Inside the Crawling T Cell: Leukocyte Function-Associated Antigen-1 Cross-Linking Is Associated with Microtubule-Directed Translocation of Protein Kinase C Isoenzymes β(I) and δ[1]

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T cells activated via integrin receptors can polarize and start crawling locomotion with repeated cycles of cytoskeletal reassembly processes, many of which depend on phosphorylation. We demonstrate that protein kinase C (PKC) activation represents an essential event in induction of active T cell motility. We find that in crawling T cells triggered via cross-linking of integrin LFA-1 two PKC isoenzymes, β(I) and δ, are targeted to the cytoskeleton with specific localization corresponding to the microtubule-organizing center (MTOC) and microtubules, as detected by immunocytochemistry and immunoblotting. Clustering of LFA-1 associated with its signaling function also occurs at the membrane sites adjacent to the MTOC. We further show that cells of a PKC-β-deficient clone derived from parental PKC-β-expressing T cell line can neither crawl nor develop a polarized microtubule array upon integrin cross-linking. However, their adhesion and formation of actin-based pseudopodia remain unaffected. Our data demonstrate the critical importance of the microtubule cytoskeleton in T cell locomotion and suggest a novel microtubule-directed intracellular signaling pathway mediated by integrins and involving two distinctive PKC isoforms. ***The Journal of Immunology*****, 1998, 161: 6487–6495.**

Lymphocyte migration and homing requires a series of ligand-receptor interactions involving adhesion molecules of the integrin family. These transmembrane proteins connect the extracellular matrix with the cell interior both physically, being linked to the cortical cytoskeleton, and functionally, serving as bi-directional signal transducers. Intracellular signaling networks are based largely on phosphorylation-dependent cascades integrated by small m.w. GTPases (rho, rac, cdc42), tyrosine kinases, focal adhesion kinase (pp125^{FAK}), phospholipase C-γ, and protein kinase C (PKC)[3] (1). However, the exact sequence of integrin-mediated signaling events resulting in cytoskeletal rearrangements and cell locomotion is not well defined.

Cross-linking of cell surface adhesion receptors by mAbs mimicking to a certain extent multivalent interactions with natural ligands (2) has been successfully used as a model to study intracellular signaling processes mediated by integrins and CD44 (3, 4). Ab-induced effects in this case are often judged by homotypic aggregation (4), clustering of cytoskeletal and signaling proteins (2), or cell motile characteristics (3). To investigate LFA-1-mediated signaling in T cells, we gave preference to the reporter system based on the induction of cell locomotion, as potentially more closely related to physiological phenomena taking place, for instance, at the stage of cell extravasation. In the present study, we used the model described earlier (5) in which cells of the human T lymphoma line HUT-78 or activated human peripheral blood T lymphocytes (PBTL) were exposed to a triggering signal via LFA-1 by immobilized mAb (mAb(i)) specific for its α_L-chain. In this system, T cells adopted a locomotion-associated phenotype on anti-LFA-1 mAb. Preactivation via TCR-CD3 complex or phorbol ester (PMA) treatment was required for the development of motile phenotype in normal PBTL (5) and represents an essential step in LFA-1-mediated lymphocyte adhesion (6).

PMA is a potent activator of the intracellular phosphorylation enzymes of the PKC family. It includes the growing number of isoenzymes grouped as follows: classical (Ca^{2+}-dependent and activated by diacylglycerol and PMA (α, β(I), β(II), and γ)), novel (Ca^{2+}-independent (δ, ϵ, η, θ, μ)), and atypical (phospholipid- and Ca^{2+}-independent (ζ, λ, ι)). PKC-β has been demonstrated to undergo translocation to the plasma membrane from the cytosolic pool and cytoplasmic vesicles containing β_2 integrins in response to phorbol ester treatment (7). This isoform also has been reported to colocalize with microtubule-associated proteins and to be physically linked with the actin cytoskeleton (7, 8). Redistribution of novel PKC isoforms δ and ϵ between cytosolic and cytoskeletal fractions can be modulated by PKC agonists and specific inhibitors (9, 10). We herein demonstrate that cross-linking of LFA-1 resulted in specific translocation of PKC-β(I) and δ isoforms to the cytoskeleton with a pattern consistent with microtubule-organizing center (MTOC) and microtubules. We analyzed this phenomenon in conjunction with the other LFA-1-induced intracellular changes from the point of view of its impact on T cell locomotory behavior.

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[3] Abbreviations used in the paper: PKC, protein kinase C; HUT-78, T lymphoma cell line HUT-78; PBTL, peripheral blood T cells; mAb(i), immobilized mAbs; MTOC, microrubule-organizing center; F-actin, filamentous actin; TRITC, tetramethylrhodamine isothiocyanate.

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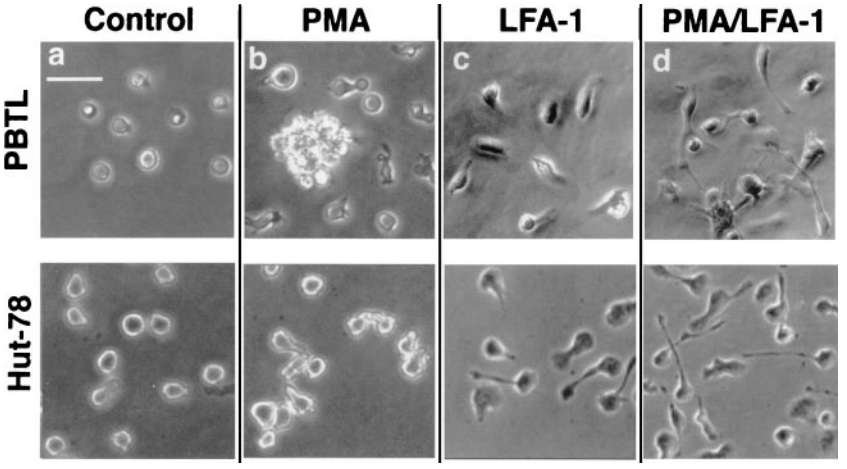

FIGURE 1. Phenotypic changes in PBTL and HUT-78 induced by PKC activation and LFA-1 cross-linking. *Upper panel*, PBTL. *Lower panel*, HUT-78. *a*, Cells exposed to isotype-matched control IgG. *b*, Cells activated with PMA on control IgG. Note that the cells in *a* and *b* are not adherent to substrate in comparison to *c* and *d. c*, Cells activated via LFA-1 cross-linking. *d*, Cells preactivated with PMA and subsequently on mAb to LFA-1. Scale bar: 20 μm. Consistent cell phenotypes were reproduced in >10 independent experiments. Ab clones SPV-L7 and YTH-81.5 (not shown) were equally potent for inducing cytoskeletal changes in PBTL and HUT-78.

