

Figure 4. Structural Analysis of the mDia1 Core FH2 Domain

(A) Ribbon diagram of the core FH2 domain. The NH₂-terminal, three-helix-bundle, and FH2 motif subdomains are colored red, blue, and green, respectively. The region corresponding to the highly conserved ⁹⁶⁷GNXMN⁹⁷¹ motif is colored purple. The NH₂ and COOH termini of the molecule are indicated by N and C, respectively. Secondary structures are numbered consecutively. Ile845, Met970, Lys989, Lys994, Lys999, Arg919, and Asp1067 are shown as ball-and-stick representation. Distance between Ile845 and Met970 and approximate locations of the FH1 domain, linker, and DAD in mDia1 are indicated.

(B) A buried salt bridge. A salt bridge between Arg919 and Asp1067 is shown as ball-and-stick representation together with surrounding hydrophobic residues, as indicated.

(C) Superimposition of the four molecules in the asymmetric unit. Molecules A, B, C, and D, colored in red, green, blue, and yellow, respectively, were superimposed on the three-helix-bundle subdomains, and are oriented as in (A). Note that the FH2 subdomain exhibited the highest conformational differences.

(D) Conserved residues. Surface representation of the mDia1-FH2 core domain with residues conserved in the six formin family proteins colored orange and those conserved in five of six colored yellow. Residues mentioned in the text are indicated. Left and right: orientation as in Figure 3B and rotated 180°, respectively.

polymerization of actin was measured in the presence of I845R or M970R as for WT (Figure 2E). The effect of the mutants on the actin polymerization was smaller than that of the wild-type mDia1-FH2. Hyperbola fit to the [M970R] dependence of the elongation rates (insert in Figure 2E) gave half saturation at $8.91 \pm 3.05 \mu\text{M}$, suggesting an approximately four times weaker effect than for the wild-type ($1.96 \pm 0.43 \mu\text{M}$). The fit also indicated that in the presence of saturating [M970R] the elongation rate decreased to $36 \pm 6\%$ of that measured in the absence of formin. The effect of I845R was even weaker than that of the M970R. Although the I845R effect did not give a well-defined hyperbola, the results are consistent with a decrease in elongation rate to $\sim 40\%$ with half saturation at $\sim 16 \mu\text{M}$, approximately eight times weaker than that of the wild-type mDia1-FH2. Accordingly, at saturating concentrations the mutants can produce the inhibition of the actin polymerization similar to that observed for the wild-type mDia1-FH2, but the half saturation concentrations were approximately four and eight times higher for the M970R and I845R mutants than for the wild-type FH2. The effect of the mutations showed that the proper interaction between mDia1-FH2 and actin requires both ends of the elongated mDia1-FH2 protein.

Discussion

In our experiments mDia1-FH2 and mDia3-FH2 did not appear to induce actin nucleation but rather inhibited the spontaneous polymerization of actin (Figure 2A). The inhibition of actin polymerization *in vitro* by mDia1-FH2 and mDia3-FH2 is consistent with an *in vivo* study, which showed that a similar FH2 construct inhibited actin polymerization induced by an activated mDia protein (Copeland and Treisman, 2002). Our data suggest that the core FH2 domain of mDia1 and mDia3 has a subset of properties of longer constructs characterized in previous studies (Pruyne et al., 2002; Pring et al., 2003; Kovar et al., 2003; Li and Higgs, 2003; Zigmund et al., 2003). The effect of mDia1-FH2 and mDia3-FH2 on the depolymerization rate of actin filaments (Figure 2B) and on the critical concentration of actin (Figure 2C) is consistent with the observation that barbed-end capping proteins decrease the dissociation rate of actin monomers from filaments and increase critical concentration of actin assembly (Wear et al., 2003). Although on the basis of our data it is not possible to exclude other mechanisms (such as for example a copolymerization mechanism similar to that observed for microtubule assembly in the presence of colchicine [Sterlicht and Ringel, 1979]), the similarities between our data and the analyses of other formin fragments (Pruyne et al., 2002; Pring et al., 2003; Li and Higgs, 2003; Kovar et al., 2003; Zigmund et al., 2003) suggests that both mDia1-FH2 and mDia3-FH2 are likely to act at the barbed end of actin filaments. Although more work is required to determine the affinity of mDia1-FH2 and mDia3-FH2 for barbed ends, the available data also suggest that the affinity may be about three orders of magnitude weaker than that of longer constructs.

The comparison of the detailed analysis of budding yeast Bni1p (Pruyne et al., 2002; Pring et al., 2003; Zig-

mond et al., 2003), fission yeast Cdc12p (Kovar et al., 2003), and mDia1 (Li and Higgs, 2003) fragments with our data indicates that mDia1-FH2 resembles properties of Bni1, which caused partial barbed-end capping (Pruyne et al., 2002; Pring et al., 2003; Zigmund et al., 2003). Both FH2 and FH1FH2 Bni1p fragments inhibited barbed-end elongation to $\sim 50\%$ and the depolymerization rate to $\sim 60\%$, while the critical concentration showed no change in the presence of the fragments (Zigmund et al., 2003), in agreement with the conclusion that mDia1 was more similar to Bni1p than to Cdc12p (Li and Higgs, 2003). It appears that both mDia1 and Bni1p act through a leaky barbed-end capping mechanism. The mDia3-FH2 fragment, however, is more similar to the construct containing the FH2 domain of the fission yeast formin, Cdc12p, which tightly caps barbed ends and acts as a classic barbed-end capping protein (Kovar et al., 2003). Cdc12p(882-1375) was shown to decrease the actin depolymerization rate to 34%, to inhibit barbed-end elongation, and to increase the critical concentration to $0.9 \mu\text{M}$. To determine the physiological significance of the differences between the observations with Cdc12p, Bni1p, and mDia, more experimental work and direct experimental comparisons are required.

While longer mDia1 fragments were previously reported to accelerate actin polymerization (Li and Higgs, 2003), our experiments showed that mDia1-FH2 and mDia3-FH2 inhibited spontaneous actin assembly. We thus set out to define a minimal length FH2-containing fragment which is able to accelerate polymerization. An mDia3 fragment containing the core FH2 domain and a linker region which connects FH2 and FH1 (mDia3-FH2+linker, see Figure 1A), whose N-terminal domain boundaries were close to those identified as being functional for actin polymerization *in vivo* (Copeland and Treisman, 2002), accelerated actin polymerization at subnanomolar concentrations under conditions previously applied for the FH2 fragments (Figure 5A). Above 2.5 nM mDia3-FH2+linker the initial parts of the pyrene transients were within the mixing time. Below this concentration the mDia3-FH2+linker dependence of the elongation rate was linear (insert in Figure 5A).

We find a tetramer of FH2 in the asymmetric unit (Figure 3A), where only the A/B and C/D molecules have a large, albeit not very complementary, surface area of interaction (2350 \AA^2). The tetramer in the crystal could be an artifact of crystal packing. Size exclusion analysis of the mDia1-FH2 indeed gives an apparent molecular mass of 67 kDa (Figure 5B), which is between that expected for a dimer and a monomer. Considering the elongated nature of the FH2, it is unlikely that a dimer of the elongated FH2 would elute later than a value calculated for a spherical particle. The longer mDia3-FH2+linker eluted much earlier than mDia1-FH2 and gave an apparent molecular mass of more than 200 kDa . Although gel filtration analysis in principle only gives the Stokes' radius of proteins, the apparent dimerization or oligomerization of the mDia3-FH2+linker construct (Figure 5B) is completely in line with previous studies on mDia1(748-1255) (Li and Higgs, 2003), Bni1p(1227-1324), and Bni1p(1348-1824), which led the authors to suggest that the ability of formins to oligomerize allows them to walk processively with the barbed end as the filament elongates (Zigmund et al., 2003). The finding

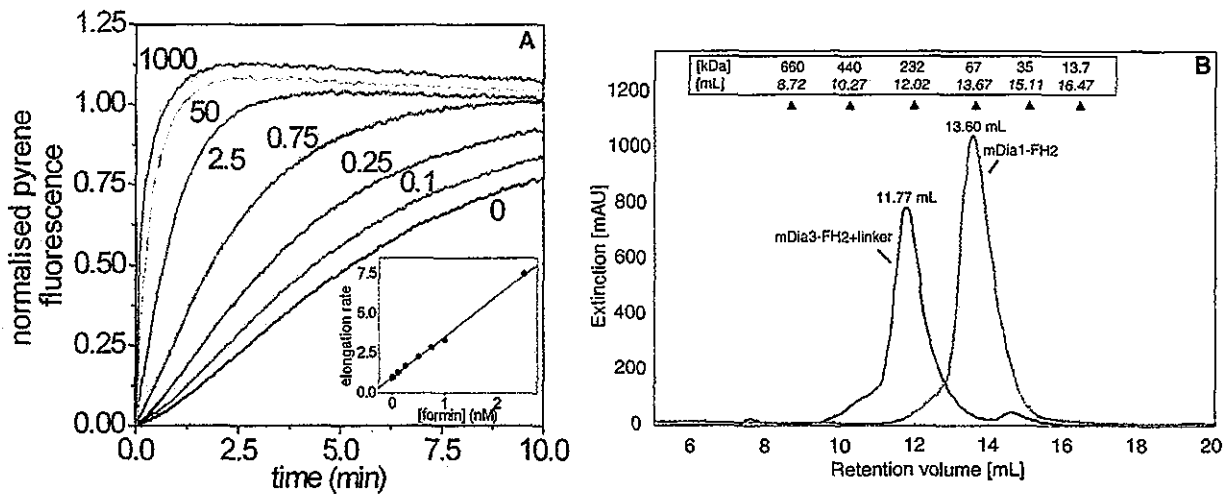


Figure 5. The Linker Dramatically Alters the Properties of the Core FH2 Domain

(A) Actin polymerization. Actin was polymerized by simultaneously adding 10 mM KCl, 0.5 mM MgCl₂, and mDia3-FH2+linker to actin monomer solutions (3.5 μM, 5% pyrene-labeled). The figure shows the normalized pyrene fluorescence transients in the absence of formin (black) and in the presence of increasing mDia3-FH2+linker concentrations in nM as indicated. The insert shows the [mDia3-FH2+linker] dependence of the relative elongation rate determined from the slopes at 50% saturation of the reaction (linear fit is shown as solid line).

