce) according to manufacturer’s instructions. Briefly, 1 mg of Thr(P)-7 peptide or (OH)Thr-7 peptide was coupled to separate amino-link columns. A total of 0.5 ml of serum was first applied to the (OH)Thr-7-peptide-coupled column to remove nonspecific binding against the (OH)Thr-7 residue of Rab5a. The pre-cleared serum was then purified with a Thr(P)-7-peptide-coupled column and tested by ELISA for specificity and sensitivity.

Cell Lysis, Co-immunoprecipitation, and Western Immunoblotting—Cell lysis was performed as described previously (27) with minor modifications. Briefly, cells were washed with ice-cold PBS and lysed in the lysis buffer containing Triton X-100 (1%) and protease inhibitors phenylmethylsulfonyl fluoride (2 mM), leupeptin (10 μg/ml), and aprotinin (10 μg/ml). The protein content of the cell lysates was determined by the Bradford or BCA assay. The immunoprecipitation was performed as described previously (28, 33). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the cellular lysates or immunoprecipitates and subsequent Western immunoblotting using indicated antibodies were performed as described (28). Where indicated, affinity-purified or crude anti-phospho Rab5α(Thr-7) antisera (1:2000 dilution in blocking buffer) was preincubated with 20 μg/ml non-phosphorylated Thr-7 peptide for 1 h at room temperature before incubation on the membranes. The immunoreactive bands were visualized using the LumiGLO® chemiluminescent detection system (Cell signaling Technology) and subsequent exposure to CL-XPosureTM light-sensitive film (Thermo Scientific). Densitometric analyses of the Western blots were performed by using ImageJ software.

In Vitro Kinase Assay—Rab5α was cloned into the GST expression vector pGEX-6p1, over-expressed in BL21(DE3)pLysS cells, and purified by using glutathione-Sepharose beads (GE Healthcare) according to the manufacturer’s instructions. PKCe-dependent phosphorylation was measured by incorporation of [32P] from [γ-32P]ATP. Purified recombinant Rab5α-GST fusion protein, GST alone, or myelin basic protein (MBP; 200 ng) was incubated in 50 μl of kinase assay buffer (40 mM Tris, pH 7.5, 40 mM MgCl2, 0.2 mM HEPES, pH 7.4, 0.2 mM DTT, 0.0002% Triton X-100, 0.3 μM [γ-32P]ATP, 1 μM phorbol 12,13-dibutyrate, and 160 μM phosphatidylserine. After 20 min of incubation at 37 °C, the reaction was stopped by adding 5× SDS-PAGE sample buffer (250 mM Tris, pH 11.5, 10% SDS, 50% glycerol, and 25% β-mercaptoethanol) and analyzed by SDS-PAGE followed by autoradiography. In vitro kinase assays were also carried out as above using non-radioactive ATP (Sigma) in place of [γ-32P]ATP. After allowing the kinase reaction to proceed, SDS-PAGE sample buffer was added, and the samples were resolved on SDS-PAGE gels and probed by Western blotting with the phospho-T7 Rab5α antiserum.

Confocal Imaging and Photoactivation—For confocal imaging and analysis, cells were seeded to rest or migrate on coverslips as described above and then fixed with 3% (w/v) paraformaldehyde in PBS (27). T-cells were permeabilized with 0.3% Triton X-100 in PBS and blocked with 5% w/v BSA in PBS for 30 min. After blocking, cells were incubated with primary antibodies for 1 h at room temperature. After washing, cells were incubated with Alexa Fluor® 488- or 568-conjugated secondary antibody for 1 h at room temperature. Cells were also stained with Hoechst to visualize nuclei or phallloidin-TRITC to visualize F-actin. After washing, cells were mounted in fluorescence mounting medium (Dako) and stored at 4 °C. Fluorescence microscopy was performed using a confocal microscope LSM 510 with a Plan-Apochromat differential interference contrast 63× oil objective and 1.4 numerical aperture (Carl Zeiss, Inc.). Images were analyzed using the LSM Imaging software (Carl Zeiss).

For photoactivation and confocal live-cell imaging, cells expressing photoactivatable fluorescently labeled proteins were stimulated to migrate on coverslips as described earlier and then placed onto a heated chamber with the internal temperature set at 37 °C (PerkinElmer Life Sciences). Photoactivation was performed with a 405-nm laser using the photobleaching function of LSM Imaging software (Carl Zeiss Inc.) in a time-lapse mode. Generally, 1 pulse of the 405-nm laser was sufficient to activate pAGFP so that it produced very bright fluorescence emission that was detected by excitation at 488 nm using a 500–530-nm band pass filter. At least 20 different microscopic fields were observed for each sample.