Materials and Methods

Cells and cell culture

Normal human PBTL, isolated as described (5), were preactivated by treatment with 25 ng/ml PMA (Sigma, St. Louis, MO) for 48–72 h at 37°C unless specifically indicated otherwise in the text and figure legends. Human T lymphoma cell line HUT-78 (American Type Culture Collection, Manassas, VA) were used nontreated or preactivated by PMA in the same concentration for 60 min in several experiments. RPMI 1640 culture medium on HEPES buffer (Life Technologies, Paisley, U.K.) supplemented with antibiotics and 10% FBS was used in all experiments, unless stated otherwise in the text.

Cell adhesion, motility, and transmigration

HUT-78 or activated PBTL (10–20 $\times$ 10^3/well) were added to 8-well Permanox plastic chamber slides (Nunc, Naperville, IL) coated with mAb to α-chain of LFA-1, clone SPV-L7 (Sanbio, Uden, The Netherlands) at 1.75 μg/ml as described (5). Control chambers were treated similarly with isotype-matched murine IgG (Dako, Bucks, U.K.). In several experiments, the chambers were coated with another locomotion-inducing mAb to human α-LFA-1 (clone YTH-81.5) at 5 μg/ml (Serotec, Oxford, U.K.). These mAbs both proved to be equally potent in inducing cytoskeletal changes in T cells. Anti-LFA-1 mAb MEM-83 were used for the same purposes at 2 μg/ml. Chimeric ICAM-1-Fc fusion protein (kindly provided by A. Craig, Oxford, U.K.) was used in motility studies at 10 μg/ml coating concentration, and cellular fibronectin from human foreskin fibroblasts (Sigma) was used at 25 μg/ml. Cell motility on different integrin ligands was also assessed using 3-μm membrane pore filters precoated with fibronectin, laminin, and collagen type I and IV by the manufacturer (Becton Dickinson Labware, Bedford, MA).

Broad spectrum kinase inhibitor staurosporine and selective PKC-α and -β inhibitor Go6976 used in functional studies were purchased from Calbiochem (Nottingham, U.K.). After 4 h of incubation in culture medium or under specific experimental conditions as described in the figure legends, unattached cells were removed by triple gentle washing of wells with warmed culture medium. The fraction of adherent cells was calculated as a percentage of cells remaining attached to substrate from the initial cell count (before washing the wells). Motility was assessed by estimating the ratio of cells undergoing cytoskeletal rearrangements and formation of uropods (locomotion-associated phenotype, Figs. 1*d* and 2, *a–f*) of the total number of adherent cells per microscopic field. At least five randomly chosen fields at $\times$400 magnification were analyzed for each experimental condition. Transmigration experiments were performed in a modified Boyden chamber assay using polyethylene terephtalate track-etched membrane filters with 3 μm pore size (Becton Dickinson Labware, Bedford, MA) in 24-well plates. Anti-LFA-1 mAb or control IgG were immobilized on the upper side of the filters in the same way as for chamber slides (5). The lower side of the filters was left uncoated. Activated PBTL (500 μl of suspension at 10^6 cells/ml) were added to the filter chambers, while the lower compartments were filled with culture medium alone or supplemented with 50 ng/ml human recombinant RANTES (Sigma) and incubated overnight at 37°C. After removing the filter chambers, transmigrated cells from the bottom of the plate wells were collected by pipetting and counted in a haemocytometer. Four grids corresponding to 0.1 mm^3 suspension volume were averaged to estimate cell number in each well. Mean values for each experimental condition were obtained from six wells.

Time-lapse video recording and image analysis

Nikon Diaphot inverted microscope (Nikon Europe, Badhoevedozp, The Netherlands) with CCD video camera (Sony Corporation, Tokyo, Japan)

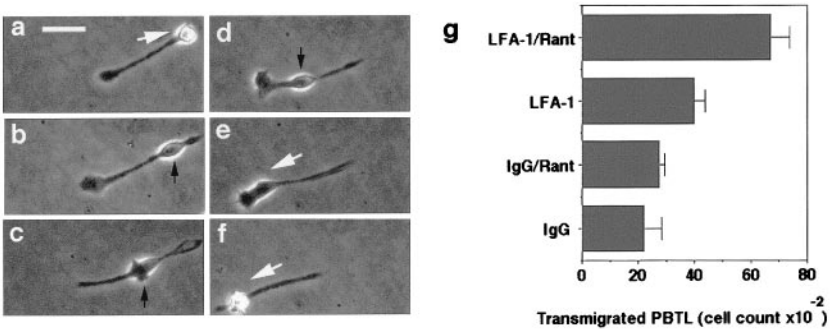

FIGURE 2. T cells triggered by cross-linking of integrin LFA-1 display crawling locomotion. *a–f*, Consecutive phase-contrast images of a single representative activated PBTL on mAb(i) to LFA-1 α_L. Frame intervals are 36 min. *a*, Direction of migration of the T lymphocyte is diagonal, left to right. *b–d*, Cell remains static relative to substrate and translocation of the nucleus precedes change in direction of migration (black arrows). *e–f*, Active locomotion in the right to left diagonal. White arrows indicate the direction of cell migration. Scale bar: 20 μm. *g*, Directed chemotaxis of preactivated PBTL through 3-μm filter pores in modified Boyden chambers. IgG, PBTL on control IgG immobilized on the upper side of the filter. RANTES, Cells exposed to isotype-matched Ig in the presence of 50 ng/ml RANTES in the lower chamber. LFA-1, PBTL triggered by mAb(i) to LFA-1 without chemotactic gradient. LFA-1/Rant, PBTL triggered by mAb(i) to LFA-1 in the presence of RANTES gradient. Data reflect mean $\pm$ SD of transmigrated cell counts from 6-plate wells. The experiment was repeated three times with similar results.

was used for image acquisition, phase contrast observations, and microphotographs. Analysis of acquired video and photographic images was performed on a Macintosh computer using the National Institute of Health Image program (developed at the U.S. National Institutes of Health and available on the Internet).

Bead preparation

Polystyrene beads (0.8 μm) (Sigma) coated with anti-LFA-1 mAb as described for chamber slides (5) were added to the chambers (1000:1 bead: cell ratio) when the cells had established a locomotory phenotype (60 min after contact with mAb(i). Following 15 min incubation, unbound particles were removed by gentle washing and refilling of the chambers with warmed culture medium.