(B) Gel permeation chromatography of mDia1-FH2 and mDia3-FH2+linker. The elution profiles of mDia1-FH2 and mDia3-FH2+linker are colored gray and black, respectively. Elution volume and corresponding calculated molecular weight are indicated above the profiles. Elution volumes (in ml) and molecular masses (kDa) of marker proteins are shown above.

that fragments covering the FH2 domain of FHOD1/Fhos bind to each other (Takeya and Sumimoto, 2003) further supports the importance of oligomerization for the function of formins.

Previous studies have shown that a dimeric protein binding to two adjacent actin monomers in a filament can bind with an affinity more than 100-fold tighter than the equivalent monomer provided that the relative geometry of the two pairs of binding sites is appropriate. The classic case is the dimeric fragment of myosin II, heavy meromyosin, which binds two actin monomers with an affinity more than 100-fold tighter than the equivalent monomeric fragment subfragment 1 (K_D of 2×10^{-10} and 2×10^{-7} M, respectively; see Goody and Holmes, 1983, and Conibear and Geeves, 1998, for a full discussion). The reduced affinity of the core FH2 domain compared to the longer FH2 plus linker can thus be accounted for by the dimerization (or higher oligomerization) of the longer fragment. This implies that the linker region is essential for nucleation and for dimerization and that the tight affinity for actin and nucleation of actin polymerization are both properties of the dimer. Crystallization of the longer fragment will be needed to address this issue.

We thus conclude that, while the core FH2 domain binds to actin and inhibits actin polymerization, it is the 72 residue linker between the FH2 and FH1 domain which dramatically changes the property of the fragment. It provides the structural framework for oligomerization, for increasing the affinity with F-actin, and for inducing actin polymerization. Further structural and biochemical studies will be necessary to clarify the exact mechanism mediated by the linker fragment.

Experimental Procedures

Protein Preparations

Complementary DNA for full-length mDia3 was isolated by polymerase chain reaction-based cloning, and its nucleotide sequence has

been deposited in GenBank (AY 312280) (S. Yasuda and S.N., unpublished data). The FH2 domains of mouse mDia1 and mDia3 were expressed as GST-fusion proteins in the *E. coli* strain BL21 (DE3) pLysS. GST-fusion proteins were loaded onto a GSH column (Pharmacia), eluted from the column after cleavage by thrombin and further purified by size exclusion chromatography (for details see Supplemental Data at <http://www.molecule.org/cgi/content/full/13/4/511/DC1>). The mDia3-FH2+linker was prepared in the same way as the other FH2 constructs described above. Rabbit skeletal actin was prepared as described previously (Spudich and Watt, 1971) and gel filtered through a Sephadex G100 column. Pyrene labeling was carried out according to Criddle et al. (1985).

We measured concentrations of mDia samples with Bradford method (using BSA as standard) and confirmed the values with a method based on extinction coefficients of proteins in guanidine hydrochloride. The deviation between values obtained from both the methods were less than 30%. We used the concentrations from the Bradford method for interpretation.

Fluorescence Experiments

Freshly prepared unlabeled and pyrene-labeled Ca-G-actin in 4 mM Tris/HCl (pH 7.4), 0.2 mM ATP, 0.1 mM CaCl₂, 0.5 mM DTT were clarified by ultracentrifugation (100,000 g, 2 hr, 4°C) before the experiments. In spontaneous actin assembly measurements mDia1-FH2 or mDia3-FH2 fragments were added to actin monomer solutions (5% pyrene-labeled) and the samples were incubated for 5 min with 200 μM EGTA and 50 μM MgCl₂ at 22°C to exchange the actin bound Ca²⁺ for Mg²⁺. The polymerization was started by addition of KCl and MgCl₂ to 10 mM and 0.55 mM, respectively. The longer mDia3 fragment (mDia3-FH2+linker) induced actin polymerization during the cation exchange. Therefore, these fragments were added to the actin solution after the cation exchange was completed together with the KCl and MgCl₂. In actin depolymerization experiments, 5 μM actin (68% pyrene-labeled) was polymerized (10 mM KCl, 0.5 mM MgCl₂) for at least 4 hr and then diluted into polymerization buffer alone, or into polymerization buffer supplemented with mDia1-FH2 or mDia3-FH2. In critical concentration experiments, actin (between 0 and 20 μM) was incubated with fixed concentrations of mDia1-FH2 or mDia3-FH2 in polymerization buffer overnight, and then the fluorescence intensity of the samples was measured. In all experiments, the pyrene fluorescence changes were monitored with excitation and emission wavelengths of 365 and 407 nm, respectively. The fluorescence experiments were carried out with a

Cary Eclipse Fluorescence Spectrophotometer (Varian) or a Perkin-Elmer LS50B Spectrofluorimeter at 22°C.

Cosedimentation Assay

Actin filaments (3 μ M) were mixed with mDia1-FH2 or mDia3-FH2 at different concentrations and incubated for 30 min at room temperature. The samples (100 μ l) were centrifuged at 600,000 g for 30 min at 4°C, and the pellets and supernatants were separated. One hundred microliter SDS-PAGE sample buffer was added to the supernatants. The pellets were dissolved in 100 μ l distilled water, and 100 μ l SDS-PAGE sample buffer was added to each tube. These samples were then applied to 12.5% SDS-PAGE gels. The affinity of FH2 for actin (K_D) was estimated from the results of polyacrylamide gel analysis (Figure 2) using the following equation (Kurzawa and Geeves, 1996):

$$[A]_0 S^2 - ([A]_0 + [FH2]_0 + K) S + [FH2]_0 = 0 \quad (1)$$

where $[A]_0$ and $[FH2]_0$ are the total actin and FH2 concentrations, respectively, and S is the fraction of FH2 bound to actin (calculated as the ratio of density of FH2 bands to those of actin bands in the pellets).

Crystallization, Data Collection, and Structure Determination

The mDia1-FH2 was purified as described above and concentrated to approximately 20 mg/ml prior to crystallization. Crystals were grown at 20°C using the hanging-drop vapor-diffusion method by mixing the protein in 1:1 volume ratio with a solution containing 20% (v/v) PEG3350 (Sigma) and 200 mM Na₂SO₄. Crystals were harvested in a solution containing 10% (v/v) PEG3350, 100 mM Na₂SO₄, and 18% (v/v) glycerol and flash-frozen in liquid nitrogen. A native data set to 2.6 Å resolution was collected at 100 K at ID14-1 beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France (Table 1). The selenomethionine (SeMet) substituted protein was expressed in the *E. coli* strain BL21 (DE3) pLysS growing in minimal medium in which methionine was replaced by SeMet. The SeMet-substituted protein crystals were grown at 20°C using the hanging-drop vapor-diffusion method by mixing the protein in 1:1 volume ratio with a solution containing 20% (v/v) PEG3350 and 200 mM KSCN. The SeMet crystals were harvested in a solution containing 10% (v/v) PEG3350, 100 mM KSCN, and 21% (v/v) glycerol and flash-frozen in liquid nitrogen.

A SAD data set with a resolution of 3.4 Å resolution was collected at the selenium edge at the ID29 beamline at the ESRF (Table 1) and processed with the HKL suite (Otwinowski and Minor, 1997) and/or XDS (Kabsch, 1993), SnsB (Weeks and Miller, 1999), SHELXD (Usón and Sheldrick, 1999), and SOLVE (Terwilliger and Berendzen, 1996) were used to identify 32 selenium sites that were refined with SHARP (de La Fortelle and Bricogne, 1997). The initial phases were calculated by SHARP using 52 selenium sites (32 plus 20 additional ones located by SHARP) and were improved by DM (CCP4, 1994), resulting in a readily interpretable map to which most of the backbone of all four molecules in the asymmetric unit could be positioned using O (Jones et al., 1991). The preliminary model of a monomer with side chains was used for molecular replacement into the native crystal (Table 1) using the program AmoRe (CCP4). The best solution had a correlation coefficient of 43.8 and R factor of 47.9% (second best: correlation coefficient 38.6, R factor 49.8%). Manual rebuilding was performed for ambiguous parts using simulated annealing omit electron density maps. Iterative rounds of rebuilding and refinement were performed, and more residues were added. Initial NCS constraints were relaxed gradually during refinement after the significant differences between the relative domain positions of the molecule pairs A/C and B/D became apparent. Three sulfate ions were also modeled into strong electron densities close to molecules A, B, and C. Refinement was done by the program CNS (Brunger et al., 1998) and REFMAC (CCP4). Figures were generated using MolScript (Kraulis, 1991), Raster3D (Merrit and Murphy, 1994), and MOLMOL (Koradi et al., 1996).

Analytical Gel Filtration

Analytical gel filtration was performed on Superdex 200 in 50 mM Tris-HCl buffer (pH 7.3) containing 50 mM NaCl, 5 mM DTE, and 5% glycerol.

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Accession Numbers

The coordinates for the core FH2 domain of mDia1 have been deposited in the Protein Data Bank under ID code 1V9D.

RhoA and ζ PKC Control Distinct Modalities of LFA-1 Activation by Chemokines: Critical Role of LFA-1 Affinity Triggering in Lymphocyte In Vivo Homing

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Summary

Chemokines regulate rapid leukocyte adhesion by triggering a complex modality of integrin activation. We show that the small GTPase RhoA and the atypical ζ PKC differently control lymphocyte LFA-1 high-affinity state and rapid lateral mobility induced by chemokines. Activation of LFA-1 high-affinity state and lateral mobility is controlled by RhoA through the activity of distinct effector regions, demonstrating that RhoA is a central point of diversification of signaling pathways leading to both modalities of LFA-1 triggering. In contrast, ζ PKC controls LFA-1 lateral mobility but not affinity triggering. Blockade of the 23–40 RhoA effector region prevents induction of LFA-1 high-affinity state as well as lymphocyte arrest in Peyer's patch high endothelial venules. Thus, RhoA controls the induction of LFA-1 high-affinity state by chemokines independently of ζ PKC, and this is critical to support chemokine-regulated homing of circulating lymphocytes.

