Cbl and Akt regulate CXCL8-induced and CXCR1- and CXCR2-mediated chemotaxis

Heather C. Lane, Appakkudal R. Anand and **Ramesh K. Ganju**

Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Room 343, Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115, USA

Abstract

CXCL8 (IL-8) plays an important role in the pathogenesis of a variety of inflammatory diseases. However, little is known about the signaling pathways that regulate CXCL8-induced chemotaxis. Here, we found that CXCL8 treatment of CXCR1- and CXCR2-over-expressing L1.2 cells (CXCR1-L1.2 and CXCR2-L1.2, respectively) induced the phosphorylation of Cbl and Akt. The tyrosine kinase inhibitor Tyrphostin A9, phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 as well as proteasome inhibitors significantly blocked the CXCL8-induced chemotaxis of L1.2 cells and human neutrophils. We further found that stimulation with CXCL8 enhanced the association of the PI3K subunit p85 with Cbl. Additionally, over-expression of wild-type Cbl and G306E-Cbl (mutation in the tyrosine kinase-binding domain) inhibited chemotaxis by ~50% as compared with the vector control, whereas the 70Z mutant (deletion in the RING finger domain) did not reduce migration. However, wild-type Cbl or its mutants had no effect on the CXCL8-induced activation of MAPK, indicating that Cbl specifically modulated CXCL8-induced chemotaxis. Furthermore, over-expression of the kinase-dead Akt mutant decreased CXCL8-induced chemotaxis by 60% and diminished Cbl phosphorylation as compared with the vector control. The CXCL8-induced phosphorylation of Cbl was also reduced when cells were pre-treated with the PI3K inhibitor LY294002. Lastly, we have shown that pre-treatment of L1.2 cells with the proteasome inhibitor Lactacystin blocks CXCL8-induced internalization of the CXCR1 and CXCR2 receptors. These studies provide new information regarding CXCL8-induced signaling pathways that may regulate chemotaxis and receptor internalization.

Introduction

Chemokines and their receptors are regulators of cell migration and growth and play an integral role in immune response. These molecules have also been shown to be involved in the pathogenesis of several diseases (1–5). CXCL8 (IL-8) is a potent chemotactic factor for neutrophils (6) and plays an important role in the pathogenesis of a variety of inflammatory diseases such as psoriasis, sepsis, asthma, rheumatoid arthritis and atherosclerosis (7–9). In addition, it has been shown to regulate the angiogenesis and metastasis of various cancers (10, 11). CXCL8 is produced by a variety of cells aside from neutrophils, including monocytes, T lymphocytes, fibroblasts, endothelial cells and epithelial cells, and binds to the chemokine receptors CXCR1 and CXCR2 (12, 13).

Despite the increasingly prominent role of CXCL8 and its cognate receptors CXCR1 and CXCR2 in various cell responses such as inflammation, embryogenesis, wound repair and metastasis (1–5, 14–16), relatively little is known about the molecular mechanisms that mediate CXCL8-induced chemotaxis. Phosphatidylinositol-3 kinase (PI3K), the GTP-binding proteins Rac, Rho and cdc42 as well as phospholipase c have been shown to regulate CXCR1-mediated chemotaxis (17–20). In the present study, we further delineated CXCR1-mediated chemotactic signaling mechanisms and found that Cbl and Akt are key components of this pathway.

Cbl, a 120-kD protein that contains a tyrosine kinase-binding (TKB) domain can function as an adaptor protein by binding to several different proteins. It also contains a RING finger domain and negatively regulates signaling by directing the ubiquitination and degradation of activated receptor tyrosine kinases (21). The Cbl protein has been shown to regulate cell spreading in response to integrin engagement and is involved in the functional organization of the actin cytoskeleton (22, 23). However, the precise role of Cbl in chemotaxis is unclear.

Akt/PKB, which is a cell survival molecule, has recently been shown to be activated by various chemokines including CXCL8 and CXCL12 (24–26). Akt is known to be regulated by PI3K and is involved in the chemotactic signaling pathways of endothelial and other cell types (27). It has also been shown to be recruited to the leading edge of the cell in *Dictyostelium* and neutrophils (28, 29).

In order to understand further the regulatory mechanism of CXCL8-induced and CXCR1/CXCR2-mediated chemotaxis, we investigated the role of various signaling molecules. Our results have shown that Cbl and Akt mediate the CXCL8-induced chemotactic pathway. These proteins most likely act via a complex composed of the p85 subunit of PI3K and Cbl. Furthermore, our work has revealed that Cbl is a negative regulator of chemotaxis and that the RING finger domain of the protein is essential for this activity.