High Content Analysis—A high content analysis protocol for T-cell morphology analysis has been optimized and established in our laboratory as described (29, 34). Briefly, cells were seeded in triplicate on 96-well flat bottom plates precoated with either poly-1-lysine or anti-LFA-1 for 2 h. After washing, cells were fixed by incubating them for 20 min with 3% (w/v) paraformaldehyde in PBS. Attached cells were then stained for F-actin using phallloidin-TRITC, and the was nucleus stained using Hoechst. Plates were scanned (9 randomly selected fields/well at 20×) using an automated microscope IN Cell Analyzer 1000 (GE Healthcare), and the acquired images were automatically analyzed by IN Cell Investigator software (Version 1.6) using multitarget analysis bio-application module (GE Healthcare).

Transferrin Internalization—Cells were serum-starved for 1 h and stimulated to migrate on anti-LFA-1 as described above before incubating with Alexa Fluor® 568-transferrin conjugate for 30 min on ice. Cells were then rinsed twice in ice-cold PBS and transferred to 37 °C incubator for 10 min to allow internalization before being fixed with 3% (w/v) paraformaldehyde and imaged.

Transwell Migration Assay—Transwell chambers (5-μm pores; Corning Costar) were precoated with 5 μg/ml rICAM-1-Fc at 4 °C overnight and blocked with 5% (w/v) BSA for 1 h at 37 °C. Serum-starved T-cells were loaded in triplicate in the upper chambers and allowed to migrate toward 50 ng/ml SDF-1α-enriched serum-free medium in the lower wells at 37 °C. After 4 h, migrated cells in the lower wells were fixed and stained with Hoechst. IN Cell Analyzer 1000 (GE Healthcare) was employed to perform whole well cell counts. Data from at least three independent experiments were pooled by normalizing the counts with corresponding controls.

Determination of Rac1 Activity in Cells (PBD Binding Assay)—The capacity of Rac1-GTP to bind to GST–PBD (p21-activated kinase binding domain) beads was used to analyze the activity of this GTPase. Serum-starved T-cells (2 × 10⁶) were stimulated...
with or without immobilized ICAM-1-Fc or SDF-1α for 10 min and lysed in 500 μl of lysis buffer as described (27). Rac1 activity in the cellular lysates was determined using Rac1 activation assay kit according to the manufacturer’s instructions (Millipore). Briefly, cleared lysates were incubated overnight at 4 °C with glutathione-Sepharose 4B beads coupled to GST-PBD. After extensive washing, beads were boiled in SDS-PAGE loading buffer, and the amount of bound Rac1-GTP was detected by Western immunoblotting with an anti-Rac1 antibody. In all cases, whole-cell lysates were also analyzed for total Rac1 for normalization purposes. Bands were visualized and quantified as described above, and the Rac1-GTP levels were normalized to the input levels of total Rac1.

Statistical Analysis—The data are expressed as mean ± S.E. For comparison of two groups, p values were calculated by two-tailed unpaired Student’s t test. In all cases p values <0.05 were considered to be statistically significant.

RESULTS

PKCε Localizes to Endosomes in Migrating T-cells and Associates with Rab5α—We investigated the subcellular distribution of PKCε in resting and migrating T-cells by transfecting the HuT-78 cell line with a plasmid construct encoding PKCε-GFP. After allowing the transfected cells to polarize on an anti-LFA-1 cross-linking antibody, we monitored the subcellular distribution of the PKCε-GFP expression by confocal microscopy. We noted that PKCε-GFP had a distinct localization, as it was distributed around the neck/centrosomal region in migrating cells and also displayed a vesicular pattern that was reminiscent of endosomes (Fig. 1A). To investigate whether PKCε was localized to endosomes in T-cells, we transfected HuT-78 cells with a PKCε-cherry expression vector in combination with a Rab5α-GFP construct that served as a marker of endosomes. Co-localization of PKCε and Rab5α was clearly evident at the neck region and throughout the cell body of migrating HuT-78 T-cells, as demonstrated by confocal microscopy and analysis (Fig. 1, B and C). Notably, both proteins also partially co-localized in resting T-cells (Fig. 1D).

