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Novel Association of the Src Family Kinases, Hck and c-Fgr, with CCR3 Receptor Stimulation: A Possible Mechanism for Eotaxin-Induced Human Eosinophil Chemotaxis

Amr El-Shazly,*[,1] Naoto Yamaguchi,† Keisuke Masuyama,* Toshio Suda,† and Takeru Ishikawa*

**Department of Otorhinolaryngology and Head and Neck Surgery, Kumamoto University School of Medicine, Honjo 1-1-1, Kumamoto 860, Japan; and †Department of Cell Differentiation, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860, Japan*

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The chemokine eotaxin is a potent and relatively eosinophil-specific chemoattractant implicated in the cell migration to inflammatory sites in allergic diseases. Eotaxin exerts its activity solely through the CCR3 receptor, but the signaling pathways are poorly defined. In this study, we show that eotaxin induces an increase in tyrosine phosphorylation of multiple cellular proteins in normal human eosinophils. Eotaxin-dependent tyrosine phosphorylation was detected 1 min after stimulation and increased for at least 15 min with kinetics similar to those of eotaxin-induced cell shape changes. Herbimycin A, a tyrosine kinase inhibitor, blocked both eotaxin-induced tyrosine phosphorylation and cell shape changes as well as chemotaxis. Immunofluorescence microscopy analyses showed that eotaxin-induced cell shape changes were accompanied by redistribution of tyrosine-phosphorylated proteins and F-actin reorganization that were sensitive to herbimycin A. Coimmunoprecipitation studies revealed that binding of eotaxin to CCR3 greatly enhanced association of the Src family kinases, Hck and c-Fgr, with CCR3 after internalization of CCR3. These results may indicate that recruitment of Hck and c-Fgr to CCR3 in a compartment triggers tyrosine phosphorylation, leading to rapid cell shape changes required for cell migration.

Eotaxin, a C-C chemokine, was originally identified in the bronchoalveolar lavage fluid from a guinea pig model of allergic airways inflammation and was later found in bronchial epithelial and endothelial cells of asthmatics and nasal mucosa of allergic rhinitis patients (1–5). In allergic inflammatory diseases such as allergic asthma and rhinitis, tissue and/or blood eosinophilia is the most striking feature (6, 7). Eotaxin, which binds only to the seven-transmembrane G-protein-coupled receptor CCR3 abundantly expressed on eosinophils (8–11), possesses the highest selectivity to eosinophils in controlling cell migration among the eosinophil chemoattractants, e.g. RANTES, macrophage inflammatory protein (MIP)-1α, monocyte chemotaxis protein (MCP)-2, MCP-3, MCP-4, eotaxin, and IL-8 (12–17). Eotaxin is therefore thought to play an important role in the pathogenesis of eosinophilic inflammation.

The chemotactic response is essential for accumulation of cells at inflammatory sites. Cell migration following exposure to chemoattractants is preceded by many processes, including cytoskeletal reorganization and cell shape changes. Rapid and reversible polymerization of globular monomeric actin into filamentous polymeric actin (F-actin) initiates cell shape changes. Although the mechanisms of cell migration are of current interest (18, 19), the molecular mechanism triggering cell migration is still unclear.

Eotaxin is reported to induce a rapid increase in F-actin contents in human eosinophils (20). Treatment with inhibitors of tyrosine kinases is shown to modulate activity of eotaxin-induced chemotaxis in human eosinophils, suggesting involvement of tyrosine kinases in eotaxin-mediated signaling (21). The recent availability of specific mAbs directed against CCR3 (9–17) allows us to explore signals generated from CCR3. In this study, we show that binding of eotaxin to CCR3 on normal eosinophils recruits the Src family tyrosine kinases, Hck and c-Fgr, to CCR3 and promotes an increase in tyrosine phosphorylation of mul-

[1] To whom correspondence should be addressed at present address: Department of Otolaryngology and Head and Neck Surgery, St. George's Hospital–London University, Blackshaw Road, SW17 0QT England. Fax: ++44-121-443 4409. E-mail: 106726.2140@compuserve.com.

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tiple cellular proteins required for cell shape changes, leading to cell migration.