Methods

Reagents and materials

Antibodies to Cbl, phosphotyrosine (PY99), Akt, p85 and Erk 1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). P-Akt antibody was obtained from Cell Signaling (Beverly, MA, USA). HA antibody was obtained from Covance (Princeton, NJ, USA). Proteasome inhibitors were obtained from Alexis Biochemicals (San Diego, CA, USA). Anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). CXCL8 was obtained from Peprotech (Rocky Hill, NJ, USA).

Cell culture

CXCR1-L1.2 or CXCR2-L1.2 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 μg ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 55 μM 2-mercaptoethanol and 0.8 mg ml^{-1} G418 (Invitrogen). CXCR1-L1.2 and CXCR2-L1.2 cells are murine pre-B cells that are stably transfected with the CXCR1 or CXCR2 receptor, respectively. L1.2 cells over-expressing CXCR1 or CXCR2 have ~80% expression of CXCR1 or CXCR2, respectively.

Transfection of cells

Cells were transiently transfected with Metafectene (Biontex), and cells were suspended at a concentration of 5 × 10^5 cells ml^{-1} in medium containing serum. In polypropylene tubes, the DNA–lipid complex was mixed with the following: 1 μg of DNA [pcDNA 3.1, wild-type Cbl, Cbl mutant 70Z, Cbl mutant G306E (kindly provided by Hamid Band of Northwestern University)], wild-type Akt or kinase-dead mutant Akt (kindly provided by Michael Greenberg of Harvard Medical School) and 4 μl Metafectene (for every 1 × 10^6 cells to be transfected), each in solution with 50 μl RPMI. The mixture was incubated for 20 min at room temperature to form the DNA–lipid complex. After 20 min, the solution was added to the cells and incubated at 37°C for 48 h. The cells were then used for the specified experiments. The transfection efficiency was observed to be ~70–80%.

Stimulation of cells

Stimulation of cells was carried out as previously described (30). Briefly, cells were washed with 1× Hank's buffered salt solution (Cellgro) and serum starved for 1 h at 37°C with 1× Hank's at a concentration of 10 × 10^6 cells ml^{-1}. The cells were then treated with 5 nM CXCL8 at 37°C for various time periods. A concentration of 5 nM CXCL8 was used because, upon performing a dose–response curve, we found that this concentration yielded a maximum chemotaxis of the cells. At the end of the stimulation, cells were harvested by centrifugation and lysed in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} antipain, 10 μg ml^{-1} chymostatin, 100 μg ml^{-1} trypsin inhibitor, 10 μg ml^{-1} pepstatin, 10 mM sodium vanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate). Protein concentrations were determined by the Bio-Rad detergent-compatible protein assay.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were carried out as previously described (30). Briefly, equal amounts of protein from the stimulated time points were clarified by incubation with protein A-Sepharose CL-4B or GammaBind™ Sepharose beads (both from Amersham Biosciences) for 1 h at 4°C. The Sepharose beads were removed by brief centrifugation, and the supernatants were incubated with different primary antibodies for 2 h at 4°C. Immunoprecipitation of the antibody–antigen complexes was performed by incubation at 4°C overnight with 50 μl of protein A-Sepharose or GammaBind™ Sepharose (50% suspension). Non-specific interacting proteins were removed by washing the beads thrice with modified RIPA buffer and once with PBS. Immune complexes were solubilized in 50 μl of 2× Laemmli buffer, boiled and subjected to SDS–PAGE. The proteins were transferred onto nitrocellulose membranes, and the membranes were blocked with 5% non-fat milk protein for 2 h at 37°C or overnight at 4°C. The membranes were then probed with primary antibody for 3 h at room temperature or at 4°C overnight. Immunoreactive bands were visualized using HRP-conjugated secondary antibody (Amersham) and the enhanced chemiluminescence system (ECL from NEN).

Western blotting

Equal amounts of protein were solubilized in 50 μl of 2× Laemmli buffer, boiled and subjected to SDS–PAGE. The proteins were transferred onto nitrocellulose membranes. The membranes were then blocked with 5% non-fat milk protein for 2 h at 37°C or overnight at 4°C and probed with primary antibody for 3 h at room temperature or at 4°C overnight. Immunoreactive bands were visualized using HRP-conjugated secondary antibody (Amersham) and the ECL system (from NEN).