We next examined the interactions between PKCε and Rab5α by live cell imaging of migrating T-cells. For this purpose, we expressed Rab5α as a photoactivatable GFP fusion protein (Rab5α-paGFP) in HuT-78 T-cells that also co-expressed PKCε-cherry (31). Time-lapse confocal microscopy revealed transient interactions between Rab5α-paGFP-positive endosomes and the PKCε-cherry expressing compartments around the neck/centrosomal region of migrating T-cells (supplemental Video S1). A similar localization pattern of Rab5α-containing vesicles has been reported in cultured human U2OS cells (35). Therefore, we focused more closely on the centrosomal region of migrating cells by selecting a population of Rab5α-paGFP positive vesicles and followed their kinetics over time concomitant with PKCε-cherry expression (supplemental Video S2). Upon irradiation, Rab5α-paGFP present around the centrosomal area was photo-activated, and in the following 30-s period, Rab5α-positive endosomes fused with a PKCε-cherry compartment before separating (Fig. 1E, white circle). Next, we applied irradiation aiming at a “single point” corresponding to a PKCε-positive vesicle, and photo-activation of Rab5α-paGFP was visualized within the confines of this structure. The two proteins interacted with each other for a further 60 s and then disengaged (supplemental Video S3). These observations of real-time protein–protein interactions in T-cells after LFA-1 stimulation imply that PKCε and Rab5α dynamically interact during the migratory process. Importantly, endogenous PKCε co-localization with endogenous Rab5α was also detected in primary human PBL T-cells migrating on ICAM-1, which is the physiological ligand for the LFA-1 integrin (Fig. 1F). To quantify the protein–protein interaction of endogenous PKCε with endogenous Rab5α, we performed co-immunoprecipitation studies utilizing primary human PBL T-cells. We observed significantly increased levels (>7-fold) of PKCε-associated Rab5α in LFA-1/ICAM-1-stimulated migrating PBL T-cells as compared with that of resting cells (Fig. 1G). Interestingly, the association of Rab5α with PKCε was not influenced by the levels of GTP on Rab5α because constitutively active GTP-bound Rab5α (Q79L mutant) and dominant-negative GDP-bound Rab5α (S34N mutant) both associated with the endogenous PKCε in migrating T-cells to similar extents (Fig. 1H). Hence, the association of Rab5α with PKCε is a nucleotide-independent interaction. Taken together, these studies confirm that PKCε localizes to endosomes in migrating T-cells and physically interacts with Rab5α after LFA-1/ICAM-1 stimulation.

PKCε Phosphorylates Rab5α on Threonine 7—We next investigated whether PKCε could phosphorylate Rab5α. An in vitro kinase assay was performed where active recombinant PKCε was incubated with a purified Rab5α-GST fusion protein in the presence of radio-labeled ATP ([32P]ATP). Incubation of recombinant PKCε with GST alone or with MBP in the presence of [32P]ATP was also used as negative or positive control, respectively. Incorporation of [32P]ATP into Rab5α, GST, or MBP was measured by SDS-PAGE followed by autoradiography. This analysis clearly indicated that PKCε was capable of phosphorylating Rab5α in vitro, whereas GST alone was not phosphorylated (Fig. 2A). In contrast, MBP was highly phosphorylated when incubated with PKCε in the presence of [32P]ATP (Fig. 2A).

Members of the PKC family generally target serine/threonine residues that lie within (S/T)(I/K/R), (K/R)(X)(S/T), or (L/R)(X)(S/T) motifs, where X indicates any amino acid (36). Using an oriented peptide library, Nishikawa et al. (37) also reported that PKCε and other novel PKC isozymes prefer hydrophobic amino acids at positions +2, +3, and +4. Based on these criteria, we identified three potential sites on human Rab5α, namely Thr-7, Ser-84, and Ser-123, that may be phosphorylated by PKCε (Fig. 2B). These three sites on human Rab5α are evolutionarily conserved, as they are present on Rab5α from all other species (supplemental Fig. S1). Thr-7 lies within the N terminus of Rab5α, a region previously shown to be critical for its functionality, whereas Ser-84 and Ser-123 fall within the highly conserved GTP/GDP binding switch regions of small GTPases (38–40).

We used a number of approaches to determine that Rab5α was phosphorylated on Thr-7 by PKCε and that phosphorylation of this site (but not at Ser-84 or Ser-123) was crucial for T-cell migration. First, we demonstrated that transfecting HuT-78 T-cells with a kinase-dead PKCε construct perturbed
PKCe-Rab5a-Rac1 Axis in T-cell Motility