Immunofluorescence microscopy

The slides with attached cells were fixed/permeabilized in acetone at −20°C. Ab to PKC isoforms α, δ, ϵ, η, θ, and ζ (Research & Diagnostic Abs, Berkeley, CA) and β(I) and β(II) (Sigma) were used with FITC-labeled secondary Ab (Sigma). Ab blocked with relevant peptide Ag were used for negative control staining. The tetramethylrhodamine isothiocyanate (TRITC) conjugate of phalloidin (Sigma) was used to stain filamentous actin (F-actin); mAb to LFA-1 (Sanbio), vimentin (Dako), and α-tubulin were used with TRITC-labeled secondary affinity-purified Ab (both from Sigma). Light and fluorescent microscopy and microphotography were performed on a Leica photomicroscope (Leica Microscopy Systems, Heerbrugg, Switzerland) using Kodak Panther 1600 or Kodak Elite II-400 reversible films (Eastman Kodak, Rochester, NY). Equal exposure times were used to photograph cells with specific and negative control staining.

Western blot analysis

To analyze distribution of PKC isoforms between the subcellular fractions, HUT-78 either kept in suspension or attached to plastic via mAb(i) were lyzed on ice in buffer A (20 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 1 mM PMSF, and 10 mg/ml leupeptin (all reagents from Sigma)), sonicated for 5 s and spun down at 600 × *g* to remove the nuclei and unlyzed cells. After centrifugation at 100,000 × *g* for 10 min the resulting supernatant was designated as the cytosolic fraction. The pellet was resuspended in buffer B (20 mM Tris-HCl, pH 7.5, containing 1% (w/v) Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and protease inhibitors), and centrifuged at 15,000 × *g* for 30 min. The supernatant was designated as the detergent-soluble membrane fraction. The pellet representing the detergent-resistant cytoskeletal fraction was dissolved in boiling buffer C (20 mM Tris-HCl, pH 7.5, 1% SDS, 150 mM NaCl, 1 mM EGTA, and 1 mM EDTA). Equal amounts of proteins were separated on a 10% SDS-polyacrylamide gel, electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), probed with mAb to PKC isoforms β and δ (Transduction Laboratories, Lexington, KY) or α-tubulin (Sigma), and visualized with Phototope-horseradish peroxidase detection system (New England Biolabs, Hertfordshire, U.K.). Densitometry of the blots was performed using National Institute of Health Image software. In a number of control experiments, similar results were reproduced using rabbit polyclonal Ab to PKC-β(I) (Santa Cruz Biotechnology, Santa Cruz, CA) and sheep Ab to PKC-δ, which were a gift from J. Lord (Department of Immunology, University of Birmingham, U.K.).

Results

Locomotory phenotype in T cells activated via LFA-1

HUT-78 and preactivated PBTL (both originally nonadherent, Fig. 1*a*) exposed to a triggering signal from anti-LFA-1 mAb(i) started spreading and subsequently underwent dramatic cytoskeletal changes resulting in a polarized phenotype with long cytoplasmic projections (up to 50–60 μm) over 60 min after the beginning of the incubation (Fig. 1*d*). Stimulation with PMA alone was not sufficient to induce these changes in either of cell types (Fig. 1*b*), but it enhanced homotypic aggregation of PBTL. Pretreatment with phorbol ester was necessary for adhesion and the development of cytoskeletal rearrangements upon LFA-1 cross-linking in PBTL, but not in HUT-78 (Fig. 1, *c* and *d*). As seen from the time-lapse video images (Fig. 2, *a–f*), the acquisition of this phenotype was directly associated with active cell body translocation, while cytoplasmic processes were represented by extended trailing cell tails (or uropods). Characteristic migratory phenotype was induced in T cells by anti-LFA-1 mAb(i) clones SPV-L7 and YTH-81.5, but not by mAb(i) to a number of other abundant cell surface proteins including CD3, CD43, and MHC class I (data not shown) or mAb to LFA-1 clone MEM-83 (5). Specific properties of various LFA-1-binding mAb in respect to cell locomotion might be dependent on the differences in the functional significance of the recognized epitopes (11) or their binding affinity (12). This question has been further addressed in this study (see below).

LFA-1 cross-linking augments chemokine-directed T cell locomotion

Locomotory T cells triggered via LFA-1 mAb(i) in the absence of obvious chemotactic gradient produced movements in an apparently random manner (Fig. 2, *a–f* and Fig. 3, *a–c*), often undergoing a complete 180° change in the direction of migration. We next examined whether LFA-1 cross-linking could affect T cell migration in response to the chemokine RANTES. We used a modified Boyden chamber assay with small, relative to PBTL, diameter pore size (3 μm), where mAb were immobilized on the upper side of the filter and human recombinant RANTES (50 ng/ml) was added to the culture medium in the lower chamber compartment. A selected concentration of the chemokine was established as optimal in preliminary experiments. As shown in the Fig. 2*g*, transmigration of PBTL triggered by mAb(i) to LFA-1 was significantly enhanced in the presence of the chemotactic gradient of RANTES in comparison to control IgG (with or without the chemoattractant). A relatively high rate of activated PBTL transmigration on mAb(i) without RANTES is evidently due to increased background cell locomotory potential.

T cell crawling locomotion involves dynamic redistribution and clustering of LFA-1 on the cell surface

Dynamic redistribution of adhesion receptors in locomotory cells was demonstrated using polystyrene beads coupled to anti-LFA-1 mAb (Fig. 3, *a–c*). The mAb-coated beads added to HUT-78 initially underwent centripetal migration away from the leading edge toward the rear of the nucleus over the MTOC (Fig. 3*a* and 4*c*, *lower panel*). Here they formed a characteristic "necklace" of aggregated particles (Fig. 3*a*) reflecting the site of integrin clustering and were subsequently redistributing in accordance with cyclic cytoskeletal changes. LFA-1 aggregation was not induced by the beads themselves, as shown by immunostaining of HUT-78 in bead-free conditions (Fig. 3*d*) and represented a specific step in cell migration, because it had not been registered using the beads coated with isotype-matched IgG or mAb to CD3 (data not shown).