Introduction

The concerted action of adhesion molecules and chemokine receptors regulates leukocyte extravasation from the blood and determines the specificity of the immune response (Kunkel and Butcher, 2002). Chemokines activate an extremely rapid and complex modality of integrin activation consisting of heterodimer high-

affinity state and of lateral mobility (Constantin et al., 2000; Laudanna et al., 2002). These modalities of integrin activation play a cooperative role in mediating LFA-1-dependent lymphocyte immediate arrest on ICAM-1 under physiologic flow conditions. In particular, induction of rapid LFA-1 lateral mobility on the plasma membrane has been shown to mediate lymphocyte arrest to surfaces presenting a low density of ICAM-1 (Constantin et al., 2000). This suggests that LFA-1 lateral mobility allows the adaptation of lymphocytes to blood vessels presenting a variable expression level of integrin ligand. In contrast, the physiological role of LFA-1 high-affinity state induction in the context of lymphocyte rapid adhesion and in vivo arrest has never been formally demonstrated.

Signaling pathways controlling LFA-1 activation are largely unknown. Recent data show the involvement of phosphatidylinositol 3-(OH) kinase (PI(3)K), Cytosolin-1, and Rap1 in LFA-1 lateral mobility induced by chemokines in lymphocytes (Constantin et al., 2000; Weber et al., 2001a; Shimonaka et al., 2003). However, signaling events controlling the rapid induction of LFA-1 high-affinity state by chemokines are completely unidentified.

Previous data have implicated the small GTPase RhoA and the atypical ζ PKC in chemoattractant-induced β 1 and β 2 integrin-mediated leukocyte adhesion (Laudanna et al., 1996, 1998). The discovery that chemokines activate in lymphocytes a complex modality of integrin activation raises the hypothesis that RhoA and ζ PKC may control specific modalities of LFA-1 triggering.

In this study we addressed the possibility that RhoA and ζ PKC may control different modalities of LFA-1 activation by chemokines. We found that RhoA and ζ PKC play diversified, yet necessary, roles in rapid LFA-1 triggering by chemokines in lymphocytes. RhoA controls both LFA-1 high-affinity state and lateral mobility induction by chemokines through the engagement of distinct RhoA downstream effector regions. In contrast, ζ PKC is involved only in heterodimer lateral mobility induction. Importantly, we show that blockade of RhoA downstream effector region specifically involved in the induction of LFA-1 high-affinity state prevents rapid arrest of circulating naive lymphocytes on ICAM-1-expressing high endothelial venules (HEV) in secondary lymphoid organs. These findings show that RhoA and ζ PKC are critical points of diversification in signal transduction pathways generated by chemokines and leading to LFA-1 activation. Moreover, the data illuminate the critical physiological role of triggering LFA-1 to high-affinity state in lymphocyte homing in vivo.

Results

A Novel Method for Analyzing RhoA Signaling Activity

Three distinct regions of RhoA have been shown to activate individual downstream effectors (Fujisawa et al., 1998). To block RhoA-dependent signaling in a region-selective manner, we generated fusion peptides

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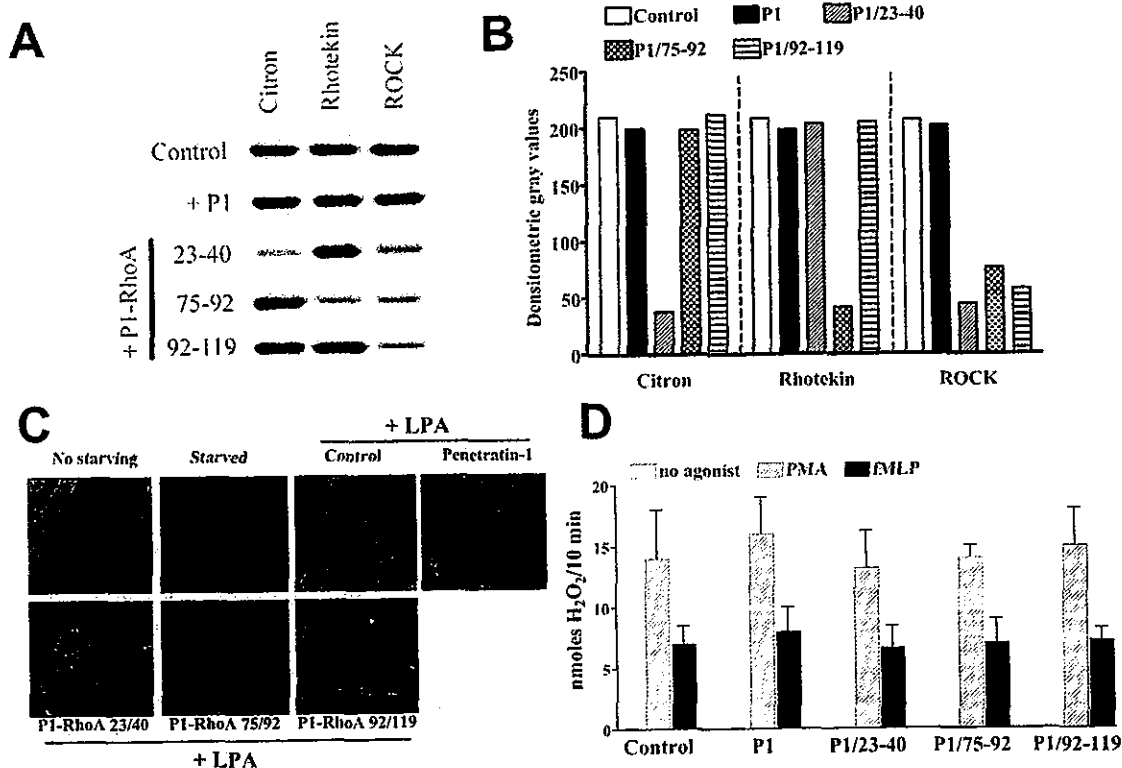


Figure 1. P1-RhoA Peptides Are Effective Inhibitors of RhoA-Dependent Signaling

(A) RhoA binding to Citron-, Rhotekin- and ROCK-RBD in the presence of buffer (Control) or 20 μ g of Penetratin-1 (P1) or 23-40, 75-92, and 92-119 P1-RhoA peptides. A protein immunoblot of anti-RhoA is shown. One representative experiment of two.

(B) Densitometric analysis of the immunoblot shown in (A).

(C) Swiss 3T3 mouse fibroblasts were treated for 4 hr at 37°C with 50 μ M of Penetratin-1 (P1) or different P1-RhoA peptides and then stimulated for 10 min with 25 ng/ml lysophosphatidic acid (LPA). Shown are confocal microscopy images.

(D) Human polymorphonuclear neutrophils were treated for 2 hr at 37°C with 50 μ M of P1 or different P1-RhoA peptides and then stimulated with 10 ng/ml PMA or 100 nM formyl-Met-Leu-Phe (fMLP). Stimulation was performed under stirring at 37°C. Shown are nmoles of released H_2O_2 . Values are the means from three experiments. Error bars are SDs.

including an N-terminal plasma membrane translocating sequence from the third helix of the homeoregion of *Drosophila melanogaster* transcription factor Antennapedia, also called Penetratin-1 (P1) (Prochiantz, 2000), fused to the three distinct downstream effector regions of human RhoA, encompassing amino acids 23-40, 75-92, and 92-119.

We first evaluated the ability of the peptides to block RhoA interaction with specific effectors. In pull-down assays, binding of RhoA to Citron was blocked by 23-40, but not 75-92 and 92-119, peptides; binding to Rhotekin was blocked by 75-92, but not 23-40 and 92-119, peptides; finally, binding to ROCK was inhibited by all the peptides (Figures 1A and 1B). These data are in agreement with a previous study (Fujisawa et al., 1998) and show that soluble RhoA-derived effector regions may block RhoA interaction with specific effectors. As the three peptides were able to significantly prevent RhoA interaction with ROCK, we could validate the biological activity of the peptides by testing the capability of P1-RhoA peptides to interfere with the accumulation of actin stress fibers induced by lysophosphatidic acid (LPA)

in fibroblasts, a phenomenon dependent on RhoA-activated Rho-kinase (ROCK) (Amano et al., 1997). Pretreatment of fibroblasts with each peptide almost completely prevented the accumulation of stress fibers upon LPA triggering. In contrast, the peptide encompassing only the P1 sequence was completely ineffective (Figure 1C). Inhibition was dose dependent with significant effects starting at 10 μ M (data not shown). These results are consistent with the known involvement of these RhoA sites in ROCK activation and with the result of our pull-down experiment. In contrast, LPA-induced membrane ruffling, which is Rac1 dependent (Ridley and Hall, 1992), was unaffected by pretreatment with the peptides (data not shown). Furthermore, in human polymorphonuclear neutrophils, the peptides were unable to block the phorbol myristate acetate (PMA)- and formyl-Met-Leu-Phe (fMLP)-induced activation of the superoxide forming complex NADPH-oxidase, whose activation relies on rac activity (Abo et al., 1991) (Figure 1D). Together, these data show that P1-RhoA fusion effector regions are effective selective inhibitors of RhoA-induced signaling events.