A

PKCe-GFP

B

PKCe-cherry

Rab5a-GFP

Merge

C

Intensity

D

PKCe-cherry

Rab5a-GFP

Merge

E

PKCe-cherry 0s 2.44s 20.1s 28.9s

Rab5a-paGFP

F

PKCe

Rab5a

Merge

G

ICAM-1

IP-IgG  IP-Rab5a

PKCe

Rab5a

IP-Rab5a WB PKCe

H

IP-GFP

Rab5a-NT

Rab5a-Q76L

Rab5a-S33N

ICAM-1

PKCe

GFP

IP-GFP WB PKCe
their ability to migrate through ICAM-1-coated filters toward the chemokine SDF-1α in transwell chambers (Fig. 2C). Second, we generated mutant constructs of Rab5a by replacing Thr-7 (Rab5a-T7A), Ser-84 (Rab5a-S84A), or Ser-123 (Rab5a-S123A) residues with alanine, transfected the constructs into HuT-78 T-cells, and analyzed the ability of the cells to migrate toward SDF-1α through transwell filters coated with ICAM-1. Expression of the Rab5a-T7A construct in HuT-78 T-cells perturbed migration in comparison to cells transfected with wild-type Rab5a (Rab5a-WT), mutant Rab5a-T7A, or Rab5a-S123A plasmid constructs and analyzed in a trans-well migration assay. E, HuT-78 cells were transfected with wild-type Rab5a (Rab5a-WT), mutant Rab5a-T7A, or Rab5a-T7E plasmid constructs and analyzed using trans-well migration assay. Data of at least three independent experiments were normalized to respective control and are presented as the mean ± S.E., *p < 0.01.

FIGURE 2. PKCe-dependent phosphorylations of Rab5a and its involvement in T-cell motility. A, an in vitro kinase assay was performed with recombinant PKCe incubated with purified GST alone (control), MBP, or Rab5a-GST. A representative blot from three independent experiments is shown. B, three potential PKCe phosphorylation motifs (underlined) were identified in Rab5a amino acid sequence. The predicted serine/threonine residues (indicated in bold) are Thr-7, Ser-84, and Ser-123. C, HuT-78 T-cells were transfected with the control plasmid, wild-type (PKCe-WT), or the kinase-dead mutant of PKCe (PKCe-KD) expression vectors, and the migratory potential of cells was analyzed using trans-well migration assays. D, HuT-78 cells were transfected with wild-type Rab5a (Rab5a-WT) or mutant Rab5a-T7A, Rab5a-S84A, or Rab5a-S123A plasmid constructs and analyzed in a trans-well migration assay. E, HuT-78 cells were transfected with wild-type Rab5a (Rab5a-WT), mutant Rab5a-T7A, or Rab5a-T7E plasmid constructs and analyzed using trans-well migration assay. Data of at least three independent experiments were normalized to respective control and are presented as the mean ± S.E., *p < 0.01.
PKCe-Rab5a-Rac1 Axis in T-cell Motility

A

Anti-phospho-Rab5a(Thr-7) antibody dilution

1:100 1:200 1:400 1:800 1:1600 1:3200 1:6400 1:12800

(p)T7 peptide + purified antibody

(OH)T7 peptide + purified antibody

(p)T7 peptide + pre-immune serum

Mean OD 560

0 1 2 3 4

B

(p)T7 peptide + purified antibody

(OH)T7 peptide + purified antibody

IB: Rab5a(p)T7

IB: Blocked Rab5a(p)T7

1:1 1:10 1:100 1:1000 1:1

C

PKCe

pRab5a(T7)

GST

Rab5a

Rab5a-WT

Rab5a-T7A

Relative densitometric values

[pRab5a(T7)]: mean ± S.E.; *; p < 0.01

FIGURE 3. Characterization of anti-phospho Rab5a(Thr-7) antibody. A, serial dilutions (1/100 to 1/12,800) of the affinity-purified anti-phosphoRab5a(Thr-7) antibody was titrated against phospho Thr-7-containingpeptide (p)T7 peptide and analyzed by ELISA. Preimmune serum and non-phospho(OH)T7-containing peptides (OH)T7 peptide were used as controls. B, various dilutions of the purified pRab5a(T7)-antibody ranging from 1:1 to 1:1000 were analyzed by immuno dot-blot assay (IB) using (p)T7 peptide. (OH)T7 peptide or pRab5a(T7)-antibody preincubated with the Thr(P)-7 peptide was used as the control. C, purified GST, GST-conjugated wild-type Rab5a (Rab5a WT), or mutant Rab5a-T7A protein was separately incubated in the presence or absence of PKCe. The products were analyzed by Western blot using the affinity-purified anti-pRab5a(Thr-7) or anti-Rab5a (loading control). Densitometry analysis of the Western blots was performed and presented (mean ± S.E.). *; p < 0.01. Data represents three independent experiments.

verified through titration experiments using phospho-T7 containing peptides as analyzed by ELISA (Fig. 3A) and immuno dot-blot assays (Fig. 3B). Furthermore, purified Rab5a-WT-GST, Rab5a-T7A-GST, or GST proteins were incubated in the presence or absence of PKCe and analyzed by Western blot using the anti-phospho-Rab5a(Thr-7) antibody. PKCe-induced phosphorylation of Rab5a was detected only in the Rab5a-WT-GST sample but not in Rab5a-T7A-GST or GST proteins (Fig. 3C).