MATERIALS AND METHODS

Reagents and antibodies. Recombinant human eotaxin was purchased from PeproTech (London, UK). Pervanadate solution (50 mM) was freshly prepared by mixing equal volumes of 100 mM Na_3VO_4 and 100 mM H_2O_2. The anti-CCR3 mAbs, 7B11 (9) and 5H12 (17), were kindly provided by Drs. W. Newman, C. R. Mackay, and P. D. Ponath (LeukoSite, Inc., Cambridge, MA). Anti-Syk (4D10), anti-Lyn (#sc-15), anti-Hck (#sc-72), and anti-c-Fgr (#sc-130) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (4G10; Upstate Biotechnology Inc., Lake Placid, NY), horseradish peroxidase-conjugated F(ab′)$_2$ of anti-mouse Ig and of anti-rabbit Ig (Amersham Corp., Buckinghamshire, UK), FITC-conjugated F (ab′)$_2$ of anti mouse IgG (BioSource International, Camarillo, CA), and rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) were also used.

Purification of eosinophils. Human normodense eosinophils were isolated from heparanized venous blood of normal volunteers by density gradient centrifugation (56%/71%/78% Percoll discontinuous layers) followed by negative selection with anti-CD16-coated magnetic beads (22), as described previously (21). Briefly, leukocytes were enriched by sedimentation with 6% dextran and subsequent Percoll gradient centrifugation at 20°C for 30 min. The granulocyte fraction was recovered from the pellet and the lowest band, and the contaminated red blood cells were removed by hypotonic lysis. The cells were incubated with CD16 immunomagnetic beads (Miltenyi-Biotec, Germany) for 30 min at 6–12°C. The CD16-negative cells were collected through a MACS column. Eosinophil purity was >98% as judged by Hinkelmann staining, and viability was typically >97%.

Chemotaxis assay. Purified eosinophils were suspended in RPMI 1640 medium containing 10% FCS at 10^6 cells/ml. Then, 50 μl of cell suspension in duplicate was placed in the top wells of a 48-well microchemotaxis chamber (NeuroProbe Inc., Cabin John, MD). The lower wells, separated from the upper by a 5-μm pore polycarbonate membrane, were filled with 29 μl of 100 ng/ml eotaxin or medium alone. After incubation at 37°C for 90 min, cells migrating through the membrane were fixed and stained with Diff-quick. Eosinophils were counted under a microscope in five selected high-power fields (magnification, ×400). Results were expressed as cell number/5 high power fields (5 hpf). Cells were treated with the following reagents: (i) cells were pretreated with 20 μg/ml anti-CCR3 mAb at 4°C for 30 min, and assayed with the same mAb; (ii) cells were preincubated with 5 μM herbimycin A at 37°C for 60 min, washed, and assayed; (iii) cells were assayed in the presence of pervanadate.

Immunofluorescence. Cells were fixed in 3.7% paraformaldehyde at room temperature for 20 min, and permeabilized with 0.1% saponin, as described previously (23). The cells were double-stained with the 4G10 mAb at room temperature for 1 h followed by FITC-anti-mouse IgG for 1 h, and with rhodamine–phalloidin for 30 min. To prevent dephosphorylation, 10 mM Na_3VO_4 was included throughout the staining procedure. Merged confocal and Nomarski differential-interference-contrast images were obtained using a Fluoview laser scanning microscope (Olympus, Tokyo, Japan). Z-series sections were recorded at 0.5-μm intervals and all sections were merged. For FACS analysis, cells were stained with the 5H12 mAb directed against CCR3 at 4°C for 1 h, followed by FITC-conjugated F (ab′)$_2$ fragment of anti-mouse IgG for 1 h, and analysed by a FACScan (Becton–Dickinson). The F-actin contents were measured by FACS analysis precisely as mentioned in Ref. 20.

Western blotting. Cells were washed with Hepes-buffered saline containing 1 mM Na_3VO_4 (pH 7.4) at 4°C, and then lysed in Triton X-100 lysis buffer (50 mM Hepes, pH 7.4, 1% Triton X-100, 4 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 50 μg/ml aprotinin, 200 μM leupeptin, 50 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Lysates were subjected to SDS–PAGE and electrotransferred onto polyvinylidene difluoride membranes for Western blotting, as described elsewhere (24). The immune complexes were washed with Triton X-100 lysis buffer at 4°C, and then analyzed, as described previously (25).

RESULTS AND DISCUSSION

Involvement of tyrosine phosphorylation in eotaxin-induced chemotaxis. To determine if the mAbs directed against the human receptor CCR3 could inhibit eotaxin-induced chemotaxis of normal human eosinophils, chemotaxis assays were performed using two mAbs, 7B11 and 5H12 (Fig. 1A). The 7B11 mAb showed complete blocking activity, whereas the 5H12 mAb showed marginal inhibitory activity (lanes 1–4), consistent with previous observations (9, 17).