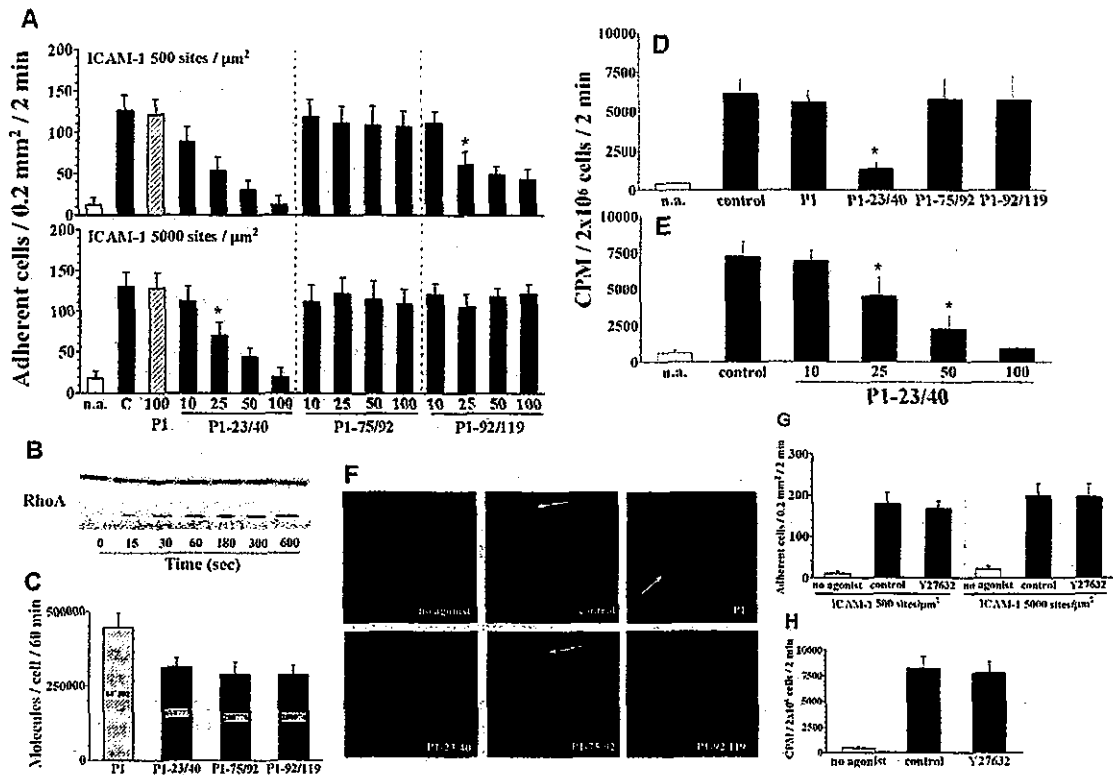


Figure 2. RhoA Controls LFA-1 High-Affinity State and Lateral Mobility Triggering by CCL21
(A) ICAM-1 was immobilized at the indicated site densities. Lymphocytes were treated at 37°C for 60 min with buffer (n.a. and C) or with the indicated μ M doses of P1 or different P1-RhoA peptides and then stimulated for 2 min with buffer (n.a., no agonist) or with 1 μ M CCL21. Values are mean counts of adherent cells in six to nine experiments. Error bars are SDs. * $p < 0.01$.
(B) Lymphocytes were stimulated at 37°C under stirring with 1 μ M CCL21 for the indicated time; time 0 corresponds to no agonist. A protein immunoblot of anti-RhoA in lysates (top) and precipitates (bottom) is shown. One representative experiment of three.
(C) Lymphocytes were treated at 37°C for 60 min with 100 μ M of 125 I-P1 or P1-RhoA peptides. Values are the mean numbers of internalized molecules per cell in three experiments. Error bars are SDs.
(D and E) RhoA controls the induction of LFA-1 high-affinity state by CCL21. Lymphocytes were treated at 37°C for 60 min with buffer (n.a. and control), with 100 μ M of P1 or different P1-RhoA peptides, or **(E)** with the indicated concentrations of P1-23/40 RhoA region, and then stimulated for 2 min with buffer (no agonist) or with 1 μ M CCL21. The mean CPM from 125 I-ICAM-1 in three experiments is shown. Error bars are SDs. * $p < 0.01$.
(F) RhoA controls the induction of LFA-1 rapid lateral mobility on the plasma membrane induced by CCL21. Lymphocytes were treated as described for **(D)**, and then stimulated at 37°C for 2 min with 1 μ M CCL21. Confocal images of LFA-1 surface distribution are shown. Arrows indicate LFA-1 clusters.
(G) ICAM-1 was immobilized at the indicated site densities. Lymphocytes were treated at 37°C 30 min with buffer (n.a. and control) or with 50 μ M Y27632 and then stimulated for 2 min with buffer (no agonist) or with 1 μ M CCL21. Values are the mean counts of adherent cells in three experiments. Error bars are SDs.
(H) Lymphocytes were treated with 50 μ M Y27632. LFA-1 affinity triggering was evaluated as described for **(D)**. Values are from three experiments. Error bars are SDs.

Distinct RhoA Effector Regions Control Chemokine-Induced LFA-1-Dependent Rapid Lymphocyte Adhesion to ICAM-1

To evaluate the role of RhoA in the complex modality of LFA-1 activation by chemokines, we first investigated the involvement of RhoA in CCL21-induced rapid LFA-1-dependent lymphocyte adhesion to ICAM-1 immobilized at either low or high site densities. When lymphocytes were stimulated to adhere to low density of ICAM-1 (~500 sites/ μ m²), both 23-40 and 92-119 P1-RhoA peptides prevented in a dose-dependent manner rapid adhesion induced by CCL21 (Figure 2A). In contrast, the 75-92 P1-RhoA peptide or the P1 peptide was unable

to block rapid adhesion triggering. However, on high density of ICAM-1 (~5000 sites/ μ m²), only the 23-40 P1-RhoA peptide blocked adhesion triggering in a dose-dependent manner, whereas P1, 75-92, and 92-119 P1-RhoA peptides were ineffective (Figure 2A). None of the used peptides interfered with intracellular calcium increase induced by CCL21, thus ruling out potential nonspecific effects (data not shown). RhoA involvement in CCL21-induced LFA-1-dependent lymphocyte adhesion was further corroborated by biochemical analysis showing that CCL21 activated RhoA within seconds, thus displaying kinetics consistent with rapid LFA-1 triggering (Figure 2B). To exclude that the difference be-

Table 1. Quantitative Analysis of the Effect of RhoA, ROCK, and ζ PKC Inhibition on LFA-1 Plasma Membrane Distribution by CCL21

	LFA-1 Distribution on Plasma Membrane	
	Disperse (%)	Clusters (%)
No agonist	88 ± 14	12 ± 8
Control	21 ± 10	79 ± 11
Penetratin-1 (P1)	30 ± 17	70 ± 13
P1-23/40	60 ± 14	40 ± 16
P1-75/92	14 ± 10	86 ± 11
P1-92/119	79 ± 13	21 ± 8
CCL21	23 ± 12	77 ± 14
Myr- α PKC	22 ± 5	78 ± 12
Myr- δ PKC	17 ± 8	83 ± 16
Myr- ϵ PKC	25 ± 10	75 ± 11
Myr- ζ scramble	19 ± 9	81 ± 19
Myr- ζ PKC	81 ± 18	19 ± 7

Lymphocytes were treated with buffer (Control), 50 μ M P1 or different P1-RhoA peptides, 50 μ M of Y27632 or with 100 μ M of PKC myristoylated pseudosubstrate peptides, and then stimulated for 2 min with buffer (no agonist) or with 1 μ M CCL21. At least 100 cells for each data point were analyzed. Values are mean \pm SD percentage of analyzed cells in three experiments.

tween P1-RhoA peptides in lymphocytes could be due to unequal accumulation of the peptides into the cell, we calculated the number of molecules of different peptides loaded per single cell. Although the P1 peptide loaded more efficiently, all three different P1-RhoA peptides showed a similar capability to accumulate inside lymphocytes (Figure 2C).

Together, these data show that RhoA controls LFA-1 activation by CCL21 through the signaling activity of two distinct effector regions.

Distinct RhoA Effector Regions Control the Different Modalities of LFA-1 Activation by Chemokines

Chemokine-triggered lymphocyte arrest in condition of variable density of ICAM-1 relies on distinct intracellular signaling pathways specifically controlling the two modalities of LFA-1 activation (Constantin et al., 2000). Thus, the previous data strongly suggest that RhoA activates both modalities of LFA-1-activation through the distinct signaling activity of 23-40 and 92-119 effector regions. To test this hypothesis, we evaluated the capability of different P1-RhoA peptides to interfere with LFA-1 high-affinity state and rapid lateral mobility triggering by chemokines. Pretreatment with the P1 control or the 75-92 and 92-119 P1-RhoA peptides did not prevent LFA-1 high-affinity state triggering (Figure 2D). In contrast, pretreatment of lymphocytes with the 23-40 P1-RhoA peptide prevented heterodimer high-affinity state induction by CCL21 (Figure 2D). Inhibition of LFA-1 high-affinity state triggering by the 23-40 P1-RhoA peptide was dose dependent and almost complete, with a maximum blockade of about 90% (Figure 2E).

We next determined the involvement of RhoA in the induction of rapid LFA-1 lateral mobility by CCL21. Pretreatment with the P1 control peptide or with the 75-92 P1-RhoA peptide did not prevent LFA-1 rapid lateral mobility induced by CCL21. In contrast, pretreatment with both 23-40 and 92-119 P1-RhoA peptides prevented rapid generation of LFA-1 clusters (Figure 2F and Table 1).

Taken together, these data show that RhoA controls both modalities of chemokine-induced rapid LFA-1 activation by means of distinct effector regions. Interestingly, the 75-92 effector region, involved in ROCK activation, does not appear to have a role in chemokine-induced integrin activation. Notably, 23-40 and 92-119 RhoA regions have been also implicated in ROCK activation (Fujisawa et al., 1998). Therefore, we wished to determine whether ROCK could be an effector in RhoA-dependent LFA-1 rapid activation by chemokines. Pretreatment of lymphocytes with Y27632, a specific ROCK inhibitor (Uehata et al., 1997), did not inhibit CCL21-induced lymphocyte adhesion to ICAM-1 immobilized either at low or high site density (Figure 2G). Furthermore, neither LFA-1 affinity triggering (Figure 2H) nor induction of lateral mobility (Table 1) were blocked by Y27632. These data rule out ROCK as a possible downstream signaling effector linking RhoA to rapid LFA-1 activation by chemokines.

The Atypical ζ PKC Is Involved in Chemokine-Induced LFA-1-Dependent Rapid Lymphocyte Adhesion to ICAM-1

Previous data proposed ζ PKC as potential RhoA effector implicated in Mac1 activation by chemoattractants in polymorphonuclear cells (PMNs) (Laudanna et al., 1998). To evaluate the role of ζ PKC in rapid LFA-1 triggering by chemokines in lymphocytes, we used myristoylated peptides with sequence identical to the pseudosubstrate inhibitory region of ζ PKC (Laudanna et al., 1998). As shown in Figure 3A, blockade of ζ PKC activity inhibited in a dose-dependent manner adhesion triggering to ICAM-1 induced by CCL21. Notably, the inhibitory effect of the ζ PKC peptide was evident only on low density of ICAM-1. Moreover, a control peptide, with a scrambled sequence (Figure 3A), as well as peptides with sequence identical to the pseudosubstrate region of α , δ , and ϵ PKCs (Figure 3B) were completely unable to prevent adhesion triggering either on low or high density of ICAM-1. To further corroborate the involvement of ζ PKC in signaling pathways generated by chemokines and leading to LFA-1 activation, we analyzed the activation state of ζ PKC upon CCL21 stimulation. As shown in Figure 3C, CCL21 induced a consistent and rapid increase of ζ PKC kinase activity. We also evaluated the intracellular distribution of ζ PKC. As shown in Figure 3D, in resting lymphocytes ζ PKC was mainly associated with the particulate fraction, whereas it was almost absent in the cytosol and plasma membrane. Upon triggering with CCL21, ζ PKC rapidly translocated to the plasma membrane fraction. These data show that the atypical ζ PKC is the only PKC isoform expressed in lymphocytes selectively involved in rapid LFA-1 triggering by chemokines. Importantly, ζ PKC seems relevant only to adhesion to low density of ICAM-1.