Next, we examined Rab5a phosphorylation at Thr-7 induced via LFA-1 signaling in cells. PBL T-cells were incubated for a range of time points on an immobilized ICAM-1-coated surface, lysed, and analyzed by Western immunoblotting using the anti-phospho-Rab5a(Thr-7) antiserum. Thr-7 phosphorylation of Rab5a was barely detectable in resting T-cells, which was significantly increased after LFA-1/ICAM-1 stimulation, reaching maximum levels within the time frame of 10–30 min, and then declined to basal level at 2 h (Fig. 4A). Similar results were obtained when PBL T-cells were stimulated with either SDF-1α or with the PKC activator phorbol 12-myristate 13-acetate (PMA; Fig. 4B), further suggesting that Rab5a Thr-7 phosphorylation was PKC-dependent.

To confirm the role of PKC in LFA-1-induced Rab5a Thr-7 phosphorylation, we pretreated PBL T-cells for 30 min with a 10 μM concentration of the PKC inhibitor bisindolylmaleimide I. Western immunoblot analysis of T-cells stimulated with or without LFA-1/ICAM-1 showed that bisindolylmaleimide I pretreatment significantly inhibited Rab5a Thr-7 phosphorylation (Fig. 4C). Furthermore, specific knockdown (>75%) of PKCe in T-cells using a siRNA approach (Fig. 4D) significantly inhibited LFA-1-mediated Thr-7 phosphorylation of Rab5a (Fig. 4E). These data clearly indicate that PKCe is the specific PKC isoform mediating Thr-7 phosphorylation of Rab5a.

T7A Mutation Partially Inhibits Rab5a Targeting to Endosomal Compartments—Because mutation of Thr-7 to alanine on Rab5a affected T-cell migration, we investigated the mechanistic basis of this migratory defect. Truncation mutants of Rab5a lacking the first 14–22 N-terminal residues have been shown to disrupt Rab5a functionalities (40–43). However, these mutants remain prenylated, membrane-associated, and functionally interact with guanine nucleotides (40, 41, 44). In addition, functional aspects of protein folding and the crystal structure also remain unaffected (44, 45). Therefore, the T7A mutation would not be predicted to interfere with the GTPase activity of Rab5a and should not affect its three-dimensional conformation. Hence, we investigated whether mutation of Thr-7 on Rab5a affected its subcellular distribution. Expression of wild-type Rab5a-GFP in HuT-78 T-cells demonstrated punctate and globular structures evenly distributed throughout the whole cell body, center, and the periphery (Fig. 5A). Although the expression pattern of Rab5a-S84A-GFP or Rab5a-Ser-123-GFP resembled that of wild type, Rab5a-T7A-GFP appeared to be more cytosolic and less punctate throughout the uropod (Fig. 5A). This distinctive pattern of Rab5a distribution was also verified by co-expressing both wild-type Rab5a-GFP and mutant Rab5a-T7A-cherry constructs in T-cells (data not shown). Some punctate Rab5a-T7A-GFP-associated structures were still associated with endocytic vesicles labeled by internalized Alexa Fluor® 568-transferrin-conjugate (Fig. 5B). These studies suggest that phosphorylation of the Thr-7 site partially influences Rab5a subcellular localization and that its mutation does not totally disrupt Rab5a targeting to the transferrin receptor-positive endosomal compartment. Consistent with the finding that the T7E mutant did not affect T-cell migration as much as the T7A mutant (Fig. 2E), the phos-
phomimetic Rab5a-T7E-GFP transfected cells expressed many small vesicular structures throughout the whole cell body co-localizing with transferrin and resembling the Rab5a-WT-like expression pattern (Fig. 5B).