A typical sequence of events reflecting a "successful" cell movement (resulting in a net translocation of the cell body to a new position) involved formation of the leading lamella (Figs. 2*d* and *e* and 3*a*), translocation of the nucleus termed as "nucleokinesis" (13) (Fig. 2, *b–d*), and extension of the trailing tail (uropod) accompanied by rearward movement of deaggregated integrins, as indicated by LFA-1-coupled beads (Fig. 3*b*). A contraction of the uropod with rear release of bound membrane integrins on the substrate (Fig. 3*e*) and centripetal recondensation of integrin clusters (Fig. 3*c*) preceded the new locomotion cycle. Rear release of LFA-1 on the substrate may in fact represent membrane "ripping" as one of the mechanisms of uropod detachment in the migrating cell determining sometimes the overall locomotion rate (14). This process has been previously described in detail for fibroblasts migrating on laminin using β_1 integrins (15).

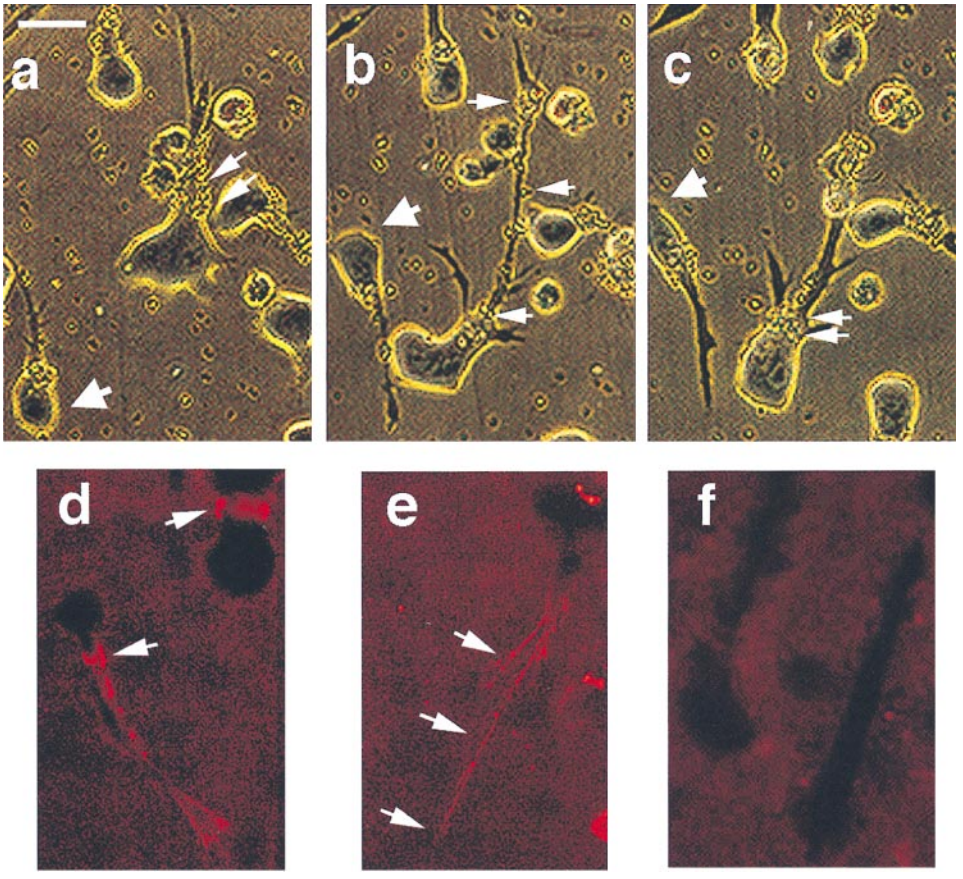

FIGURE 3. Migration of T cells involves dynamic recirculation and clustering of integrin LFA-1. *a–c*, Redistribution of polystyrene beads coated with anti-(α_L)LFA-1 mAb on locomotory HUT-78. Consecutive phase-contrast microphotographs taken with 40-min intervals. Small arrows indicate the location of the beads. Large arrow indicates the cell undergoing complete (180°) change in the direction of migration accompanied by nucleokinesis (13). *d*, Ring-like concentration of LFA-1 at the rear of the nucleus in crawling HUT-78 (arrows) detected by immunofluorescent staining of formalin-fixed nonpermeabilized cells. Cell bodies are seen as black shades. *e*, Rear release of LFA-1 on substrate. Integrin "trail" left by the cell is indicated by arrows. *f*, Negative control staining with isotype-matched Ig. Scale bar: 20 μm. Figures demonstrate microscopic fields representative of three experiments.

Cytoskeletal rearrangements in migrating T cells involve actin, microtubules, and intermediate filaments

At the intracellular level, induction of motility in HUT-78 affected the actin cytoskeleton and microtubules as well as vimentin intermediate filaments (Fig. 4). Actin-containing filopodia were present at the leading edge and trailing tails (Fig. 4*a*). The cell body and axial longitudinal cytoskeleton contained thick vimentin filaments (Fig. 4*b*). The characteristic array of microtubules displayed that the MTOC was located at the side of the nucleus opposing the direction of cell migration and from here long microtubules extended to the uropods, reflecting the state of cell polarization (Fig. 4*c*).

LFA-1-mediated intracellular signaling targets PKC isoforms β and δ to the microtubule cytoskeleton

We analyzed the redistribution of PKC isoforms representing all three groups of isoenzymes (classical (α and β(I)), novel (δ, ϵ, η, and θ), and atypical (ζ)) expressed in HUT-78 and PBTL in conjunction with major cytoskeletal components. Resting HUT-78