Pretreatment of cells for 1 h with herbimycin A, a tyrosine kinase inhibitor, significantly blocked eotaxin-induced chemotaxis (Fig. 1B, lanes 5–7), a comparitive studies using other tyrosine kinases; erbstatin and genestin, showed similar results (Table 1). By contrast, exposure to pervanadate, a tyrosine phosphatase inhibitor, strengthened the activity of eotaxin-induced chemotaxis (compare lane 8 with lane 6). Treatment of cells with pervanadate alone, however, had no effect on random migration activity (Fig. 1B, lanes 9–12). Incubation with herbimycin A or pervanadate under these conditions did not affect cell viability (data not shown). These results suggest that tyrosine kinase activities are involved in eotaxin-induced signal transduction through CCR3 in eosinophil chemotaxis, and that eotaxin-induced signaling is mediated by tyrosine kinases and negatively regulated by tyrosine phosphatases.

Eotaxin-dependent tyrosine phosphorylation potentiated by pervanadate. To determine whether eotaxin indeed induced tyrosine phosphorylation of cellular proteins, normal human eosinophils were stimulated with eotaxin for the indicated times in the presence or absence of pervanadate, and tyrosine phosphorylation was analyzed by Western blotting using the anti-phosphotyrosine mAb 4G10 (Fig. 2A). Without pervanadate we could not detect an increase in tyrosine phosphorylation induced by eotaxin (compare lanes 1–4 with lanes 5–8). In the presence of pervanadate, tyrosine phosphorylation was detected 1 min after stimulation with eotaxin and continued to increase at least 15 min after stimulation (lanes 1–4). It is of note that proteins of smaller molecular sizes (<40 kDa, triple asterisks) were heavily tyrosine-phosphorylated after stimulation. In addition, tyrosine phosphorylation of proteins of larger molecular sizes (>40 kDa, single asterisks) was clearly enhanced. These results demonstrate that eotaxin induces tyrosine phosphorylation of multiple cellular proteins immediately following stimulation, and that inhibition of tyrosine

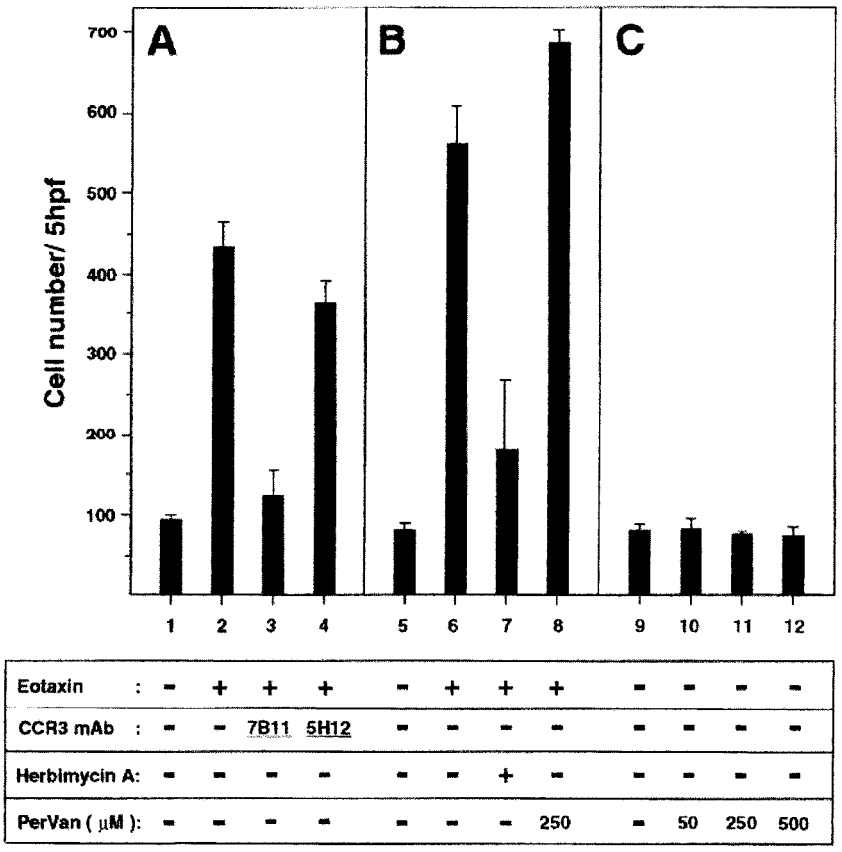

FIG. 1. Modulation of eotaxin-induced chemotactic response. Normal human eosinophils were treated with various reagents and assayed as described under Materials and Methods. Data are the number of cells per 5 high-power fields (5 hpf; ×400) and presented as the mean ± SEM of 7 different experiments (A, B), and of 3 different experiments (C, D), counted from duplicate wells. PerVan, pervanadate. $p < 0.01$, by paired t-test (lanes 7 and 8 versus lane 6).

phosphatase activities by pervanadate ensures the biochemical detection of eotaxin-dependent tyrosine phosphorylation.