We next employed photoactivation in combination with time-lapse microscopy to directly visualize and compare the dynamics of wild-type Rab5a and Rab5a-T7A at the centrosomal region in transfected cells. After transfection with Rab5a-paGFP or Rab5a-T7A-paGFP constructs, HuT-78 cells were seeded on coverslips coated with a cross-linking anti-LFA-1 antibody for 1 h to allow extended polarization. A pulse of 405-nm laser light aimed around the centrosomal region of the polarized cell resulted in immediate photoactivation of paGFP fusion proteins within the confines of this area. In cells expressing wild-type Rab5a-paGFP, a patch of photoactivated endosomes at the centrosomal region could be seen to rapidly disperse from this area over the following 5 min (Fig. 6A, supplemental Video S4). Conversely, the dispersal rate of photoactivated Rab5a-T7A from the centrosomal region was relatively slow (Fig. 6A, supplemental Video S5). Quantifying a total of 30 Rab5a-paGFP or 40 Rab5a-T7A-paGFP-expressing cells from 3 independent experiments showed that the dispersal rate of Rab5a in phospho-deficient mutant cells was significantly slower than wild-type Rab5a-paGFP-expressing cells in the first 100 s (Fig. 6, B and C). When the dynamics of the photoactivated population of Rab5a-paGFP was tracked for a longer period of time, fluorescence was rapidly dimmed to background levels (Fig. 6C), and this was also accompanied by the appearance of budding vesicles at the leading edge of the polarized cell. This observation was in accordance with a previous study suggesting the half-life of Rab5a-positive early endosomes to be in the range of 2 min (47). In a parallel experiment, we also observed that a majority (80%) of Rab5a-paGFP-expressing vesicles continuously redistributed from large structures in the cell centrosomal region, to allow the recycling of Rab5a onto budding endosomes at the leading edge (Fig. 7A, Supplemental Video S6). On the other hand, although the pool of Rab5a-T7A-associated endosomes gradually lost its fluorescence signal (Fig. 7A, supplemental Video S7), the number of cells with emerging and budding vesicles at the leading edge was significantly reduced to 33% (Fig. 7B).

**Rab5aT7A Disrupts Actin Rearrangement during Migration**—By means of time-lapse microscopy, we observed that T-cells co-expressing Rab5a-GFP and Lifeact-ruby, a 17-amino acid peptide that stains filamentous actin networks (48), displayed highly motile lamellipodia structures extending in a vibrant umbrella-shaped fast protrusion followed almost immediately by active ruffling and retraction in a highly dynamic cycle in the course of moving forward (supplemental Video S8). We investigated if loss of Thr-7 phosphorylation of Rab5a could interfere
with actin remodeling at lamellipodia during migration. For this purpose, HuT-78 T-cells expressing wild-type Rab5a, Rab5a-T7A, or Rab5a-T7E were incubated on a cross-linked anti-LFA-1 antibody for 2 h and fixed. These cells were then stained with phalloidin-TRITC, which binds filamentous actin, and Hoechst, which stains the nucleus (Fig. 8A). Looking closely at the leading edge, we observed that in 25% of cells expressing Rab5a-T7A, filamentous actin bundles were disrupted and condensed around the plasma membrane accompanied by asymmetrically shaped lamellipodia and/or irregular ruffling (Fig. 8B, supplemental Video S9). Such disrupted lamellipodia phenotypes were present only in 12% of the wild-type Rab5a or 5% of the Rab5a-T7E-expressing and -migrating T-cells (Fig. 8B). As migrating T-cells rapidly change shape and the lamellipodia are highly dynamic, it was difficult to compare and quantify cell-to-cell differences. Therefore, these measurements reflect gross differences observed at the lamellipodia of only those crawling T-cells that displayed typical amoeboid phenotypes.

We employed objective quantification of cytoskeletal change using a previously reported cell-based method of high content analysis that entailed population analysis of the morphology of cells expressing GFP fusion protein (34). Utilizing this approach, we observed that although cells expressing the Rab5a-T7A-GFP mutant were polarized after anti-LFA-1 stimulation, the cell polarization index based on the actin cytoskeleton was significantly reduced ($p = 0.003$) as compared with cells expressing wild-type Rab5a-GFP or Rab5a-T7E-GFP (Fig. 8C). These results suggest that within the population of Rab5a-T7A-GFP-expressing T-cells, a relatively higher proportion of cells with less elongated or irregularly shaped cytoskeleton is present, implying that they have deficiencies in their ability to reorganize their cytoskeleton in response to a migratory stimulus.

A number of previous studies have established Rac1 as the main driving force in the formation of actin protrusions that are essential for cell migration (47, 48). A crucial role of Rab5a in the activation of Rac1 has also been demonstrated (17, 18).
These findings prompted us to investigate if Thr-7 phosphorylation of Rab5a is important for Rac1 activation in migrating T-cells. To address this question, we transfected HuT-78 T-cells with plasmid constructs encoding wild-type, T7A, or T7E forms of Rab5a, and then cells were incubated on ICAM-1 to trigger LFA-1-induced migration. The levels of Rac1 activation in these cells were determined by a PBD binding assay. Consistent with the above observed role of Rab5a in actin remodeling and T-cell polarity, significantly high Rac1 activity (∼2-fold) was detected in T-cells expressing Rab5a-WT or Rab5a-T7E after LFA-1 stimulation in this migration-triggering model system (Fig. 8D). In contrast, expression of Rab5a-T7A perturbed this increase in Rac1 activation after LFA-1/ICAM-1 stimulation (Fig. 8D). Similar results were observed when these cells were stimulated with SDF-1α (data not shown). Taken together, these results clearly demonstrate that PKCε-mediated Thr-7 phosphorylation of Rab5a is a necessary step in the regulation of T-cell cytoskeletal reorganization and migration.

