

Myosin light chain kinase mediates eosinophil chemotaxis in a mitogen-activated protein kinase-dependent manner

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Background: Eosinophil migration in the tissue is one characteristic feature of allergic diseases. The CC chemokine eotaxin plays a pivotal role in local accumulation of eosinophils.

Myosin light chain kinase (MLCK) is known to regulate cytoskeletal rearrangement and cell motility by means of phosphorylation of myosin light chain (MLC).

Objective: We have previously shown that mitogen-activated protein (MAP) kinases are important for eosinophil migration. In the present study we hypothesized that MLCK is downstream of MAP kinases, thereby linking the MAP kinase pathway to the activation of cytoskeletal components required for eosinophil chemotaxis.

Methods: Blood eosinophils were purified by using Percoll and anti-CD16 antibody-coated magnetic beads. We investigated the phosphorylation of MLCK and MLC by using the phosphorous 32-orthophosphates-labeled eosinophils. The kinase activity of MLCK was determined by measuring the phosphotransferase activity for the MLCK-specific peptide substrate. The chemotaxis assay was performed in a 48-well Boyden microchamber.

Results: The phosphotransferase activity of MLCK for a substrate peptide was enhanced in eotaxin-stimulated eosinophils. We also found that eotaxin induced phosphorylation of MLCK in vivo in phosphorous 32-orthophosphate-labeled eosinophils. PD98059 (MAP/extracellular signal-regulated kinase inhibitor) or SB202190 (p38 MAP kinase inhibitor) abrogated the eotaxin-induced phosphorylation of MLCK. The phosphorylation of MLC was upregulated by eotaxin. Eosinophil chemotaxis was inhibited by means of pretreatment of the MLCK inhibitor ML-7.

Conclusion: These results suggest that eotaxin regulates MLCK through both extracellular signal-regulated kinase 1/2 and p38 MAP kinase. MLCK activation is a critical step in the cytoskeletal rearrangements leading to eosinophil migration. (*J Allergy Clin Immunol* 2003;111:113-6.)

Key words: Chemotaxis, eosinophil, eotaxin, mitogen-activated protein kinase, myosin light chain kinase

Eosinophils play a pivotal role in the mechanism of allergic diseases, including asthma.¹ Chemotaxis of eosinophils is the single most important event in the pathogenesis of allergic inflammation. CC chemokines, such as eotaxin, RANTES, and monocyte chemoattractant peptide 3, play a crucial role in eosinophil migration into the tissue. The action of eotaxin on eosinophils is mostly mediated by the CC chemokine receptor 3 (CCR3). Eotaxin activates extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein (MAP) kinases in eosinophils, and these kinases are indispensable for eosinophil chemotaxis.² The substrates for MAP kinases that are responsible for eotaxin-induced eosinophil functions have not been determined.

The cellular locomotive function is initiated by the rapid reorganization of the cytoskeletal components actin and myosin, resulting in the generation of the contractile force.³ During this process, the cell is propelled forward by the formation of the anterior pseudopod and the release of adhesive contacts at the rear. Polymerized actin filaments are enriched in the leading edge, whereas myosin provides propulsive force by means of its interaction with actin. The ability of myosin to associate with actin and mediate contraction is modulated by means of the phosphorylation of regulatory light chain by myosin light chain kinase (MLCK), as well as dephosphorylation by myosin phosphatase. MLCK is indispensable for locomotion of many cell types. However, its involvement in eotaxin signaling of eosinophils has not been previously studied.

In the present study we investigate the activation and functional relevance of MLCK in eosinophils after eotaxin stimulation. We show that MLCK is activated through ERK1/2 and p38 MAP kinase and that it is critical for eosinophil chemotaxis.

METHODS

Eosinophil purification

Eosinophils were purified from subjects with mild eosinophilia (4%-7% of peripheral blood leukocytes). Eosinophils donors were either healthy or had mild allergic rhinitis, and blood was obtained at a time when they were relatively asymptomatic and off medication. Eosinophils were isolated by means of sedimentation with 6%

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Abbreviations used

CCR:	CC chemokine receptor
ERK:	Extracellular signal-regulated kinase
HSA:	Human serum albumin
MAP:	Mitogen-activated protein
MLC:	Myosin light chain
MLCK:	Myosin light chain kinase
ROCK:	Rho-associated coiled-coil-forming protein kinase

dextran, followed by centrifugation on 1.088 Percoll density gradient (Amasham Pharmacia Biotech AB, Uppsala, Sweden). The cells were further purified by means of negative selection with anti-CD16 immunomagnetic beads and the magnetic cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). Eosinophils (>99% purity) were then suspended in HBSS (Life Technologies, Grand Island, NY) with 2% FCS in polypropylene tubes coated with 3% human serum albumin (HSA).