Cell shape changes take place before cell migration. To examine whether normal human eosinophils changed their morphology at early stimulation periods, cells were stimulated with eotaxin for the indicated times in the presence of pervanadate and observed under a Normarski differential-interference-contrast microscope. Eotaxin was found to induce cell shape changes as early as 1 min after stimulation and the extent of the shape changes continued to increase for at least 15 min (Fig. 2B, lower panels). These kinetics corresponded to those of an increase in eotaxin-induced tyrosine phosphorylation of cellular proteins (Fig. 2A, lanes 1–4). On the other hand, pervanadate alone essentially had no effect on cell morphology (Fig. 2B, upper panels), nor did pervanadate alone stimulate random migration (Fig. 1C, lanes 9–12).

TABLE 1

Effects of Herbimycin A, Genistein, and Erbstatin on Eotaxin-Induced Normal Human Eosinophils Chemotaxis

	Number of migrated cells/5 hpf	
Treatment of cells	Buffer	Eotaxin (100 ng/ml)
Control	95 ± 2.1	546.6 ± 6.4*
Herbimycin A (5 μM)	100 ± 4.6	222.7 ± 9.1*
Genistein (1 μM)	99 ± 3.6	140 ± 6.1*
Erbstatin (3 μM)	85 ± 8.8	200 ± 4.4*

* $P < 0.01$ by paired t test. Results represent means ± SEM of six independent experiments performed in duplicate.

Blockade of tyrosine phosphorylation and shape changes by herbimycin A. To explore the role of tyrosine phosphorylation in morphological changes, the effects of herbimycin A were analyzed on normal human eosinophils stimulated with eotaxin for 15 min in the presence of pervanadate. As shown in Fig. 3A, when herbimycin A was included in culture medium during the preincubation and subsequent stimulation periods, eotaxin-induced tyrosine phosphorylation of cellular proteins (lane 2) was almost completely blocked (lane 3) to a control background level as seen in