The Atypical ζ PKC Controls LFA-1 Lateral Mobility but Not High-Affinity State Induction by Chemokines

The previous data suggest a role for ζ PKC in LFA-1 lateral mobility but not high-affinity state induction by CCL21. To validate this hypothesis we determined the

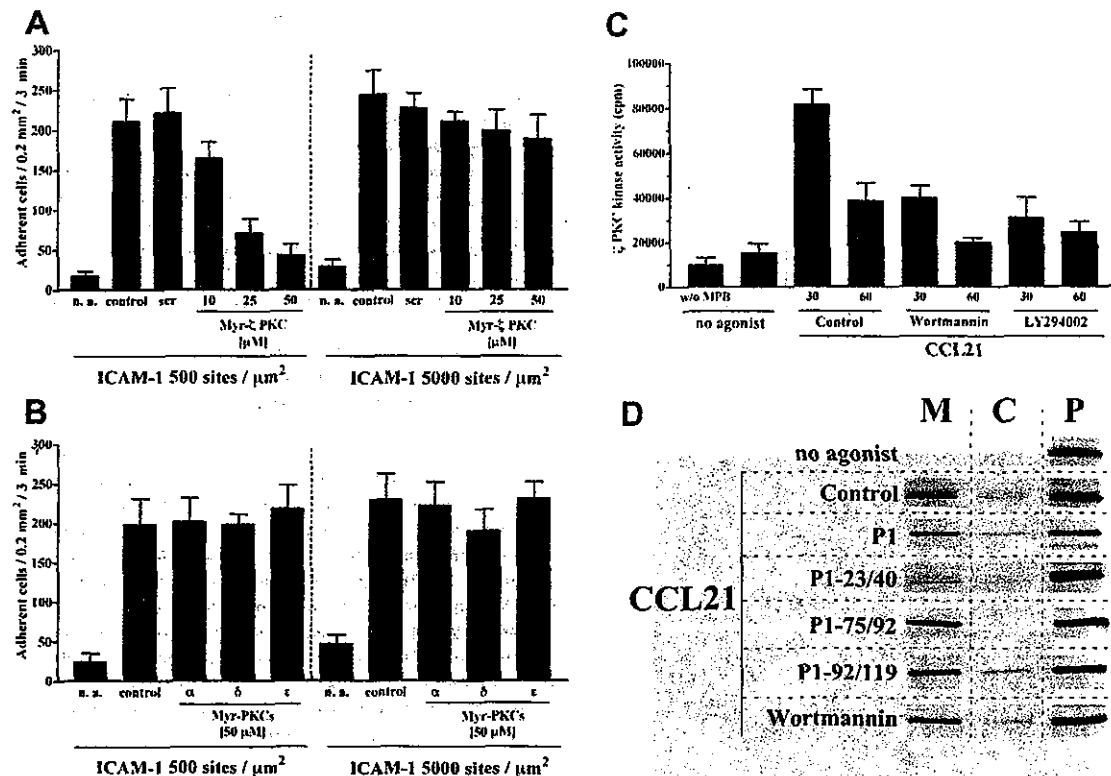


Figure 3. ζ PKC is involved in LFA-1 activation by CCL21

(A) ICAM-1 was immobilized at the indicated site densities. Lymphocytes were treated at 37°C for 60 min with buffer (n.a. and control) or with 50 μM of a scramble peptide (scr) or with the indicated doses of ζ PKC myristoylated pseudosubstrate peptides and then stimulated for 3 min with buffer (n.a., no agonist) or with 1 μM CCL21. Values are the mean counts of adherent cells in five experiments. Error bars are SDs. (B) ICAM-1 was immobilized at the indicated site densities. Lymphocytes were treated at 37°C for 60 min with buffer (n.a. and control) or with the indicated dose of various PKC myristoylated pseudosubstrate peptides and then stimulated for 3 min with buffer (no agonist) or with 1 μM CCL21. Values are the mean counts of adherent cells in four experiments. Error bars are SDs. (C) Lymphocytes were treated at 37°C for 30 min with buffer (no agonist and control) or with 150 nM Wortmannin or 30 μM LY294002 and then stimulated at 37°C under stirring for 30 or 60 s with buffer (no agonist) or with 1 μM CCL21. No agonist w/o MBP is radioactive in the absence of exogenous substrate and is a measurement of ζ PKC autophosphorylating activity in nonstimulated lymphocytes. Values are the mean counts of two experiments performed in duplicate. Error bars are SDs. (D) Lymphocytes were treated at 37°C for 30 min with buffer (no agonist and control), with 100 μM of P1 or P1-RhoA peptides, or with 150 nM Wortmannin and then stimulated at 37°C under stirring for 30 s with buffer (no agonist) or with 1 μM CCL21. Shown are protein immunoblots of cytosolic (C), light membrane (M), and particulate (P) fractions separated on sucrose gradient and probed with anti- ζ PKC Ab.

role of ζ PKC in LFA-1 high-affinity state and lateral mobility induction by CCL21. As shown in Figure 4A, pretreatment of lymphocytes with ζ PKC inhibitory peptides or with a scrambled peptide did not prevent the rapid and transient induction of LFA-1 high-affinity state induced by CCL21. α , δ , and ϵ PKC inhibitory peptides were also unable to block LFA-1 high-affinity state induction (data not shown). However, confocal microscopy analysis showed that pretreatment of lymphocytes with the ζ PKC inhibitory peptide, but not with a scrambled peptide, prevents the accumulation of large LFA-1 clusters rapidly induced by CCL21 (Figure 4B and Table 1). α , δ , and ϵ PKC blocking peptides were completely unable to inhibit LFA-1 cluster formation induced by CCL21 (Table 1).

Together, these data show that classical, novel, and atypical PKCs isoforms expressed in lymphocytes are not involved in LFA-1 high-affinity state triggering by

CCL21. In contrast, the atypical isoform ζ PKC is necessary for LFA-1 lateral mobility on the plasma membrane induced by CCL21.

The Role of PI(3)K and RhoA in ζ PKC Activation by Chemokines

PI(3)K has been previously implicated in LFA-1 lateral mobility (Constantin et al., 2000) as well as in ζ PKC activation (Le Good et al., 1998). As shown in Figure 3C, pretreatment of lymphocytes with Wortmannin or with LY234002, two PI(3)K specific inhibitors, partially prevented the increase of ζ PKC kinase activity induced by CCL21 (about 51% for Wortmannin and 62% for LY294002). In contrast, pretreatment with PI(3)K inhibitor did not affect ζ PKC translocation to the plasma membrane (Figure 3D). Thus, PI(3)K partially mediates CCL21-induced increase of kinase activity but not trans-

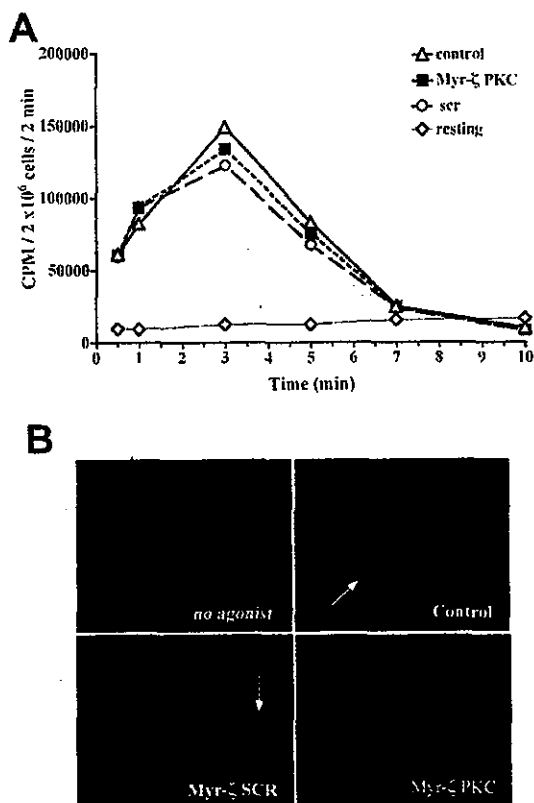


Figure 4. ζ PKC Control LFA-1 Lateral Mobility but Not High-Affinity State Triggering by CCL21

(A) Lymphocytes were treated at 37°C for 60 min with buffer (no agonist and control), or with 50 μ M scramble peptide (scr) or ζ PKC myristoylated pseudosubstrate peptide and then stimulated for 2 min with buffer (no agonist) or with 1 μ M CCL21. The mean CPM from 125 I-ICAM-1 is shown. Values are counts from a representative experiment of three.

(B) Lymphocytes were treated as in (A) and then stimulated at 37°C for 2 min with 1 μ M CCL21. Confocal images of LFA-1 surface distribution are shown. Arrows indicate LFA-1 clusters.

location of ζ PKC to the plasma membrane in lymphocytes.

Chemoattractant-induced ζ PKC translocation to the plasma membrane relies on RhoA activity in PMNs (Laudanna et al., 1998). Having identified RhoA downstream effector regions critical to rapid LFA-1 triggering, we had the possibility to investigate the involvement of distinct RhoA effector regions in ζ PKC translocation induced by chemokines in lymphocytes. As shown in Figure 3D, pretreatment of lymphocytes with P1 peptide or with 75–92 or 92–119 P1-RhoA peptides did not prevent ζ PKC translocation to the plasma membrane. However, pretreatment with the 23–40 P1-RhoA peptide blocked ζ PKC translocation to the plasma membrane; the densitometric analysis showed a blockade of about 82%.