Preparation of cytosolic cell extracts and immunoprecipitation

Purified eosinophils (1×10^7 cells/mL) were incubated with phosphate-free RPMI 1640 (Life Technologies) with 2% FCS for 2 hours and then labeled with 1 mCi/mL phosphorous 32-orthophosphates (NEN, Boston, Mass) in the phosphate-free medium for 1 hour. The cells were washed 5 times with HBSS with 2% FCS and incubated with and without PD98059 (Cell Signaling, Beverly, Mass) or SB202190 (CalBiochem, La Jolla, Calif) for 1 hour. The eosinophils (2×10^6 cells) were then stimulated with 10 nmol/L human eotaxin (R&D, Minneapolis, Minn) for 5 minutes and lysed in a lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1 mmol/L Na_3VO_4 , 1 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 1 $\mu\text{g/mL}$ aprotinin, leupeptin, and pepstatin). To avoid spontaneous activation of eosinophils, we used 3% HSA-coated polypropylene tubes for all reactions up to this step. The lysates were incubated with the anti-MLCK (Sigma, St Louis, Mo) or the anti-myosin (Biomedical Technologies, Stoughton, Mass) antibody for 1 hour, followed by means of immunoprecipitation with 20 μL of protein A/G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, Calif) for 2 hours at 4°C. The immunoprecipitates were then subjected to SDS-PAGE and autoradiography.

In vitro kinase assay

The cells were placed in the HSA-coated tubes containing an MLCK kinase buffer (40 mmol/L HEPES [pH 7.0], 5 mmol/L Mg acetate, 0.55 mmol/L CaCl_2 , 1 mmol/L Na_3VO_4 , 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1% Tween-80, 10 $\mu\text{g/mL}$ aprotinin, leupeptin, and pepstatin) after stimulation with eotaxin for 5 minutes. The samples were probe sonicated on ice for 12 seconds 2 times and centrifuged. Each lysate was divided into 2 equal aliquots, one to receive substrate and one to receive no substrate. The kinase reaction was performed by incubating the lysates with 250 $\mu\text{mol/L}$ ATP and 10 μCi [γ -phosphorous 32]-ATP (NEN) in the presence or absence of 300 $\mu\text{mol/L}$ MLCK substrate peptide (H-KKRAARATSNVFA-NH₂; Alexis, San Diego, Calif) for 30 minutes at 30°C. The reaction mixture was added onto Whatman P81 filters (Whatman, Kent, United Kingdom). After drying, the filters were rinsed 10 times with 1% phosphoric acid. The filters were air-dried, and the radioactivity was measured by using a liquid scintillation counter. The MLCK activity was calculated by means of subtraction of the radioactivity without the MLCK substrate from that with the substrate.

Chemotaxis assay

The chemotaxis assay was performed in a 48-well Boyden microchamber (Neuro Probe, Bethesda, Md). Eotaxin was diluted in Gey buffer (Life Technologies) with 0.02% BSA and placed in the lower wells (100 μL) at 10 nmol/L concentration. After incubation of eosinophils at 37°C with and without ML-7 (CalBiochem) for 30 minutes, 100 μL of the cell suspension at 2×10^6 cells/mL was added to the upper well of the chamber, which was separated from the lower well by a 5- μm pore-size, polycarbonate, polyvinylpyrrolidone-free membrane (Nucleopore, Pleasanton, Calif). The chamber was incubated for 60 minutes at 37°C. Then the membrane was removed, followed by fixation and staining for 5 minutes in May-Grünwald solution. The cells that migrated and adhered to the lower surface of the membrane were counted from 10 fields by means of light microscopy. The chemotactic response to buffer was subtracted from that induced with eotaxin with or without the inhibitors.