**DISCUSSION**

In this study we establish a novel mechanism involving a PKCε-Rab5a-Rac1 axis in the regulation of actin cytoskeletal remodeling and T-cell migration. Specifically, we uncover a key biological function of a previously undescribed phosphorylation site on Rab5a at Thr-7. We show that Rab5a interacts with PKCε in migrating T-cells in a nucleotide-independent manner and that Rab5a is phosphorylated on Thr-7 by PKCε in response to integrin or chemokine stimulation. We also demonstrate that phosphorylation of this site on Rab5a is required for T-cell migration, as ectopic expression of a phospho-deficient T7A mutant of Rab5a resulted in a significant reduction in the ability of T-cells to migrate across ICAM-1-coated membranes toward SDF-1α. This defect in T-cell motility appears to be due to inefficient trafficking of endosomes toward the leading edge of the cell and correlates with impaired Rac1 activation and actin cytoskeletal remodeling in the lamellipodia, events that are crucial for cell migration.

Rab5 exists as three closely related molecules (Rab5a, Rab5b, and Rab5c) in mammalian cells (40, 49), sharing 88.7% of protein sequence identity (supplemental Fig. S2). Although there is evidence to suggest that Rab5a is involved in chemokine receptor recycling, cell motility, actin organization, Rac distribution, and lamellipodia formation in various cell types (14, 16, 17, 50, 51), its role in the process of T-cell migration has not previously been reported. During migration, the leading edge of the T-cell actively produces filamentous actin-rich lamellipodia that likely facilitate the interaction of LFA-1 with its counter-ligand ICAM-1 on endothelial cells (52). In doing so, T-cells search for a suitable extravasation site on the venular surface that facilitates rapid directional changes that are characteristic of migrat...
ing T-cells. The LFA-1 signal in T-cells has been shown to transiently activate Rac1 and induce actin polymerization (53). Here, we found that signaling through LFA-1 or CXCR4 results in Thr-7 phosphorylation of Rab5a, which in turn is critical for Rac1 activation, subsequent actin remodeling, and T-cell motility. This is consistent with previous findings where Rab5 was shown to regulate trafficking of Rac and its guanine nucleotide exchange factor in T-cell lymphoma invasion and metastasis (16, 17, 54). Whether Tiam1 or other guanine nucleotide exchange factors regulate Rac1 activation downstream of Rab5a or PKC requires further investigation.

PKC isoforms have been implicated in T-cell signal transduction including the regulation of migration. For example, our group has demonstrated a role for PKCβ and PKCδ in regulating the microtubule cytoskeleton and active T-cell locomotion induced by LFA-1 integrin or CD44 receptors (21, 55, 56). PKCδ has also been reported to induce integrin-mediated Thr-566 phosphorylation of phospholipase D and direct cell spreading and migration in COS-7 cells (57). In addition, several studies have suggested the involvement of PKCe in cell migration in many other model systems. Overexpression of PKCe resulted in a highly motile and invasive phenotype in various cancer models (58, 59), and its disruption caused inactivation of Rho family GTPases (60). It has also been shown that PKCe can bind directly to both filamentous and globular forms of actin via its actin binding motif (61, 62) or interact indirectly via integrins and scaffolding proteins such as receptors for activated protein kinase C (also known as RACK) (63, 64). In murine embryonic fibroblasts, PKCe was shown to phosphorylate vimentin and regulate β1 integrin recycling contributing to cell motility (24, 25). Thus, our identification of Rab5a phosphorylation on the N terminus at Thr-7 represents a novel regulatory mechanism by which PKCe integrates extracellular signals via integrins and chemokine receptors and coordinates signal transduction in T-cell migration. Of note, our studies do not exclude the possibility that PKCe may also phosphorylate other Ser/Thr residues on Rab5a that could contribute to diverse lymphocyte functions.