These data show that induction of ζ PKC kinase activity by CCL21 in lymphocytes partially depends on PI(3)K, whereas ζ PKC translocation to the plasma membrane is mainly controlled by a restricted subset of RhoA-

dependent signaling activated by the 23–40 downstream effector region.

RhoA-Dependent LFA-1 High-Affinity State Is the Modality of Integrin Activation Controlling Lymphocyte Homing In Vivo

The data presented above establish diversified roles for RhoA and ζ PKC in controlling distinct modalities of LFA-1 activation. Previous data suggested a role for LFA-1 triggered to high-affinity state in lymphocyte homing to secondary lymphoid organs. However, a formal demonstration has never been provided. The definition of the role of 23–40 RhoA region in LFA-1 high-affinity state triggering by chemokines prompted us to pursue a formal demonstration of the role of LFA-1 triggering to high-affinity state in the recruitment of circulating lymphocytes in vivo.

Pretreatment of lymphocytes with the P1 control peptide or with the 75–92 or 92–119 P1-RhoA peptides did not affect rolling and arrest of circulating lymphocytes on high endothelial venules in the secondary lymphoid organ Peyer's patch (PP-HEV) (Figure 5). Pretreatment with the 23–40 P1-RhoA peptide did not influence lymphocyte tethering and allowed normal interaction with vessels (data not shown). However, this peptide consistently inhibited the stable arrest of lymphocytes on PP-HEV, with about 75% of inhibition ($p < 0.01$); the percentage of cells displaying only rolling increased, as expected. Notably, the site density of ICAM-1 presented to the interacting lymphocytes on PP-HEV was previously shown to be extremely high (about 14,000 site/ μ m²) (Constantin et al., 2000), a condition in which LFA-1 accelerated lateral mobility is not required to rapid arrest. Indeed, the 92–119 P1-RhoA peptide, which only affected LFA-1 rapid lateral mobility induced by CCL21, had no effect on lymphocyte arrest in PP-HEV. As the 23–40 RhoA effector region controls the induction of LFA-1 conformational change by CCL21 (see Figure 2), these data demonstrate that RhoA-controlled heterodimer high-affinity state is the modality of LFA-1 activation critically required for rapid arrest of circulating lymphocytes in PP-HEV.

We also wished to test the role of ROCK kinase and ζ PKC in rapid lymphocytes recruitment to HEV. Lymphocytes pretreated with Y27632 rolled and adhered normally in HEV. Moreover, pretreatment of lymphocytes with the ζ PKC inhibitory peptide did not affect the capability of lymphocytes to roll and arrest on HEV (Figure 5). These data are consistent with the inability of these inhibitors to prevent LFA-1 affinity triggering by CCL21 and exclude a participation of ROCK and ζ PKC in signaling events leading to lymphocyte rapid arrest in PP-HEV.

Discussion

Chemokines are the most powerful physiological activators of lymphocyte integrins (Laudanna et al., 2002). We have previously shown that chemokines play a dual role in LFA-1-mediated rapid lymphocyte adhesion by inducing LFA-1 high-affinity state and lateral mobility (Constantin et al., 2000). Here we have identified intracellular signaling events differently controlling this complex

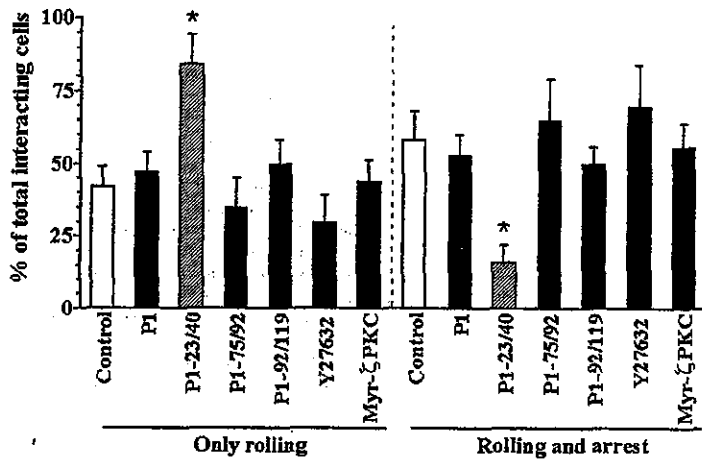


Figure 5. LFA-1 High-Affinity State Mediates Lymphocyte Homing to HEV in Peyer's Patches

Intravital microscopy was performed in Peyer's patch high endothelial venules. Lymphocytes were treated with buffer (control) or with 50 μ M P1 and P1-RhoA peptides, with 50 μ M Y27632, or with 100 μ M of ζ PKC myristoylated pseudosubstrate peptide. Values are the mean percentage of total interacting cells in three experiments. Error bars are SDs. * $p < 0.01$.

phenomenon. We presented data regarding the CCR7 ligand CCL21 which directs T lymphocyte arrest in secondary lymphoid organs (Wamock et al., 2000). However, identical results have been obtained with CCL19 and with the CXCR4 ligand CXCL12 (data not shown), thus supporting the general significance of our findings.

We show that: (1) RhoA controls LFA-1 high-affinity state triggering by chemokines; (2) RhoA also controls LFA-1 lateral mobility induced by chemokines; (3) the signaling activity of two distinct RhoA effector regions controls LFA-1 activation by chemokines; (4) the atypical ζ PKC is critical to LFA-1 lateral mobility but not to high-affinity state triggering; (5) chemokine-induced ζ PKC kinase activity and translocation to the plasma membrane depend, respectively, on PI(3)K and on the signaling activity of the 23-40 RhoA effector region; and (6) rapid arrest of circulating lymphocytes on HEV in secondary lymphoid organs critically depends on the induction of LFA-1 high-affinity state.

RhoA and the Modality of LFA-1 Activation by Chemokines

We showed that plasma membrane translocating RhoA-derived effector regions are useful tools for studying RhoA-dependent signaling events in a region-selective manner. The inhibitory, more than agonistic, activity of the regions is likely due to interference with the plasma membrane docking function of small GTPases, a step required to full activation of downstream effectors (Stokoe et al., 1994). By using these tools, we analyzed the role of RhoA in LFA-1-dependent rapid lymphocyte adhesion. Our data show that RhoA controls LFA-1 conformational change and lateral mobility by chemokines through the distinct, yet complementary, activity of two effector regions, encompassing amino acids 23-40 and 92-119. The 92-119 region is exclusively involved in LFA-1 lateral mobility induction. In contrast, the 23-40 RhoA region participates in heterodimer lateral mobility regulation and is also critical to the induction of LFA-1 high-affinity state. This latter finding is of particular importance. Indeed, the intracellular signaling events controlling LFA-1 high-affinity state triggering by chemokines and the physiological meaning of this event have

never been clarified. The blocking activity of the P1-23-40 peptide on LFA-1 affinity triggering by CCL21 highlighted the critical regulatory role of RhoA in LFA-1 high-affinity triggering. Importantly, blockade of 23-40 RhoA effector region prevented LFA-1-dependent arrest of naive lymphocytes on PP-HEV. Although 23-40 RhoA region also controls LFA-1 lateral mobility, blockade of the 92-119 region, which is only involved in heterodimer lateral mobility, did not interfere with arrest of naive lymphocytes on PP-HEV. This shows that lymphocytes rely on activation of LFA-1 high-affinity state to home to secondary lymphoid organs. This provides the definitive demonstration of the physiological role of the inside-out signal-dependent induction of LFA-1 high-affinity state *in vivo*.

Biochemical analysis showed a rapid and prolonged RhoA activation by CCL21, with RhoA remaining in an active state for at least 10 min. Notably, the induction of LFA-1 high-affinity state by chemokines displays transient kinetics, with integrin affinity completely downmodulated within a few minutes (Constantin et al., 2000). This suggests that downmodulation of LFA-1 high-affinity state, which temporally correlates with downmodulation of lymphocyte rapid adhesion to ICAM-1, does not simply rely on RhoA inactivation. Thus, it is possible that chemokines generate signaling pathways able to actively counterbalance RhoA-dependent pathways leading to LFA-1 conformational changes. Notably, the activation of H-ras and the dependent MAP-kinase prevents the induction of LFA-1 high-affinity state by the chemokine CXCL12 (Weber et al., 2001b). Thus, it is possible that a temporally and spatially regulated balance between the signaling activities of H-ras and RhoA could regulate the dynamics of LFA-1 high-affinity state activation and dependent rapid adhesion.

ζ PKC and the Modality of LFA-1 Activation by Chemokines

To deepen the investigation, we determined the involvement of the atypical ζ PKC in LFA-1 activation by chemokines. ζ PKC was found to be critical for LFA-1 lateral mobility but not high-affinity state triggering by CCL21. Notably, we have previously shown that LFA-1 lateral

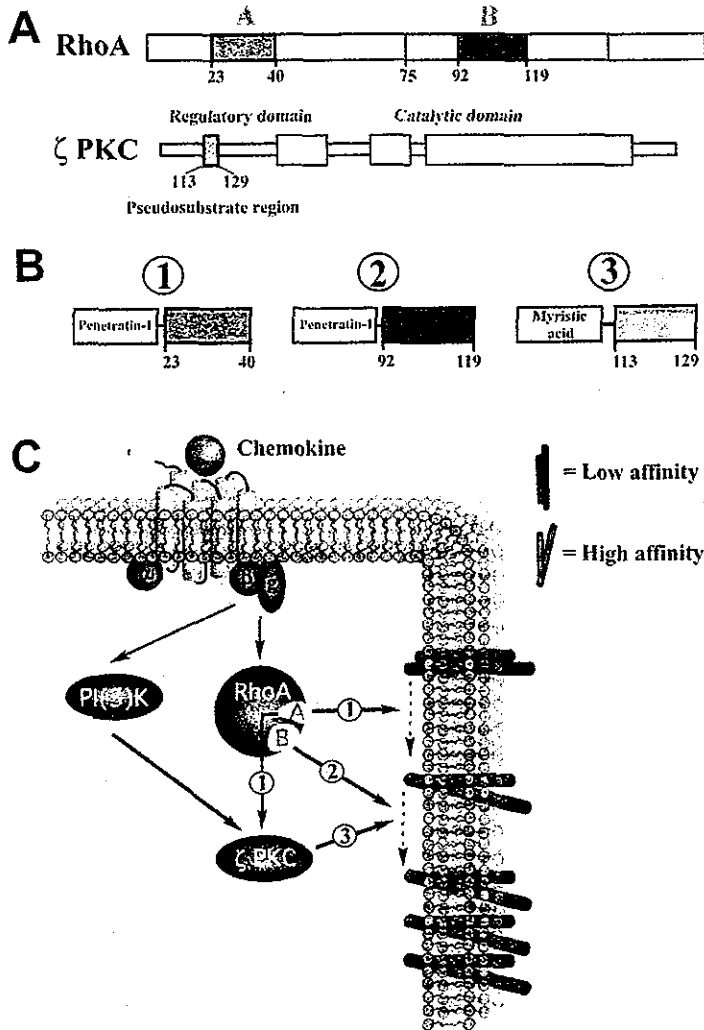


Figure 6. The Distinct Roles of RhoA and ζ PKC in the Different Modalities of Rapid LFA-1 Activation by Chemokines

(A) Site organization of RhoA and ζ PKC showing the effector regions of RhoA (aa 23–40 [A], 75–92 and 92–119 [B]), and the inhibitory pseudosubstrate region of ζ PKC (aa 113–129).