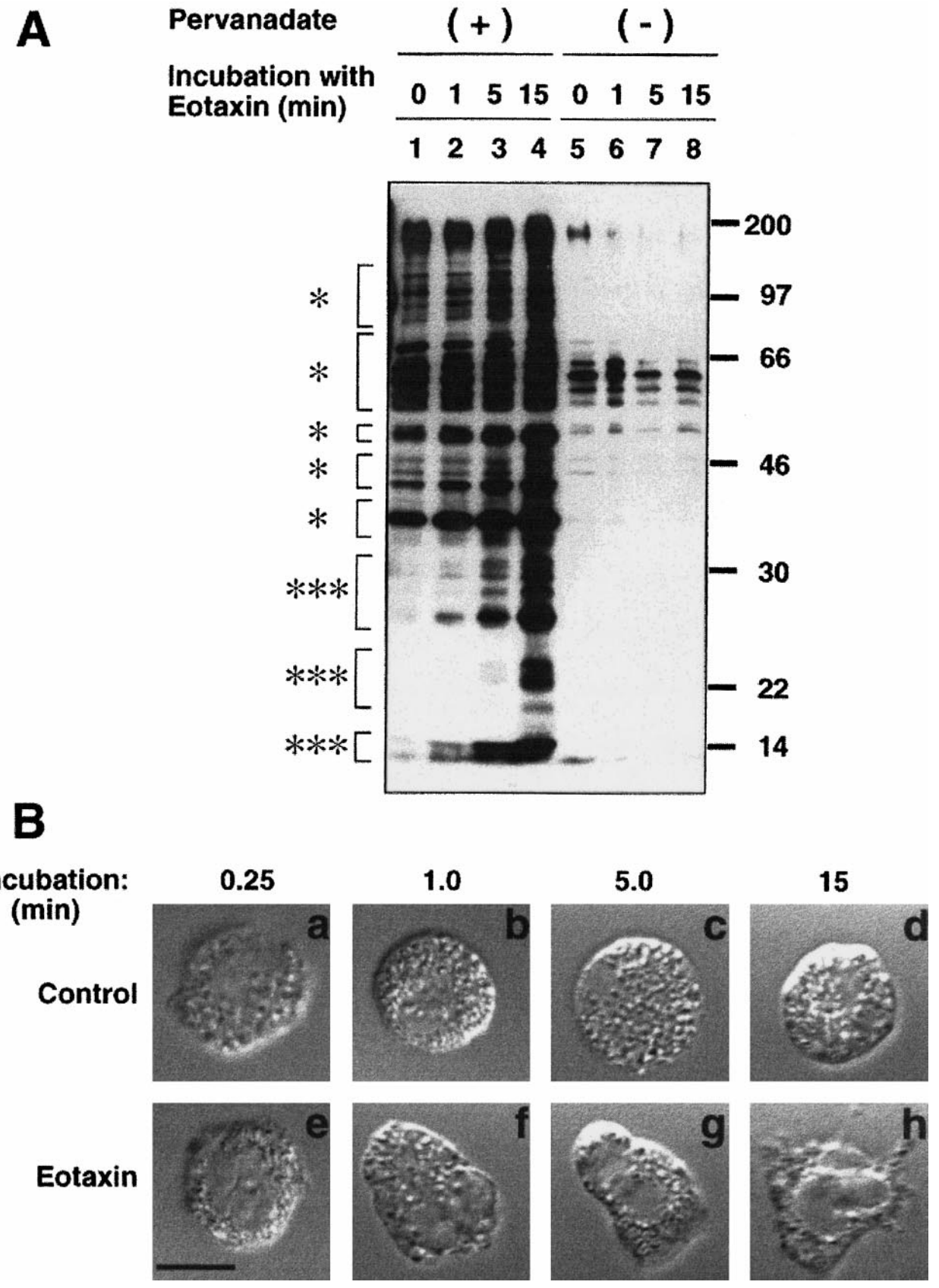

FIG. 2. Relationship between eotaxin-induced tyrosine phosphorylation and morphological changes. (A) Normal human eosinophils were incubated for 0 min (lanes 1 and 5), 1 min (lanes 2 and 6), 5 min (lanes 3 and 7), and 15 min (lanes 4 and 8) at 37°C, with 100 ng/ml eotaxin in the presence (lanes 1–4) or absence (lanes 5–8) of 250 μM pervanadate. Equal amounts of Triton X-100 cell lysates were analyzed by Western blotting with the 4G10 mAb for phosphotyrosine. Molecular size markers are indicated on the right (kDa). (B) Normal human eosinophils were incubated for 0.25 min (a and e), 1.0 min (b and f), 5.0 min (c and g), and 15 min (d and h) at 37°C, with (lower panels) or without (upper panels) 100 ng/ml eotaxin in the presence of 250 μM pervanadate. Cells were fixed and imaged by Nomarski differential-interference-contrast. Bar, 5 μm.