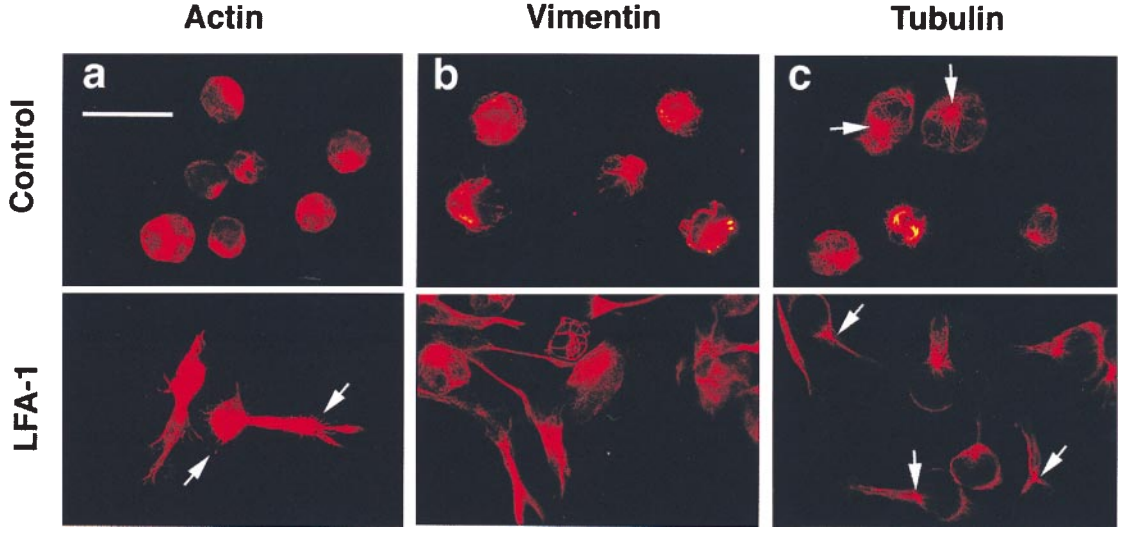

FIGURE 4. Cytoskeletal rearrangements in migrating T cells. Control (*upper panel*), HUT-78 on immobilized isotype-matched control Ig. LFA-1 (*lower panel*), HUT-78 migrating on mAb(i) to (α_L)LFA-1. Staining is shown for (*a*) F-actin, (*b*) vimentin, and (*c*) α-tubulin. Arrows indicate (*a*) filopodia and (*c*) MTOC. Scale bar: 20 μm. Similar phenotypic changes were developing in >90% of cells in four experiments.

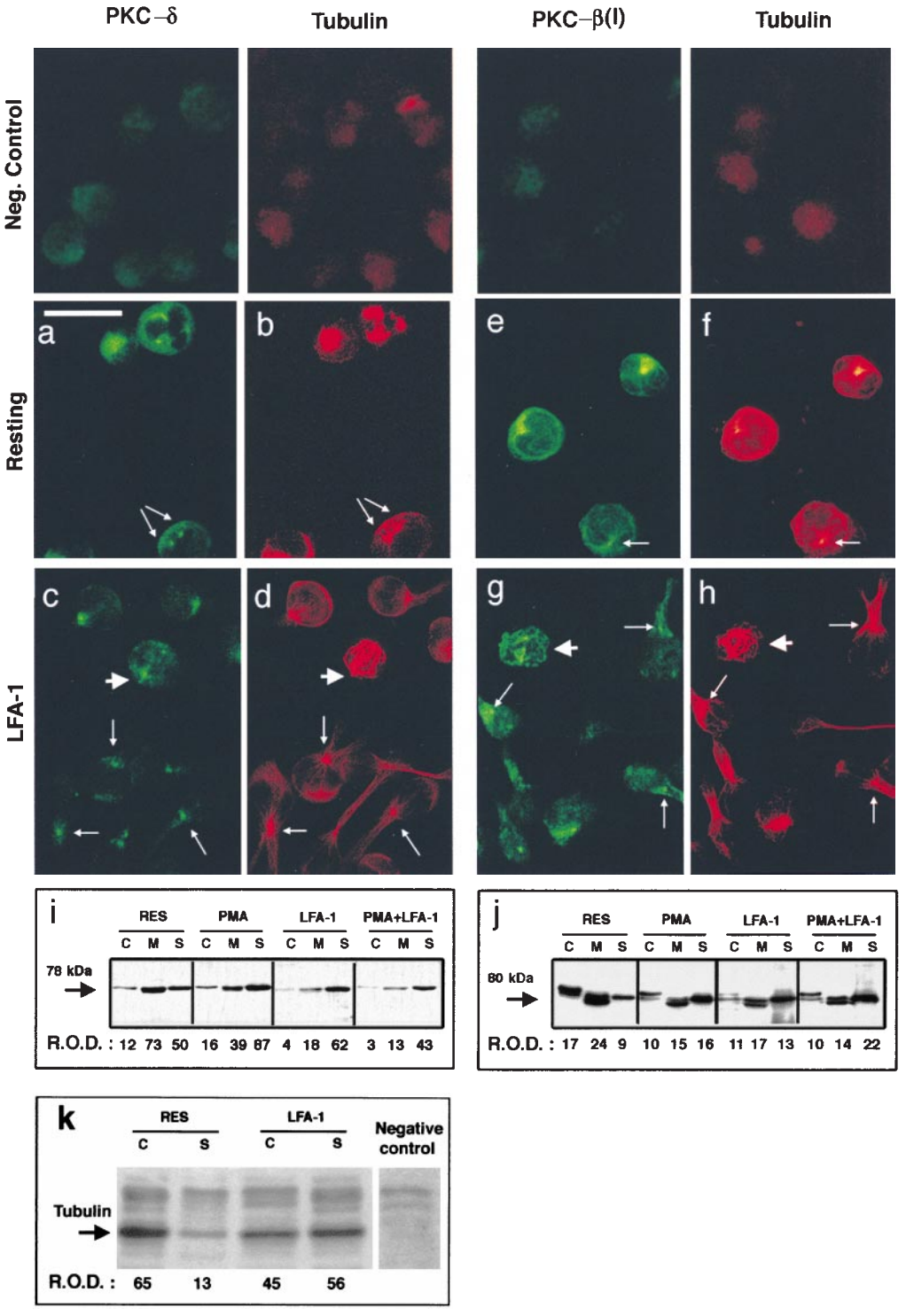

FIGURE 5. Locomotion-associated specific translocation of PKC isoforms β(I) and δ to MTOC and microtubules. Negative control (*upper panel*), Intracellular staining for PKC in the presence of isoform-specific blocking peptides and isotype-matched IgG (tubulin control). Resting, HUT-78 on immobilized isotype-matched control Ig. LFA-1, Migrating HUT-78 activated by LFA-1 cross-linking. *a*, Diffuse cytoplasmic localization of PKC-δ with spots at the centrosomes; and *e*, predominantly diffuse staining for PKC-β(I) in resting HUT-78. *b* and *f*, α-Tubulin in corresponding cells. *c*, Staining for PKC-δ attributed almost exclusively to the MTOC in locomotory HUT-78. *d*, Same field as in *c*, localization of α-tubulin. *g* and *h*, Identical fields stained for PKC-β(I) and α-tubulin. Similar PKC translocation patterns were observed in PBTL (data not shown). Fine arrows indicate positions of MTOC. Thick arrows are pointing to the cells undergoing mitosis. Scale bar: 20 μm. Similar staining patterns were registered for >90% cells in three experiments. *i* and *j*, Western blot analysis of HUT-78 subcellular fractions demonstrating distribution of PKC-δ and -β, respectively. Arrows indicate the position of specific bands with corresponding molecular mass. Labels above the lanes reflect: Res, HUT-78 on control immobilized Ig; PMA, cells activated with PMA alone; LFA-1, cells migrating on mAb(i) to LFA-1; PMA+LFA-1, migrating cells pre-activated with PMA; and C, M, S, encode cytosolic, membrane, and cytoskeletal cell fractions, respectively. Numbers below the lanes (R.O.D.) reflect the relative optical density of specific bands. Because the exposure times were different, the intensity of the bands should be compared separately for *i* and *j*. Experiments in *i*–*k* were repeated three times with similar results. *k*, Western blot analysis of HUT-78 subcellular fractions for α-tubulin. Labels above and below the lanes represent the same as in *i* and *j*. Negative control, Blot probed with control IgG. Arrow indicates the position of specific bands and corresponds to estimated molecular mass of 52 kDa. Membrane fraction was not tested in this experiment.