(B) The plasma membrane translocating peptides displaying inhibitory capability on LFA-1 activation. The 23–40 (1) and 92–119 (2) RhoA effector regions were fused to Penetratin-1. A myristic acid was added N-terminal of the pseudosubstrate region of ζ PKC (3).

(C) Induction of LFA-1 high-affinity state by chemokines is controlled by the signaling activity of 23–40 downstream effector region of RhoA (marked with A). Induction of LFA-1 lateral mobility is controlled by ζ PKC and by further signals generated by the 92–119 downstream effector region of RhoA (marked with B). The capability of ζ PKC to control LFA-1 lateral mobility depends on translocation to the plasma membrane, which is controlled by the RhoA 23–40 downstream effector region, as well as by ζ PKC kinase activity. ζ PKC appears to be an effector of both PI(3)K and RhoA mediating LFA-1 lateral mobility induced by chemokines. (1) Inhibition by the P1-RhoA 23–40 peptide. (2) Inhibition by the P1-RhoA 92–119 peptide. (3) Inhibition by the myristoylated peptide with sequence identical to ζ PKC pseudosubstrate region.

mobility is not involved in lymphocyte *in vivo* homing (Constantin et al., 2000). Therefore, it is not surprising that ζ PKC has no role in *in vivo* lymphocyte homing to PP. However, as we previously suggested for PI(3)K, ζ PKC could have a role in lymphocyte recruitment to vessels expressing low density of integrin ligand, a situation in which heterodimer lateral mobility is required for lymphocyte arrest under flow.

Of interest, we also found that the slow LFA-1 clustering induced by PMA is sensitive to ζ PKC blockade (data not shown). Accordingly, we previously showed that PMA, although not an allosteric activator of ζ PKC (atypical PKCs have incomplete C1 region), may trigger ζ PKC translocation to the plasma membrane through RhoA activation (Laudanna et al., 1998). Notably, ζ PKC has a constitutive kinase activity, and this implies that ζ PKC translocation to the plasma membrane may be sufficient to generate ζ PKC-dependent signaling events even in the absence of augmented kinase activity.

The ability of the 23–40 P1-RhoA peptide to block ζ PKC translocation suggests that ζ PKC may mediate,

at least partially, the RhoA capability to control LFA-1 clustering. Moreover, this data suggests a direct interaction between RhoA and ζ PKC, thus establishing ζ PKC as a potential direct downstream effector of RhoA. This possibility is also supported by a previous report (Slater et al., 2001). However, it is also possible that ζ PKC translocation is mediated by other RhoA-dependent signaling events. For instance, PLD is activated by direct binding with the RhoA switch I region (encompassed by the 23–40 sequence) (Dae Bae et al., 1998), and PLD-derived phosphatidic acid (PA) is a powerful ζ PKC activator (Limatola et al., 1997). Thus, it is possible that the inhibitory effect of the 23–40 P1-RhoA peptide on ζ PKC translocation is due to PLD inhibition. Further studies are required to test these possibilities.

The Complexity of the Chemokine-Induced Proadhesive Signaling Network

The data presented here, together with previous reports, further highlight the bewildering complexity of the signaling network generated by chemokines and leading

to integrin activation. Chemokine-triggered LFA-1 lateral mobility is controlled at least by PI(3)K, Cytohesin-1, Rap1, ζ PKC, and RhoA. This brings into question the network relationship that these signaling molecules have. Recruitment of Cytohesin-1 to the plasma membrane requires PI(3)K activity (Nagel et al., 1998). Moreover, PI(3)K-derived PIP3 increases ζ PKC activity, and the PIP3-dependent kinase PDK-1 is a direct activator of ζ PKC (Le Good et al., 1998). Thus, PI(3)K appears to control both Cytohesin-1 and ζ PKC signaling activity leading to LFA-1 lateral mobility and clustering. However, regulation of ζ PKC does not seem to be dependent on only PI(3)K. Indeed, inhibition of PI(3)K does not totally prevent the increase of ζ PKC kinase activity induced by CCL21 and does not block translocation of ζ PKC to the plasma membrane. In contrast, ζ PKC translocation to the plasma membrane is critically dependent on signaling activity of RhoA 23–40 effector region. Altogether, these data show that ζ PKC translocation to the plasma membrane and increase of kinase activity are independently controlled by distinct signaling pathways.

Are both of these regulatory mechanisms of ζ PKC equally relevant to the activation of LFA-1 lateral mobility on the plasma membrane? Inhibition of PI(3)K blocks chemokine-induced, but not PMA-triggered, LFA-1 clustering (Constantin et al., 2000). In contrast, blockade of ζ PKC by inhibitory peptides prevents LFA-1 clustering induced by chemokines as well as by PMA. Thus, ζ PKC translocation to the plasma membrane (which is RhoA dependent in PMA as well as chemokine-triggered signaling) is a very critical biochemical event in LFA-1 clustering. What is the role of the increased ζ PKC kinase activity triggered by chemokines? It is important to emphasize that chemokines trigger within seconds formation of big LFA-1 clusters in lymphocytes, whereas PMA induces smaller LFA-1 clusters over several minutes (Constantin et al., 2000). In this context, we previously hypothesized that PI(3)K, which is not activated by PMA, could be responsible for acceleration in LFA-1 lateral mobility induced by chemokines (Constantin et al., 2000). The critical role of ζ PKC in LFA-1 clustering and the capability of PI(3)K to partially mediate the increase of ζ PKC activity induced by CCL21 allow a further refinement of our previous hypothesis. Thus, it is possible that RhoA-mediated ζ PKC translocation triggered by PMA in the absence of any PI(3)K activation (and thus without increase of ζ PKC activity) may be sufficient to induce a slow LFA-1 lateral mobility on the plasma membrane. In contrast, the very rapid LFA-1 mobility induced by chemokines may require both RhoA-mediated ζ PKC translocation and a concurrent PI(3)K-dependent increase of ζ PKC kinase activity. We then hypothesize that a consistent increase of the kinase activity of ζ PKC is critical to the acceleration of LFA-1 lateral mobility on the plasma membrane observed upon chemokine stimulation and required for rapid lymphocyte arrest in conditions of low density of integrin ligand. It will be of great interest to test this hypothesis under experimental conditions allowing a more accurate evaluation of the kinetics of LFA-1 lateral mobility at the single molecule level.

Importantly, RhoA controls LFA-1 lateral mobility also through the engagement of the 92–119 effector region,

which is not involved in ζ PKC translocation, suggesting that further RhoA-activated signals are necessary to allow LFA-1 mobility on the plasma membrane. Furthermore, recent reports show that additional signals may derive from the activity of the small GTPase Rap1 (Shimonaka et al., 2003). Thus, chemokines trigger at least three independent, yet necessary, signaling pathways controlled by PI(3)K, Rap1, and RhoA, whose integration generates a signaling network controlling LFA-1 accelerated lateral mobility on the plasma membrane.

In conclusion, we show that RhoA and ζ PKC are critical components of the signaling network controlling the complex dynamic of activation of the β 2 integrin LFA-1. The small GTP binding protein RhoA is a central point of diversification of signaling pathways controlling both the modalities of LFA-1 activation induced by chemokines. In contrast, ζ PKC is a point of convergence of signaling events controlling only LFA-1 lateral mobility (Figure 6). Importantly, we formally demonstrate the critical role of LFA-1 affinity triggering in lymphocyte in vivo homing to PP. Our data suggest that a generic, region-nonspecific, pharmacological inhibition of RhoA would not be the most rational approach for a potential pharmacological intervention in RhoA-dependent phenomena. Indeed, a global inhibition of RhoA-dependent signaling activity may prevent LFA-1 high-affinity state triggering and, thus, block lymphocyte recirculation in secondary lymphoid organs, an event that is likely to result in undesired side effects. In contrast, selective blockade of the 92–119 RhoA region could exclusively inhibit LFA-1 lateral mobility without affecting lymphocyte homing to secondary lymphoid organs. This modality of LFA-1 activation was previously postulated to be relevant in the case of variable density of ICAM-1 expressed by the microvasculature, such as during inflammation (Constantin et al., 2000). Thus, the identification of discrete RhoA effector regions controlling distinct modalities of LFA-1 activation may help to devise a more effective pharmacological approach to control leukocyte recruitment during inflammation.

Experimental Procedures

Materials

PKC myristoylated pseudosubstrate peptides (synthesized at Stanford University PAN-facility) were dissolved before use at 1 mM concentration in PBS (pH 7.2). PMA, ketamine, and xylozine were from Sigma; FCS was from Irvine; murine CCL21 and CXCL12 were from Peprotech (London, United Kingdom); CMFDA, CMTMR, and Alexa 488 labeling kit were from Molecular Probes; Texas red-conjugated goat anti-rat antibody was from Jackson ImmunoResearch; murine ICAM-1 was purified from spleens (Constantin et al., 2000).