Migration assays

Migration assays were performed according to procedures described previously (30). Briefly, cells were re-suspended at 6.6 × 10^6 ml^{-1} in RPMI 1640 medium containing 2.5% FCS. Twenty-four-well plates containing 5 μm porosity inserts (CoStar Corp, Kennebunk, ME, USA) were used for the assays. 600 μl of medium containing 5 nM CXCL8 was added to the bottom wells, and 150 μl of cells was placed in the inserts. A concentration of 5 nM CXCL8 was used because,

upon performing a dose–response curve, we found that this concentration yielded maximum chemotaxis. In one set of experiments, CXCR1-L1.2 cells or isolated neutrophils were untreated or treated with the proteasome inhibitors Epoxomicin and Lactacystin, the tyrosine kinase inhibitor Tyrphostin A9 (31), the tyrosine phosphatase inhibitor Vanadate or the PI3K inhibitors Wortmannin and LY294002. Controls were treated with appropriate solvents and CXCL8 under similar conditions. In another set of experiments, cells were transiently transfected with either vector control (pcDNA3.1) or various vectors containing wild-type Cbl or wild-type Akt and Cbl or Akt mutants. Forty-eight hours after transfection, the cells were used for a migration assay. In all cases after 4 h (1 h for neutrophils), cells that migrated to the bottom wells were collected and counted on a hemacytometer.

Preparation of neutrophils

Neutrophils were isolated by discontinuous Ficoll-Hypaque gradient centrifugation of heparinized blood obtained from disease-free volunteers (32).

Statistical analysis

Statistics were performed using the Student's *t*-test.

Results

Proteasome, phosphatase and tyrosine kinase inhibitors reduce CXCL8-induced chemotaxis

Although CXCL8 is known to be a cytokine important in immune response, to be a potent chemoattractant, and to play a role in a variety of diseases (1, 2), little is known about its chemotactic signaling. Thus, we analyzed stably transfected CXCR1-L1.2 and CXCR2-L1.2 cells to determine their receptor expression. We observed ~80% CXCR1/CXCR2 receptor expression in these cells using flow cytometry analysis (Fig. 1A and B). To delineate the pathways involved in CXCL8-induced, CXCR1- and CXCR2-mediated signaling, we pretreated the CXCR1- and CXCR2-L1.2 cells, as well as human neutrophils, with several types of inhibitors. We found that the specific proteasome inhibitors Lactacystin and Epoxomicin caused a significant, dose-dependent reduction in the chemotaxis of CXCR1-L1.2 cells (Fig. 2A), CXCR2-L1.2 cells (Fig. 2C) and neutrophils (Fig. 2E). At the highest concentration of both Lactacystin and Epoxomicin in neutrophils (50 µM), migration was reduced to <25% of the vehicle control ($P < 0.01$), which was dimethyl sulfoxide (DMSO) for both inhibitors. Similar results were seen in both types of L1.2 cells, although at the highest concentration Epoxomicin reduced CXCR1-mediated migration to 12% of the vehicle control ($P < 0.002$), while Lactacystin reduced migration to 25% of the vehicle control ($P < 0.004$). CXCR2-mediated migration was reduced by Lactacystin and Epoxomicin to 10% ($P < 0.002$) and 5% ($P < 0.001$), respectively, compared with the vehicle control. The tyrosine kinase inhibitor Tyrphostin A9, that has been shown to block the activity of RAFTK (31), also reduced the CXCL8-induced migration of CXCR1-L1.2 cells (Fig. 2B), CXCR2-L1.2 cells (Fig. 2D) and neutrophils (Fig. 2F) in a dose-dependent manner. In both neutrophils and CXCR1-L1.2 cells, the highest concentration of Tyrphostin A9 reduced migration
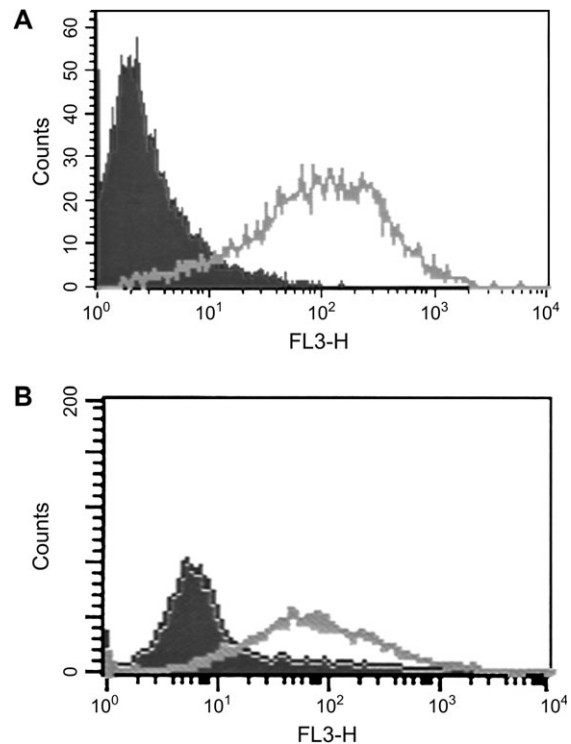
Fig. 1. Receptor expression of CXCR1-L1.2 and CXCR2-L1.2 cells. CXCR1-L1.2 cells (A) or CXCR2-L1.2 cells (B) were stained with cy-chrome antibody (BS Pharmingen) for CXCR1 and CXCR2, respectively, or with cy-chrome control antibody. Cells were then analyzed by flow cytometry. The filled line represents the antibody control and the unfilled line represents the receptor expression.