RESULTS

We investigated whether MLCK was activated in eotaxin-stimulated eosinophils. To this goal, we immunoprecipitated MLCK from eosinophils and performed an immune complex kinase assay by using an MLCK-specific peptide substrate. As shown in Fig 1, A, eotaxin upregulated MLCK activity nearly 4-fold. This increase in MLCK activity was completely inhibited by the MLCK inhibitor ML-7 ($n = 3$). MLCK is activated *in vivo* by means of phosphorylation of critical serine-threonine residues by a group of serine-threonine kinases, including MAP kinases; the latter kinases phosphorylate threonine 43 of MLCK.⁴ We have previously found that ERK1/2 and p38 MAP kinase are activated by eotaxin and that these kinases are critical for eosinophil chemotaxis.² Thus we investigated whether MLCK was downstream of MAP kinases in the eotaxin signaling pathway. To this goal, we metabolically labeled eosinophils with phosphorous 32-orthophosphates and examined MLCK phosphorylation *in vivo*. Eotaxin induced MLCK phosphorylation in eosinophils, which was inhibited by pretreatment with PD98059 (MAP/ERK kinase inhibitor) or SB202190 (p38 MAP kinase inhibitor; Fig 1, B). We have detected an approximately 150-kd form of MLCK, which was phosphorylated *in vivo*. Two low-molecular-weight forms are likely to be degradation products of MLCK and have previously been detected by others.^{5,6} Our results suggest that MLCK is indeed phosphorylated in eosinophils and that this molecule functions downstream of ERK1/2 and p38 in the eotaxin signaling pathway.

Activated MLCK phosphorylates MLC, which results in its association with actin and its subsequent contraction. Next, we examined myosin light chain (MLC) phosphorylation in eotaxin-stimulated eosinophils. As shown in Fig 2, A, eotaxin clearly induced MLC phosphorylation in eosinophils. The results suggest that MLCK activation is functionally linked to the phosphorylation of its immediate target substrate. MLCK is critical for regulation of cell motility.⁷ We therefore studied its involvement in eotaxin-induced eosinophil chemotaxis. The pretreatment of ML-7 significantly reduced eotaxin-stimulated eosinophil chemotaxis in a dose-dependent manner ($n = 3$; Fig 2, B).

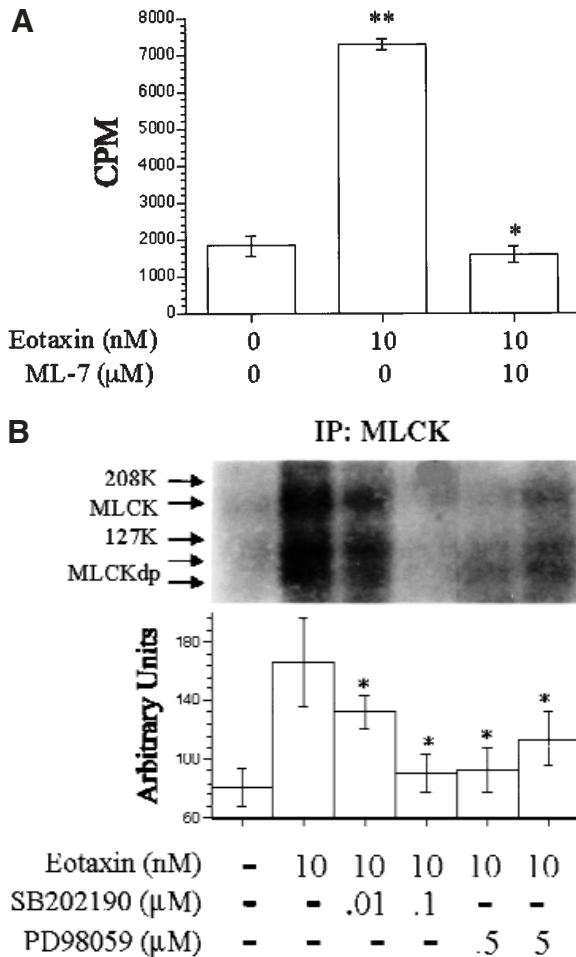


FIG 1. A, Kinase activity of MLCK in eotaxin-stimulated eosinophils. Data are expressed as means $\pm$ SD ($n = 3$). * $P < .05$ versus without inhibitor; ** $P < .05$ versus control (ANOVA). **B**, Effect of PD98059 and SB202190 on eotaxin-induced phosphorylation of MLCK in eosinophils. The upper blot was representative of 3 independent experiments. The lower panel represents the data from the densitometric analysis expressed as means $\pm$ SD ($n = 3$). * $P < .05$ versus eotaxin stimulation (ANOVA).