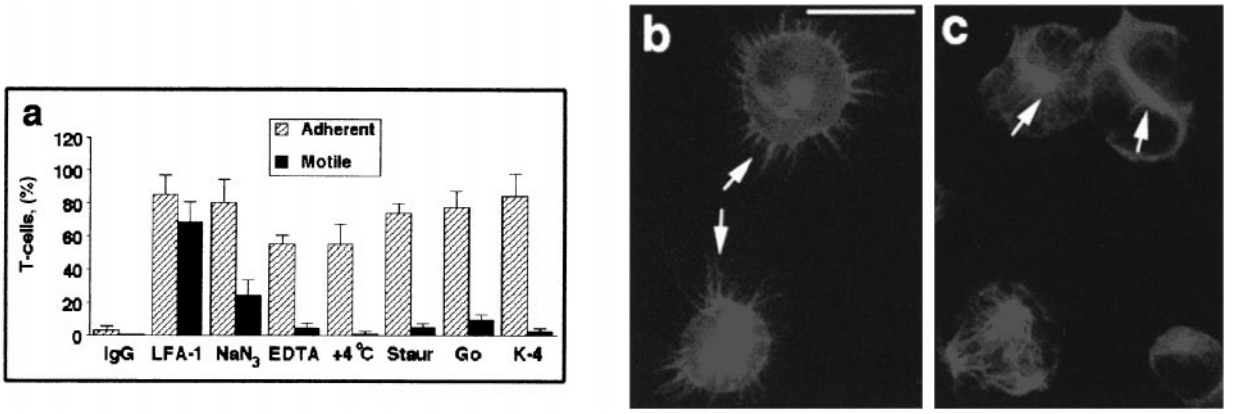

FIGURE 6. Down-regulation of PKC selectively affects T cell locomotory potential, but not cell adhesion. *a*, Percent HUT-78 displaying motility (black columns) upon activation by mAb(i) (LFA-1) is dramatically decreased by 10^{-7} M staurosporine (Staur), by a selective blocker of classical PKC isoforms Go6976 at 10^{-5} M (Go) (19), and to a lesser extent, by 0.1% sodium azide (NaN_3), though the fraction of cells attached to the substrate (striated columns) remains almost unaffected. Cells of a PKC-β-deficient clone K-4 (21) in contrast with parental HUT-78 retain adhesion, but not the locomotory potential (K-4). Low temperature (4°C) and depletion of divalent cations (EDTA) reduce both cell adhesion and motility. HUT-78 are neither adherent nor motile on control IgG (IgG). Data reflect mean ± SD from five microscopic fields of view (minimum of 200 cells) and are representative of three independent experiments. *b* and *c*, Intact functioning of actin cytoskeleton, but failure to develop polarized microtubule array in PKC-β(I)-deficient clone K-4 upon LFA-1 cross-linking. *b*, Staining for F-actin. Actin-based filopodia are indicated by arrows. *c*, Staining for α-tubulin demonstrates nonpolarized microtubule cytoskeleton in K-4 cells. Positions of MTOC are indicated by arrows. Scale bar: 20 μm.

stained for PKC-δ developed a diffuse granular cytoplasmic pattern with clearly distinguishable spots at the centrosomes of mitotic cells (Fig. 5, *a* and *b*). PKC-δ displayed a dramatic translocation with a loss of diffuse cytoplasmic pattern and localized to a compact spot corresponding to the MTOC in migrating HUT-78 (Fig. 5, *c* and *d*). Classical PKC-β(I) demonstrated granular cytoplasmic staining as well as a translocation pattern consistent with the position of MTOC and microtubules in the uropods of locomotory cells in comparison to relatively diffuse distribution in resting HUT-78 (Fig. 5, *e–h*).

By contrast, the other PKC isoforms in our study did not translocate to the MTOC and the patterns of their staining were not as clearly defined (data not shown). None of the PKC isoforms displayed evident colocalization with actin-based structures at the leading edge or uropod of migrating cells, suggesting that the formation of filo- and lamellipodia possibly develops apart from direct PKC activation and is regulated by other mechanisms such as via small protein GTPases, as demonstrated recently (16). Alternatively, PKC involvement in this processes may be characterized by transient interactions of highly dynamic nature beyond the sensitivity of our detection methods.

Western blot analysis of fractionated HUT-78 lysates confirmed the increased association of PKC-β isoenzyme with the detergent-insoluble cytoskeletal fraction in motile cells in response to stimulation through PMA and anti-LFA-1 (Fig. 5*j*). Interestingly, there were apparently three closely related species of PKC-β specifically distributed between cytosol, membrane, and cytoskeletal fractions and originally almost indistinguishable in resting HUT-78. Accumulation of 83-kDa species of PKC-β in detergent-resistant skeleton of motile cells occurred concomitantly with the losses in the 85-kDa cytosolic and 80-kDa membrane-associated bands. This phenomenon could be possibly due to the existence of alternatively spliced PKC-β variant forms with selective substrate specificity or, on the other hand, could be reflecting different degrees of phosphorylation of the PKC-β molecule, by analogy to the data reported for classical PKC-α (17). PKC-δ was detected predominantly in the cytoskeleton of the HUT-78 migrating on mAb(i) to LFA-1 (Fig. 5*i*). This translocation was significant and comparable to that induced by the potent PKC activator, PMA. The overall reduction of detectable PKC-δ in migrating HUT-78 induced by cross-linking of LFA-1 (either alone or following pretreatment with PMA) does not appear to represent a nonspecific proteolytic cleavage event as this was not induced by PMA alone nor was it observed for PKC-β isoform.