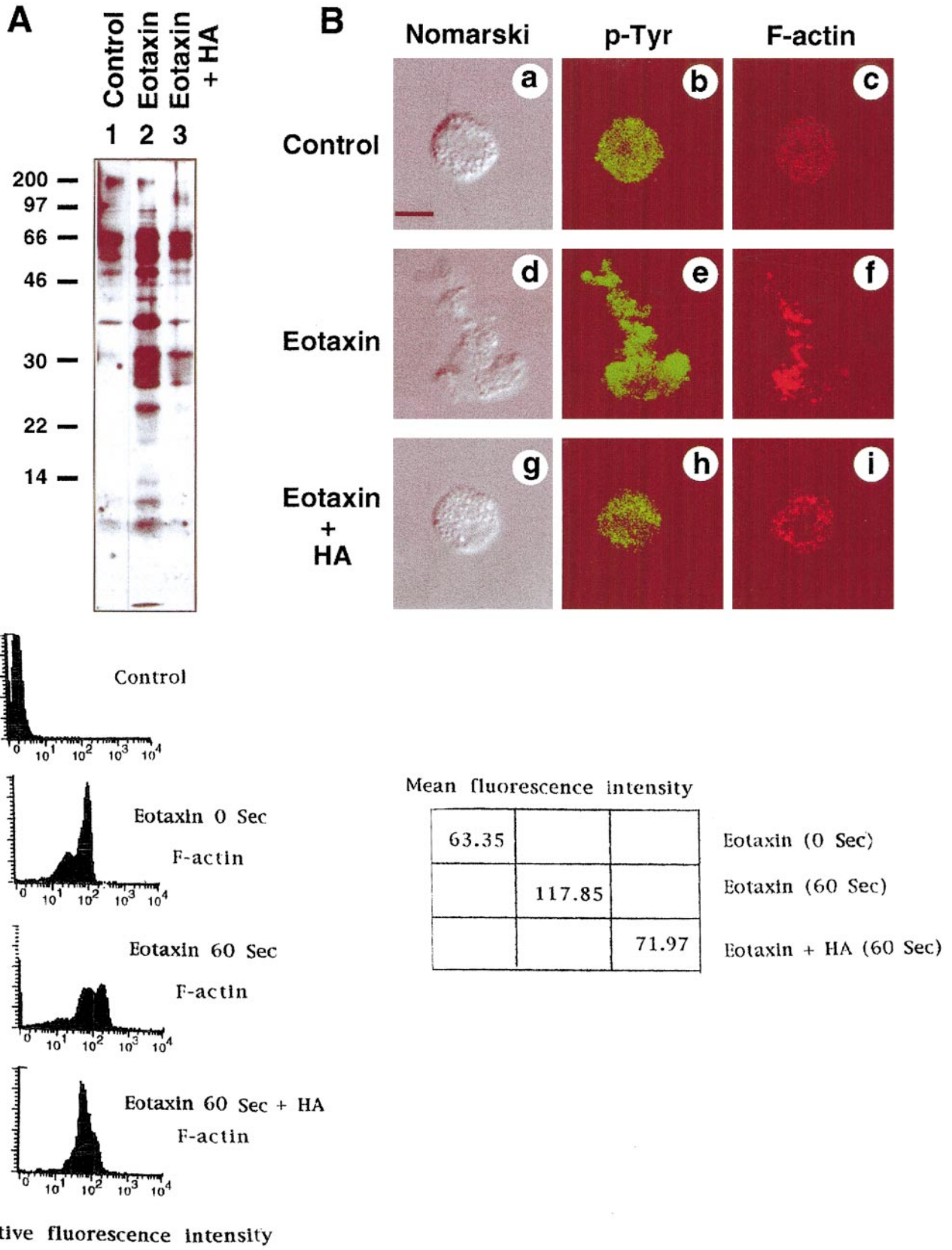

FIG. 3. Effect of herbimycin A on eotaxin-induced tyrosine phosphorylation and morphological changes. (A) Normal human eosinophils were preincubated with 5 μM herbimycin A (lane 3) or DMSO alone (lanes 1 and 2) for 1 h at 37°C, and further incubated for 15 min at 37°C in the presence (lanes 2 and 3) or absence (lane 1) of 100 ng/ml eotaxin. Tyrosine-phosphorylated proteins were analyzed as described in Fig. 2. Molecular size markers are indicated on the left (kDa). (B) Normal human eosinophils were incubated with eotaxin (d, e, and f), eotaxin plus herbimycin A (g, h, and i), or with medium alone (a, b, and c), as described above. The cells were fixed, permeabilized, and dual-stained with anti-phosphotyrosine (green channel; p-Tyr, b, e, and h) and phalloidin (red channel; F-actin, c, f, and i). Nomarski differential-interference-contrast images are shown in a, d, and g. Bar, 5 μM. (C) F-actin contents were measured for the indicated intervals in the presence or absence of herbimycin A.