to <17% of the vehicle control ($P < 0.01$), while in CXCR2-L1.2 cells it reduced migration to <3% of the DMSO vehicle control ($P < 0.003$). In addition, the phosphatase inhibitor Vanadate significantly decreased CXCL8-induced migration, with the highest concentration (10 µM) reducing the migration of CXCR1-L1.2 cells to 17% of the vehicle (water) control ($P < 0.005$) (Fig. 3A) and CXCR2-L1.2 cells to 57% of the vehicle control ($P < 0.03$) (Fig. 3B). Treatment of cells with the PI3K inhibitor LY294002 also caused a block in migration, with both CXCR1-L1.2 and CXCR2-L1.2 cells reduced to ~30% of the vehicle control (DMSO) ($P < 0.02$ for both cell lines) (Fig. 3C and D). However, the MAPK inhibitor PD98059 had no effect on the CXCL8-induced migration of the CXCR1-L1.2 and CXCR2-L1.2 cells as compared with the DMSO vehicle control (Fig. 3E and F). The inhibitor-treated cells were also subjected to viability tests at the end of the migration assay to rule out the possibility that the inhibited cell migration was an artifact resulting from cell death following the inhibitor treatments. All cells were stained with trypan blue to test for the possible toxicity of the inhibitors. However, we found no significant differences between the inhibitor-treated and DMSO control-treated cells, as ~4–7% cell death was seen under both conditions (data not shown). We determined the ED50 values for the toxicity of these inhibitors using various inhibitor concentrations (data not shown). The ED50 values for the inhibitors were as follows: Epoxomicin (400 µM), Lactacystin (600 µM), PD98059 (100 µM), LY294002 (160 µM), Vanadate
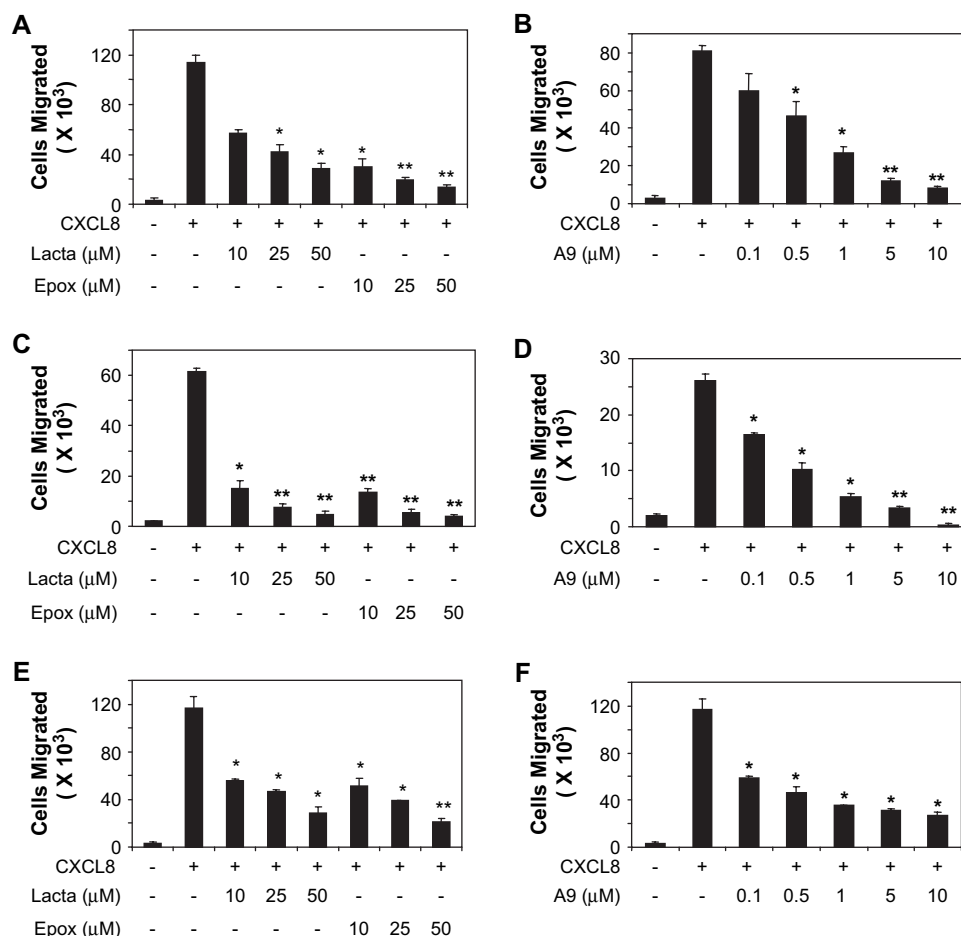
Fig. 2. Effect of proteasome and tyrosine kinase inhibitors on CXCL8-induced chemotaxis. CXCR1-L1.2 cells (A and B), CXCR2-L1.2 cells (C and D) or neutrophils (E and F) were pre-incubated with the indicated concentrations of the proteasome inhibitors Lactacystin (Lacta) and Epoxomicin (Epox) (A, C and E) or Tyrphostin A9 (B, D and F) for 1 h. The cells were then treated with CXCL8 (5 nM) and subjected to chemotaxis assay. The experiments were done in duplicate and are represented as the mean ± SEM; *$P < 0.05$, **$P < 0.005$. The experiments were done three times and representative graphs are shown.