Generation of Penetratin-1-RhoA Peptides

The Penetratin-1 (P1) fusion protein expression vector pTm3Hb was kindly donated by A. Prochiantz (CNRS, France). Oligonucleotides encompassing human RhoA bases 67–120 (aa 23–40), 223–276 (aa 75–92), and 274–357 (aa 92–119) were inserted between the BamHI and KpnI cloning sites. Recombinant proteins expressed in *E. coli* BL21(DE3)pLysS Gold were purified on heparin columns, dialyzed against PBS, and stored at -80°C . Alternatively, P1, P1-23–40, and P1-75–92 peptides were synthesized by Sigma-Genosys. A glycine was inserted between P1 and RhoA regions to allow flexibility of the peptides. Peptides displayed the following properties: P1, aa 18, mw 2246.78; P1-RhoA 23–40, aa 34, mw 4430.29; P1-RhoA 75–92, aa 34, mw 4254.06; P1-RhoA 92–119, aa 44, mw 5529.83. Lyophilized peptides were dissolved before the experiments.

Specificity of RhoA Peptides and Measurement of RhoA Activation

The specificity of P1-RhoA peptides was evaluated by affinity-precipitation assay using the rho binding region (RBD) from Citron, Rhotekin, and ROCK, as described (Ren et al., 1999; Kimura et al., 2000). Recombinant Val14-RhoA (donated by Dr. A. Hall) was loaded with 1 mM GTP at 37°C for 90 min. Ten micrograms of Citron, Rhotekin, or ROCK GST-RBD conjugated with glutathione beads was mixed with 5 µg of GTP-Val14-RhoA in 50 µl of 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.5 mM GTP, 2 mg/ml BSA (binding buffer). Binding was for 60 min at 10°C. The beads were washed twice with binding buffer and subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel. RhoA background binding to glutathione beads was negligible. In the case of experiments in the presence of P1-RhoA peptides, the regions were previously dissolved in binding buffer at 1 mg/ml.

RhoA activation by CCL21 was evaluated by using the rho binding region (RBD) from Rhotekin. Lymphocytes were lysed on ice in 0.5 ml of 100 mM HEPES buffer (pH 7.5), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 2 mM EGTA, 2 mg/ml BSA, 20 mM benzamide, containing the Complete Protease Inhibitor Cocktail from Roche. Equal amounts of lysates were incubated with GST-RBD (20 µg) beads for 45 min at 4°C. Bound GTP-RhoA was identified by Western blotting using a monoclonal antibody from Santa Cruz.

Evaluation of Actin Stress Fibers Content

Swiss 3T3 mouse fibroblasts were maintained in DMEM containing 10% FCS for 6 days and then starved for 12 hr. Actin stress fibers content was evaluated in permeabilized cells with TRITC-labeled phalloidin as described (Ridley et al., 1992). Analysis was performed by using a Zeiss LSM confocal microscope.

NADPH-Oxidase Activation

Activation of neutrophil NADPH-oxidase was evaluated by measuring reduction of dihydrorhodamine induced by superoxide anion-derived hydrogen peroxide, as previously reported (Laudanna et al., 1998). Human neutrophils were stimulated under stirring at 37°C. Time course of hydrogen peroxide-induced dihydroRhodamine reduction was evaluated using the spectrofluorimeter with 505 of excitation wavelength and 534 of emission wavelength.

In Vitro Rapid Adhesion Assay on ICAM-1

ICAM-1 was purified from mouse spleens and adhesion assays were performed as previously reported (Constantin et al., 2000). In brief, primary naive lymphocytes (about 70% T, 30% B cells) were isolated from peripheral lymph nodes and Peyer's patches from young BALB/c mice (Hartan, Italy). Cells were resuspended at 4×10^6 /ml in PBS, CaCl₂, MgCl₂, 1 mM, 10% FCS (pH 7.2). Adhesion assays were performed on 18-well glass slides coated overnight at 4°C with purified mouse ICAM-1; site density per square micrometer of immobilized ICAM-1 was calculated as reported (Lawrence and Springer, 1991). Twenty microliters of cell suspension was added to the wells and stimulated at 37°C with 5 µl of agonists prior to washing, fixation, and computer-assisted enumeration of bound cells.

Calculation of Internalization Efficiency of the Penetratin-1-RhoA Peptides

Preliminary experiments carried out with fluorescent peptides showed that P1 and P1-RhoA peptides accumulated in a comparable manner in about 95% of lymphocytes (data not shown), as also previously reported (Fenton et al., 1998). P1 and P1-RhoA peptides were labeled with ¹²⁵I using the Bolton-Hunter reagent (Pierce) following instructions from the manufacturer. After loading, cells were rapidly washed three times in PBS, mildly treated with trypsin to remove peptides eventually adsorbed to the outer plasma membrane (Richard et al., 2002), and the internalized radioactivity was measured with a γ counter. Treatment with trypsin removed no more than 5%–10% of total radioactivity. Specific activity was converted in CPM/molecules and then the number of molecules per cell was calculated. Loading efficiency at room temperature or 37°C was identical but was reduced about 40% at 4°C. Loading was linear

between 10 and 150 µM. Peptide loading was detected after 30 min and reached a plateau within 4–8 hr, depending upon peptide concentration. The presence of 10% serum did not affect protein loading.

Measurement of LFA-1 High-Affinity State

Induction of LFA-1 high-affinity state by CCL21 was evaluated by measuring binding of soluble ¹²⁵I-ICAM-1, as we previously described (Constantin et al., 2000). In brief, ICAM-1 was iodinated with ¹²⁵I-NaI by the Chizzonite method. The binding assay was performed at 37°C in a 500 µl Eppendorf tube. Forty microliters of lymphocyte suspension (5×10^7 /ml in PBS containing 1 mg/ml BSA, 2 mM MgCl₂, 1 mM CaCl₂, 1 mM D-glucose [pH 7.2]) was directly layered on 100 µl oil cushion of 2/1 dibutyl/dioctyl phthalates. Lymphocytes were stimulated with 10 µl of PBS containing chemokine (8 µM) and ¹²⁵I-ICAM-1 (5×10^6 CPM corresponding approximately to 2 µg of ¹²⁵I-ICAM-1). The binding reaction was stopped by rapid centrifugation in microfuge. Radioactivity bound to lymphocytes was counted with a γ counter.

Evaluation of LFA-1 Distribution on Plasma Membrane

Analysis and quantification of chemokine-induced LFA-1 surface distribution was determined by confocal microscopy, following the same procedure previously described (Constantin et al., 2000). In brief, lymphocytes were stimulated in suspension under stirring and then immediately fixed in 1% ice-cold paraformaldehyde in PBS (pH 7.4) for 10 min. Cell were washed and incubated with 10 µg/ml of TB213 rat anti-mouse LFA-1 (ATCC) for 30 min on ice, washed three times and then incubated 30 min with Texas red-conjugated goat anti-rat secondary antibody. The washed cells adhered for 30 min at 4°C on 0.1% poly-L-lysine coated round 13 mm glass coverslips and were analyzed with a Carl Zeiss LSM 510 confocal imaging system, with a 63× C-Apochromat objective (NA 1.2). Definition and quantitative analysis of "disperse" and "clustered" morphologies of LFA-1 distribution was as previously described (Constantin et al., 2000).

Measurement of ζ PKC Kinase Activity

The assay was performed as previously described (Laudanna et al., 1998). In brief, lymphocytes were stimulated under stirring with agonists at 37°C. Stimulation was stopped with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.01% SDS, 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 µM phenylarsine oxide, containing the Complete Protease Inhibitor Cocktail from Roche. After 30 min on ice, lysates were centrifuged at 16,000 g for 1 min to remove cell debris. Rabbit polyclonal anti- ζ PKC (1 µg) or control rabbit serum was added to an equal amount of cell lysates, followed by immunoprecipitation with trisacryl protein A. Equal amounts of ζ PKC were immunoprecipitated as confirmed by Western blot analysis (data not shown). After four washings, immunoprecipitates were subjected to the kinase reaction for 30 min at 30°C in 50 µl of kinase buffer containing 0.5 mM EGTA, 10 mM MgCl₂, 20 mM HEPES (pH 7.4), 50 µM ATP, 5 µCi [γ -³²P]ATP, and 2 µg myelin basic protein (MBP) as a substrate. The reaction was stopped by addition of 5% TCA, and the reaction mixture was filtered through phosphocellulose paper. After four rinses with 1% phosphoric acid radioactivity on the filter was determined with a scintillation counter.

Evaluation of ζ PKC Evaluation of Intracellular Distribution

The assay was performed as previously described (Laudanna et al., 1998). In brief, lymphocytes were stimulated under stirring with agonists at 37°C. Stimulation was stopped by diluting the cells in a 10× larger volume of ice-cold PBS. Cells, resuspended in 1 ml of ice-cold PBS containing 8% sucrose, containing the Complete Protease Inhibitor Cocktail from Roche, were sonicated, and the homogenates were centrifuged at 800 × g/10 min to remove nuclei and unbroken cells. The postnuclear supernatant was loaded on discontinuous sucrose gradient (60% sucrose, 30% sucrose) and centrifuged for 120 min at 100,000 × g. The light membrane fraction (plasma membrane) was collected in the 30% layer. Following SDS-PAGE on 10% acrylamide, proteins were electroblotted on nitrocellulose filters, probed with rabbit polyclonal antibodies anti ζ PKC

(Santa Cruz Biotechnology), followed by goat polyclonal anti-rabbit HRP conjugated (Sigma), and developed using ECL (Amersham).

Intravital Video Microscopy Analysis of Lymphocyte-High Endothelial Venule Interaction in Peyer's Patches

Lymphocytes (5×10^6 /ml in DMEM without sodium bicarbonate supplemented with 20 mM HEPES, 5% FCS, pH 7.1) were labeled with either CMFDA or CMTMR for 30 min at 37°C. 30×10^6 labeled cells were injected iv. In situ videomicroscopic analyses were carried out in high endothelial venules (HEV) in the secondary lymphoid organ Peyer's patch as described (Constantin et al., 2000). Cell behavior was analyzed over a period of 20–30 min starting at 2 min after iv injection. Interactions of ≥ 1 s were considered significant and were scored. Cells were considered to be interacting whether they rolled, arrested, or both. Lymphocytes that remained firmly adherent on venular wall for ≥ 10 s were considered arrested.

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