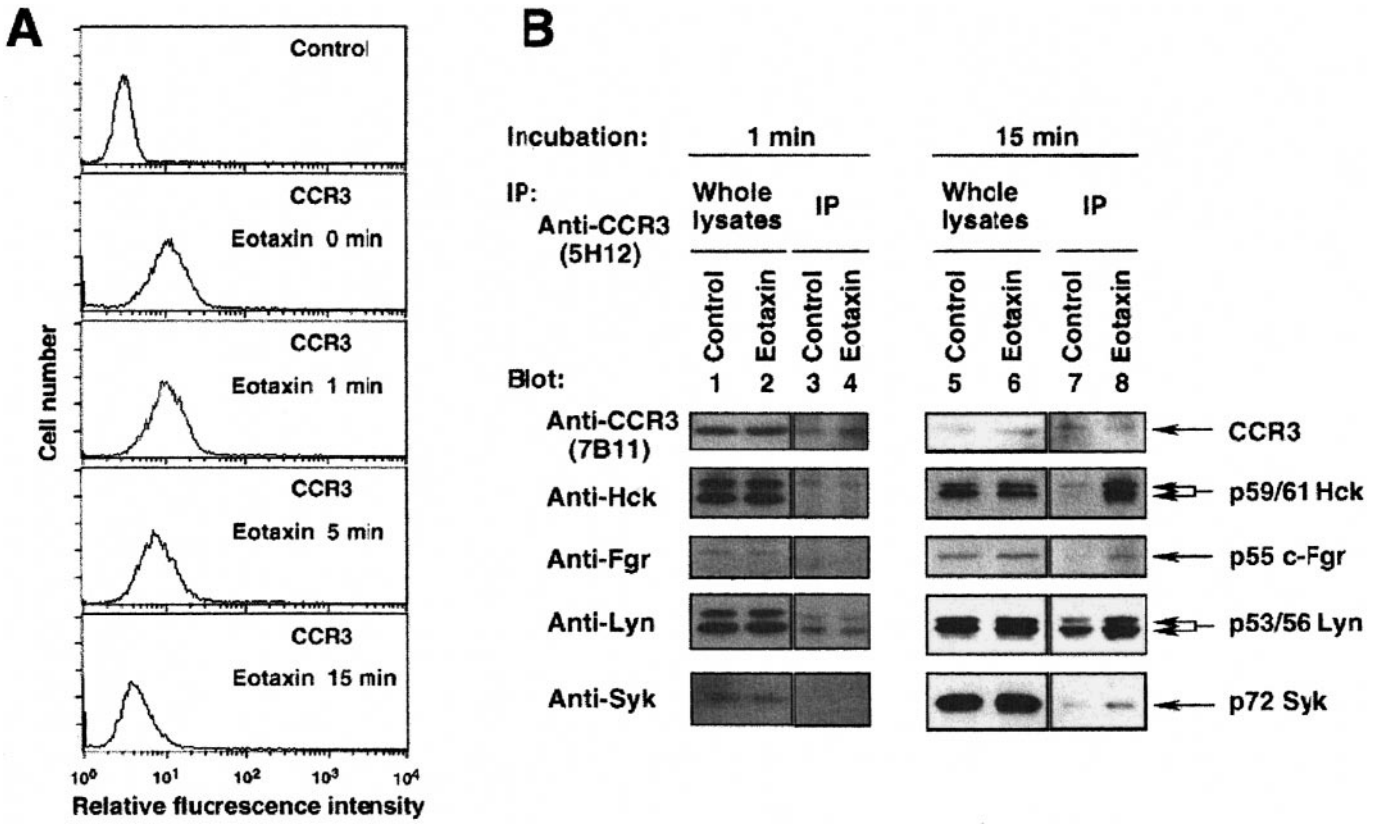

FIG. 4. Association of tyrosine kinases with CCR3. (A) Normal human eosinophils were incubated for 0, 1, 5, and 15 min at 37°C with 100 ng/ml eotaxin in the presence of 150 μM pervanadate. The levels of CCR3 expressed on the cell surface were analyzed by flow cytometry. Control, staining with secondary antibody alone; CCR3, anti-CCR3 (5H12) staining. (B) Normal human eosinophils were incubated for 1 min (lanes 1–4) or 15 min (lanes 5–8) at 37°C. With (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) 100 ng/ml eotaxin in the presence of 250 μM pervanadate. Equal amounts of Triton X-100 lysates (whole lysates, lanes 1, 2, 5, and 6) were analyzed by Western blotting, probed with anti-CCR3 (7B11) and reprobed with anti-Hck, anti-Fgr, anti-Lyn, and anti-Syk. Immunoprecipitates (IP, lanes 3, 4, 7, and 8) with anti-CCR3 (5H12) from the Triton X-100 lysates were analyzed by Western blotting, probed and reprobed as indicated above. CCR3, 45 kDa; Hck, 59/61 kDa; c-Fgr, 55 kDa; Lyn, 53/56 kDa; Syk, 72 kDa.

pervanadate alone (lane 1). Herbimycin A was particularly efficient in inhibiting eotaxin-dependent tyrosine phosphorylation of proteins of smaller molecular sizes (<40 kDa). In addition, cell viability was unaffected under these conditions (data not shown).

Next confocal immunofluorescence microscopic analyses with double staining showed that eotaxin-induced morphological changes (Fig. 3B, d) were accompanied by intracellular redistribution of tyrosine-phosphorylated proteins and F-actin, as well as increase in both the level of tyrosine phosphorylation of cellular proteins and the level of F-actin (compare e and f with b and c & Fig. 3C). Intriguingly, treatment with herbimycin A as described in the legend to Fig. 3A completely blocked eotaxin-induced shape changes (Fig. 3B, g). Furthermore, the distribution of tyrosine-phosphorylated proteins and F-actin in herbimycin A-treated cells were quite similar to those seen in control cells incubated with pervanadate alone (compare h and i with b and c). These results indicate that eotaxin-induced morphological changes, which are accompanied by reorganization of F-actin, require signaling mediated by tyrosine phosphorylation, suggesting that tyrosine phosphorylation induced by eotaxin leads to actin reorganization accompanying cell shape changes.