DISCUSSION

Myosin II is a well-known myosin isoform in non-muscle cells.³ It is concentrated at posterior regions of motile cells and along actin stress fibers in the leading lamellae. The function of myosin II is mainly regulated by means of phosphorylation of the regulatory MLC. Phosphorylation of MLC is critical for the generation of a fully functional actin-myosin motor unit. The latter is involved in generating contractile force necessary for cell motility. Phosphorylation of MLC is catalyzed by a number of calcium-dependent and calcium-independent serine-threonine protein kinases, including protein kinase C, protein kinase A, p21-activated kinase, death-associated protein kinase, Rho-associated coiled-coil-forming protein kinase (ROCK), and MLCK.⁸ We have investigated the importance of MLCK for eosinophil locomotion and

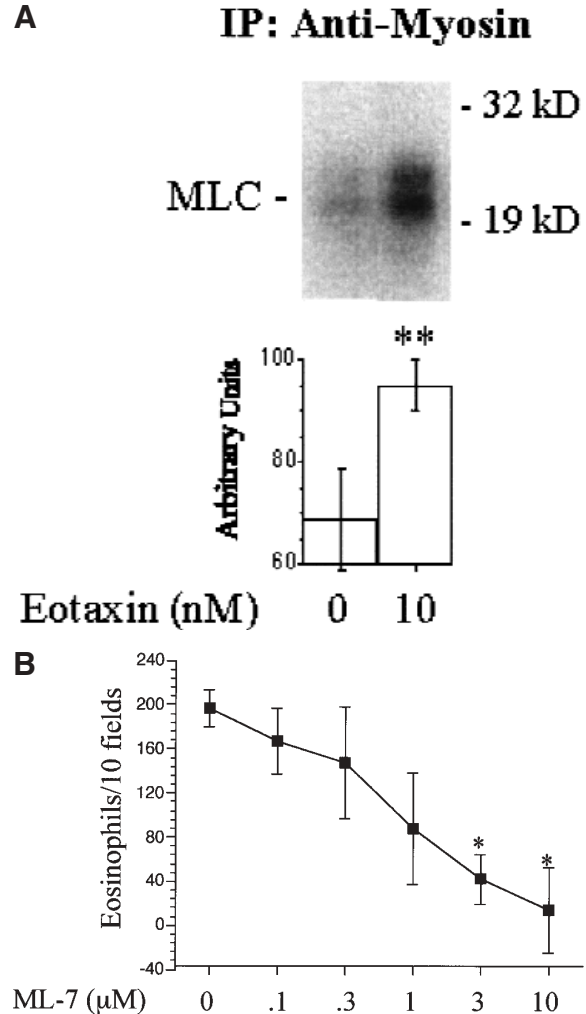


FIG 2. A, Phosphorylation of MLC in eosinophils. The upper blot was representative of 3 independent experiments. The lower panel represents the data from the densitometric analysis expressed as means $\pm$ SD ($n = 3$). ** $P < .05$ versus no eotaxin. **B**, Effect of ML-7 on eotaxin-induced eosinophil chemotaxis. Data are expressed as means $\pm$ SD ($n = 3$). * $P < .05$ versus without inhibitor (ANOVA).

its involvement in CCR3 signaling. Several investigators have reported the involvement of MAP kinases in regulating MLCK function. The activation of ERK1/2 is required for cell migration on a collagen substrate on the basis of its ability to directly phosphorylate MLCK.⁵ In polymorphonuclear leukocytes phagocytosis was mediated by MLCK after activation of MAP kinase.⁹ In accordance with the previous findings, our results reveal an important role of ERK1/2 in modulating MLCK activation in eotaxin signaling. Furthermore, this is the first report showing the regulation of MLCK by p38 MAP kinase. We have previously found that the Rho-ROCK pathway is activated by eotaxin and is important for eosinophil chemotaxis.¹⁰ Interestingly, Rho partially activates ERK1/2, as well as ROCK, in eosinophils. Thus eosinophil migration is regulated by multiple comple-

mentary signaling pathways. The identification of important CCR3 signaling molecules might help develop new strategies for the treatment of allergic diseases.

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