Redistribution of PKC isoenzymes to the cytoskeleton in motile cells was concordant with the enrichment of the microtubule content in this subcellular fraction (Fig. 5*k*). By contrast, resting HUT-78 lysates yielded a high amount of soluble (unpolymerized) tubulin in the cytosol. This observation can be possibly explained as related to the increased microtubule stability properties in LFA-1-triggered locomotory T cells, potentially required, for instance, at the stage of the extension of trailing uropods. On the other hand, the increased skeleton:cytosol ratio of tubulin may be due to a higher overall number of long polymerized microtubules present in the motile (vs resting) cells. Consequently, a substantial pool of assembled tubulin polymers is retained in the cytoskeletal fraction even under the cold extraction conditions used in our experiments to minimize PKC degradation. However, these data do not exclude a possibility that the cytoskeleton-directed redistribution of PKC may be also mediated via its isoform-specific interactions with certain microtubule-associated proteins.

Taken together, these findings correlate well with MTOC- and microtubule-attributed immunohistochemical staining patterns of PKC-β(I) and δ isoforms and suggest their involvement in integrin-mediated cytoskeletal rearrangement processes likely depending on phosphorylation (1).

Selective PKC-β blocking affects T cell locomotory potential but not adhesion on immobilized mAb to LFA-1

We further elucidated regulatory factors of T cell motility (Fig. 6). Induction of T cell locomotion upon cross-linking of LFA-1 proved to be energy- and temperature-dependent and required the presence of divalent Ca^{2+} and Mg^{2+} cations, consistent with their participation in classical PKC activation and magnesium ion-regulated integrin functioning (18). Neither a broad-spectrum protein kinase inhibitor staurosporine nor selective PKC-α and -β blocker Go6976 (19) significantly changed cell adhesiveness to substrate,

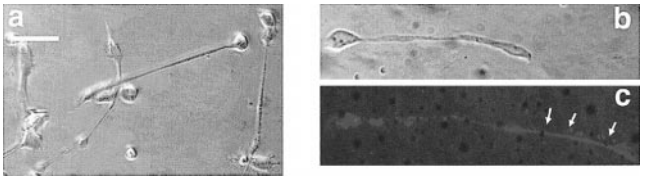

FIGURE 7. Activated human T cells plated on recombinant ICAM-1-Fc acquire a locomotory phenotype similar to that induced by anti-LFA-1 Abs. *a*, Hoffman modulation contrast (HMC) image of PBTL preactivated by PMA and exposed to human chimeric ICAM-1-Fc fusion protein after 6-h incubation. *b* and *c*, respective phase contrast and fluorescent images of the same T cell migrating on recombinant ICAM-1-Fc stained for LFA-1. Note the integrin clustering at the rear of the nucleus and the trailing integrin-positive "trail" left by the cell on the substrate (arrows) similarly to shown of Fig. 3, *d* and *e*. The microscopic pictures are representative of four experiments. Scale bar: 20 μm.

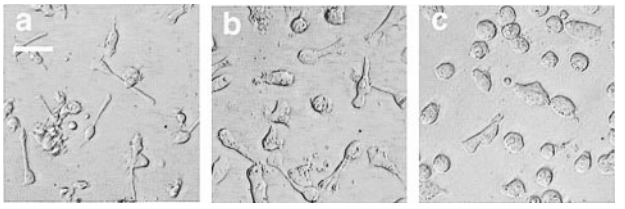

FIGURE 8. T cell locomotion is not selectively dependent on β_2 integrins but requires PKC participation. *a* and *b*, Hoffman modulation contrast images of PBTL and HUT-78, respectively, migrating on β_1 integrin-specific (fibronectin-coated) substrate following 5-h incubation. Both cell types develop a motile phenotype similar to that shown on Fig. 1*d*. *c*, Cells of a PKC-β-deficient clone K-4 adhere and spread on fibronectin but remain nonlocomotory. The experiment was performed three times with consistent results. Scale bar: 30 μm.

while they both abrogated HUT-78 locomotion. Therefore, we assume that acquisition of a migratory phenotype in T cells in our model is primarily dependent on LFA-mediated PKC activation, while adhesion modification via high-affinity LFA-1 molecules induced by inside-out signaling pathways (20) may develop as a secondary event.

Pretreatment of HUT-78 with specific blocker of classical PKC group Go6976 effectively inhibited cell motility. Of the four isoenzymes comprising this group—α, β(I), β(II), and γ—only the first two are expressed in HUT-78 cells; therefore, we used a PKC-β-deficient clone K-4 derived from parental HUT-78 line (21) to determine the PKC isoform playing the leading role in T cell locomotion by exclusion method. K-4 cells proved to be nonmotile on mAb(i) to LFA-1, though their adhesion remained unaffected (Fig. 6*a*). These cells were capable of spreading and generating multiple filopodia, thus reflecting that the function of actin-based locomotory mechanisms was not abrogated (Fig. 6*b*). At the same time, PKC-β-deficient K-4 cells failed to develop a highly polarized microtubular array (Fig. 6*c*) typically displayed by parental HUT-78 upon LFA-1 cross-linking. This provides strong supporting evidence for the role of PKC-β in the regulation of the tubulin-based cytoskeleton and hence cell motility. As the actin-based cytoskeleton appeared to function normally in K-4 cells, it is entirely possible that the defects in microtubule reorganization seen in these cells could be due to aberrations in PKC-β-mediated actin-microtubules link-up.

T cell locomotion patterns on different integrin-binding substrates are characterized by ligand and epitope specificity

This part of study was aimed to investigate the correlation of the T cell locomotion-associated phenomena observed for anti-LFA-1 Abs with the events induced by other integrin-specific ligands. As seen from the Fig. 7*a*, PBTL preactivated by PMA and exposed to immobilized chimeric ICAM-1-Fc protein imitating a native LFA-1 ligand are indeed characterized by a phenotype similar to those described above that are induced by anti-LFA mAb (compare with Figs. 1 and 2). PBTL in this system also produced active crawling movements with net cell body translocation, as confirmed by video recording (data not shown). Of note, staining of the fixed slides with anti-LFA-1 mAb (Fig. 7, *b* and *c*) demonstrated the frequent presence of integrin-positive tracks left behind by the migrating cells, by analogy to the ripping release of LFA-1 taking place when the crawling substrate was provided by immobilized Abs (Fig. 3*e*). This may potentially represent one of the mechanisms regulating T cell migration enabling the uropod detachment from the supporting matrix and described earlier for non-T cell types (14).