The GTP/GDP binding switch regions of Rab proteins are the main features for GTPase activity and are the most conserved structures among family members (38, 39, 65). On the other hand, the N- and C-terminal regions of Rab proteins are variable with the most divergent sequences lying at the extreme N terminus (40, 49). Although the isoprenylated C-terminal motif is required for membrane targeting (66, 67), functions of the extreme N-terminal region are still unknown. Given that deletion of the first 14–22 amino acid residues at the N-terminal domain alone is enough to disrupt Rab5a functionalities (42), the N-terminal region is not only an important element for Rab5a function; it might also act as the domain that specifies isoform functionality. Taking it a step further, we have shown that when phosphorylation of Rab5a on the N-terminal Thr-7 site is disrupted, T-cell migration through ICAM-1-coated...
membranes toward SDF-1α is significantly repressed. By replacing Thr-7 with glutamic acid (T7E), which mimics constitutive phosphorylation, we were able to show that this mutant also partially inhibits T-cell migration. Although we do not have a clear explanation for this observation, it is possible that Rab5α dynamically cycles between phosphorylated and dephosphorylated states to function in T-cell migration. This is consistent with the observed dynamic interactions between PKCε and Rab5α (Fig. 1E, supplemental Video S1). It may be that replacement of Thr-7 with T7E could alter the structure or folding of the Rab5α protein necessary for its function in T-cell migration. Future studies on the kinetics of Rab5α recycling in the context of phosphorylation events in T-cells will further clarify this.

The level of Rab5 dynamically fluctuates on individual early endosomes linked by fusion and fission events into a network in time. Although the bulk of early endosomes are not subjected to rapid turnover, the half-life of Rab5-containing early endosomes has been estimated to be in the range of 2 min (46). Moreover, the amount of Rab5 associated with a given endosome varies considerably over time (46). Utilizing a photoactivation-based imaging approach to track the dynamics of Rab5α after LFA-1 stimulation in motile T-cells, we observed that Rab5α-paGFP-expressing vesicles continuously redistribute from the cell centrosomal region (the site of PKCε-Rab5α interaction) to the leading edge. However, the dispersal rate of the phospho-deficient mutant Rab5α-T7A-paGFP was significantly slower than the wild-type Rab5α-paGFP in the first 100 s,
and fluorescence dimmed to almost background levels by 250 s. Thus, the dynamic turnover of Rab5a seems to be essential to allow its recycling onto budding endosomes in the cell periphery. In support of this concept, earlier studies suggest that the driving force for cell migration is a polarized endocytic/exocytic cycle that delivers adhesion receptors and proteins from the rear of the cell after focal adhesion disassembly to the leading edge of the migrating cell for extension and substrate attachment. Such events are required for driving the cell forward (68, 69). This could also be due to extraction of Rab5a from the membrane via guanine nucleotide dissociation inhibitors (15, 70, 71). Based on these observations, it can be speculated that Thr-7 phosphorylation may be important in the regulation of GD1-mediated extraction of Rab5a from the endosomal membrane.

The recycling of integrins, which involves their endocytosis from cell surface and recycling back to the plasma membrane, is important for their activities as well as for the regulation of their surface levels (72–74). Previous studies have shown clathrin-dependent or -independent endocytosis of integrins at the leading edge of migrating cells (75). In neutrophils, LFA-1 is associated with detergent-resistant membrane microdomains and is internalized in a filipin-sensitive and dynamin-dependent manner (76). Consistent with these findings, we observed that intracellular vesicles expressing Rab5a-T7A-paGFP were less efficient in redistributing from the centrosomal region to the leading edge. Another Rab5 GTPase family member, Rab5c, has recently been reported to enhance β1-integrin recycling in EGF-induced cancer invasion (74). In addition, LFA-1 recycling has been shown to be independently regulated by Rap2 GTPase as well as Gα11 proteins in T-cells (72, 73). Surprisingly, internalization of LFA-1 was not found to be inhibited by a dominant-negative Rab5 construct (N133I) in a Chinese hamster ovary cell model (76). What becomes obvious from all of these studies is that different intracellular pools exist for Rab5a, and its Thr-7 phosphorylation and redistribution may be an important mechanism in the process of cytoskeletal remodeling and lymphocyte migration. It should be noted that although we examined the role of Rab5a mutants in T-cells that still express endogenous Rab5a, it would be interesting to examine these mutants in cells where endogenous Rab5a has been depleted. However, performing such experiments is technically very challenging in T-cells.

In summary, this study provides a new mechanistic insight into the regulation of T-cell migration by a PKCe-Rab5a-Rac1 axis. PKCe-dependent phosphorylation of Rab5a on Thr-7 regulates its intracellular dynamics and Rac1 activation, which in turn controls actin cytoskeletal remodeling at the lamellipodia and cell migration (Fig. 9). Based on our data, we propose that the Thr-7 phosphorylation of Rab5a might be a missing piece of the structure-function puzzle of this 24-kDa protein and hence may provide new information regarding downstream pathways. Given that Rab5a is central to many fundamental cell processes, this new finding has profound biological implications across cellular organisms. Our results also highlight the concept that LFA-1 not only acts as an adhesion receptor, a role for which it has been known for many years, but also plays an important role in downstream protein-protein interactions and consequent signaling.

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