Recruitment of Src family kinases to CCR3 upon stimulation. Since CCR3, which is the seven transmembrane G protein-coupled receptor for eotaxin, does not possess known kinase motifs (9, 10, 15), it is possible that CCR3 is associated with non-receptor-type tyrosine kinases. Two different anti-CCR3 mAbs, 7B11 and 5H12, were well-characterized as a complete blocker of eotaxin binding to CCR3 and a non-(marginally) blocking mAb, respectively (Refs. 9, 17; Fig. 1A, lanes 3 and 4 in this study). To examine the dynamics of internalization of CCR3, normal human eosinophils were stimulated with eotaxin for the indicated times in the presence of pervanadate, and the levels of CCR3 present on the cell surface were analyzed by flow cytometry using the 5H12 mAb (Fig. 4A).

The values of mean fluorescent intensity were 10.5 at 0 min, 10.75 at 1 min, 8.06 at 5 min, 4.37 at 15 min, and 3.28 as a control. These results indicate that eotaxin induces rapid internalization of CCR3.

Next, expression of typical non-receptor-type tyrosine kinases in normal human eosinophils was examined. Western blotting analyses showed that Syk tyrosine kinase and Src family tyrosine kinases, Hck, c-Fgr, and Lyn, were detected in Triton X-100 lysates (Fig. 4B). CCR3 was also detected in the lysates by Western blotting using 7B11 mAb (Fig. 4B, upper panels). After cells were stimulated with eotaxin for 1 min or 15 min in the presence of pervanadate, protein levels of CCR3 and each tyrosine kinase were unchanged from those seen in control cells incubated for 1 or 15 min with pervanadate alone (compare lane 1 with lane 2, and lane 5 with lane 6), indicating that eotaxin has no effect on the rates of synthesis and degradation of these proteins during incubation period.

To examine whether these tyrosine kinases physically associated with CCR3, CCR3 was immunoprecipitated using the 5H12 mAb from Triton X-100 lysates of control cells incubated with pervanadate alone or of cells stimulated with eotaxin in the presence of pervanadate. The recovery of CCR3 immunoprecipitated from eotaxin stimulated cells was generally comparable to that from control cells (Fig. 4B, upper panels, compare lane 3 with lane 4, and lane 7 with lane 8). Intriguingly, after 15 min stimulation, increasing amounts of Hck and c-Fgr were present in the immune complex of CCR3 prepared from eotaxin-stimulated cells (compare lane 8 with lane 7). The amounts of Lyn and Syk in the CCR3 immunoprecipitate slightly increased after stimulation (lanes 7 and 8). However, after 1 min stimulation, no apparent increase in the amounts of these kinases was observed in the immune complex of CCR3 (lanes 3 and 4). These results suggest that CCR3 forms a complex with these kinases, and that binding of eotaxin to CCR3 greatly enhances the physical association of Hck and c-Fgr with the CCR3 complex in an intracellular compartment. It is therefore likely that following stimulation of normal eosinophils with eotaxin, rapid internalization of CCR3 and subsequent recruitment of Hck and c-Fgr to the CCR3 complex trigger signal transduction through tyrosine phosphorylation mediating cell shape changes leading to chemotaxis.

Cell migration along a chemokine gradient plays a major role in directed cell movement to inflammatory sites. Chemokine-mediated signal transduction is believed to involve (i) Ca^{2+} mobilization, protein kinase C, and heterotrimeric GTP-binding proteins in a classical view (26), and (ii) kinases and phosphatases, adapter proteins, and small GTP-binding proteins in an alternate view (18, 19, 27, 28). Although the chemokine-signaling pathways have not been fully elucidated. Our recent studies with a variety of inhibitors suggest that eotaxin-induced chemotaxis of normal human eosinophils may require tyrosine kinase activities (21).

In this study, we provide the first evidence for the significance of tyrosine phosphorylation in CCR3-mediated chemotaxis. Sufficient association of Hck and c-Fgr with CCR3 in an intracellular compartment may be critical to trigger eosinophil migration by eotaxin. Furthermore, it is likely that a putative protein-tyrosine phosphatase(s) rapidly downregulates eotaxin-dependent tyrosine phosphorylation. Thus, the balance between tyrosine phosphorylation and dephosphorylation of specific proteins is fundamentally important for eotaxin-induced eosinophil chemotaxis. Identification of eotaxin-dependent tyrosine phosphorylated proteins will help us to further understand the molecular mechanism. Finally, we believe that the eotaxin-mediated tyrosine phosphorylation as well as the eotaxin/receptor interaction might be targeted for therapeutic applications aimed at selective prevention of eosinophil accumulation at inflammatory sites in allergic diseases.

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