Fibronectin representing a β_1 integrin ligand induced similar phenotypic changes and motile behavior in phorbol ester-stimulated PBTL (Fig. 8*a*). Clearly developed but less pronounced locomotory phenotype (with shorter uropods) was induced by this ligand in constitutively activated HUT-78 (Fig. 8*b*). HUT-78 also displayed motile behavior with similar phenotypic patterns on laminin and collagen IV, but not on collagen I (data not shown). Cells of the PKC-β-deficient HUT-78-derived subclone K-4 were able to attach and spread on fibronectin, but remained nonmotile (Fig. 8*c*). Therefore, cell motility could not be induced via the β_1 or β_2 integrins studied (see Fig. 6*b*) in the absence of this particular PKC isoform.

To further elucidate the specificity of PKC-regulated intracellular signaling triggered by integrins, we used an alternative clone of anti-LFA-1 mAb, MEM-83, previously reported to induce a marked homotypic aggregation but not a significant locomotory phenotype in normal human T cells (5). HUT-78 did adhere and spread on this mAb(i) but were significantly less polarized (Fig.

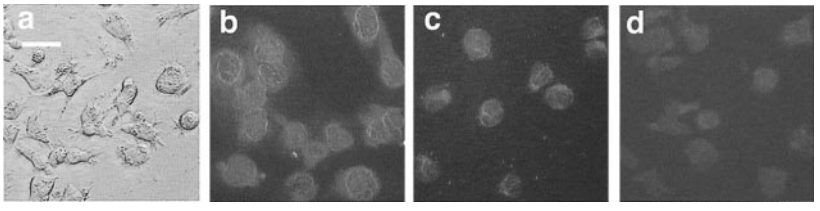

FIGURE 9. HUT-78 cells adhering to immobilized nonmotility inducing anti-LFA-1 mAb do not display the isoform-specific PKC redistribution. *a*, Hoffman modulation contrast image of HUT-78 adhering and spreading on mAb clone MEM-83. The majority of the cells do not undergo a marked polarization and are lacking clearly developed uropods. Less than 5% cells of the whole population underwent a noticeable net body translocation over a 4-h observation. *b* and *c*, HUT-78 under similar experimental conditions, stained for PKC isoforms β(I) and δ, respectively. A predominantly diffuse and granular cytoplasmic staining pattern is seen for PKC-β(I) and diffuse with spots for PKC-δ. *d*, Negative control staining. The figure shows representative microscopic fields of three experiments. Scale bar: 30 μm.

9*a*) and did not acquire a typical locomotory phenotype. At the same time, as shown on Fig. 9, *b* and *c*, respectively, neither PKC-β(I) nor PKC-δ underwent a dramatic redistribution demonstrated above for motile cells (compare to Fig. 5, *g* and *c*). In these experimental conditions, a substantial portion of PKC-attributed staining remained in the cytosol in a pattern characteristic of resting (static) cells (as on Fig. 5, *a* and *e*).

Our findings demonstrate that the patterns of T cell locomotion associated with LFA-1 cross-linking can be also reproduced using different native integrin ligands. Development of the motile behavior in T cells requires isoform-specific participation of PKC. However, the extent to which the motile phenotype is induced may depend on both specific epitopes within integrins and their ligands and their respective affinities.

Discussion

In previous studies using different cellular systems, it has been demonstrated that integrin-mediated signal transduction pathways triggered by receptor occupancy and clustering (2) can be selectively targeted to certain cytoskeletal components via isoform-specific involvement of PKC. Thus, PKC-β has been shown to associate with microtubule- and actin-based cytoskeletons in leukemic cells and fibroblasts (10, 22) and to colocalize with an actin-binding protein, spectrin, and membrane anchorage protein, ankyrin, following T cell activation (23). Interestingly, PKC-β(I) has been also reported to localize to pericentriolar region in the neck of mammalian spermatozoa and was detected in extracts of their microtubule-based tails, thus suggesting a possible participation of this isoform in flagellar type of microtubule-associated motility (24). The specific colocalizing of PKC-δ with centrosomes demonstrated in the present study is in agreement with its previously shown importance for mitotic division (25) and can reflect the involvement of this enzyme in MTOC-orchestrated cell functions. PKC recruitment in regulation of microtubule-dependent motility systems deserves special attention in view of their impact on polarization and direction of pseudopodial activity in epithelial cells (26), neutrophils (27), and T cells (28). Microtubule rearrangement can also generate forces contributing to nucleokinesis in migrating neurons, for example (29). PKC engagement in cytoskeletal assembly induced by phorbol esters has been shown earlier in human T lymphocytes (30) and neutrophils (31). From this point of view, the selective accumulation of PKC-β and -δ in the cytoskeletal fraction commencing upon LFA-1 cross-linking suggests distinct cellular functions of individual PKC isoenzymes (32).

Taken together, our data demonstrate that cross-linking of LFA-1 in HUT-78 and activated PBTL by mAb(i) induces dynamic redistribution of PKC isoenzymes and cytoskeletal rearrangements and is accompanied by cell locomotion. Redistribution of PKC isoforms β(I) and δ may play a critical role in the regulation of microtubule-driven events in T cell motility. This can involve either direct phosphorylation of microtubule proteins or closely associated molecules that regulate their assembly. Potential specific functions of other PKC isoforms in lymphoid cell locomotion still remain to be identified. Disclosure of these functions will contribute to the dissection of the mechanisms of cell motility and to the understanding of factors affecting both the migration of normal cells and the metastatic potential of malignant cells possibly leading to the development of new therapeutic strategies.

The nature of LFA-1 interaction with its potential physiological ligands remains to be examined. Motility-inducing properties of purified ICAM-1 on reconstituted lipid bilayers have been shown earlier in large granular lymphocytes (33). It has been also reported that adhesion and cytoskeletal rearrangements in T cells can be triggered by recombinant soluble ICAM-1-Fc fusion protein (18, 34, 35). The phenotypic changes demonstrated for T lymphoblasts adhering to ICAM-1 (18, 36) are different from those described in this study. In our experiments, maximal length of trailing uropods was usually reached after 3–4 h incubation on immobilized Abs and 5–6 h following the T cell exposure to ICAM-1 or extracellular matrix components. Therefore, the short-term (30–40 min) incubations commonly used in adhesion assays may be not sufficient for developing the advanced picture of motility-associated phenomena. Of note, the phenotype closely resembling that registered in our model system was documented for the human Ag-specific T cell line CFTS 4:2.80 exposed to a number of extracellular matrix components 5 h after triggering with anti-β_1 and β_2 integrin Abs (3). The potential physiological significance of the observed phenomena is further supported by the finding that LFA-1 cross-linking augments RANTES-directed chemotaxis of PBTL. These data suggest the possibility that signaling through LFA-1 in activated T cell could potentially facilitate its migration through vascular endothelium along a chemotactic gradient.

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