(10 μM) and Tyrphostin A9 (20 μM). These values were at least 3–10 times greater than the highest inhibitor concentration used in our chemotactic assays, with the exception of the value for Tyrphostin A9, which was two times greater than the highest inhibitor concentration used. These inhibitor studies suggest that different signaling pathways play a role in CXCL8-induced chemotaxis.

CXCL8 induces the phosphorylation of Cbl and Akt

We next decided to explore the activation of Cbl and Akt in CXCL8-induced signaling pathways. First, we examined the CXCL8-induced phosphorylation of Cbl. Cbl is known to be an E3 ubiquitin ligase that mediates the ubiquitination of proteins leading to proteasomal degradation (21, 33). CXCR1/CXCR2-L1.2 cells or human neutrophils were treated with CXCL8 (5 nM) and then analyzed for Cbl phosphorylation. We observed that stimulation of CXCR1-L1.2 and CXCR2-L1.2 cells with CXCL8 resulted in the rapid phosphorylation of Cbl (Fig. 4A and B, top panels, respectively). Cbl has been shown to be principally phosphorylated at three different sites: Tyr 700, 731 and 774 (21). We observed increased Cbl phosphorylation at

Tyr 700 in neutrophils following stimulation with CXCL8 by flow cytometry using a phospho-specific Cbl antibody (Fig. 4C). We employed the more sensitive flow cytometry methods using phospho-specific antibodies since we had difficulty in detecting Cbl phosphorylation in activated neutrophils using immunoprecipitation and western blotting procedures. Similar technical difficulties in detecting Cbl phosphorylation in neutrophils have been reported by other authors (34). We further found that CXCL8 induces Akt phosphorylation in the CXCR1-L1.2 and CXCR2-L1.2 cells (Fig. 4D and E, top panels, respectively), as well as in neutrophils (Fig. 4F, top panel). Equal amounts of protein were present in each lane (Fig. 4A–B and D–F, bottom panels).

Cbl and PI3K associate in response to CXCL8 stimulation

In order to further elucidate the signaling mechanisms that govern CXCL8-induced chemotaxis, we analyzed whether Cbl and PI3K associate upon activation by CXCL8. CXCR1-L1.2 and CXCR2-L1.2 cells were treated with CXCL8, and cell lysates were analyzed for the association of Cbl with p85. Figure 5 shows the increased association of Cbl with p85 in
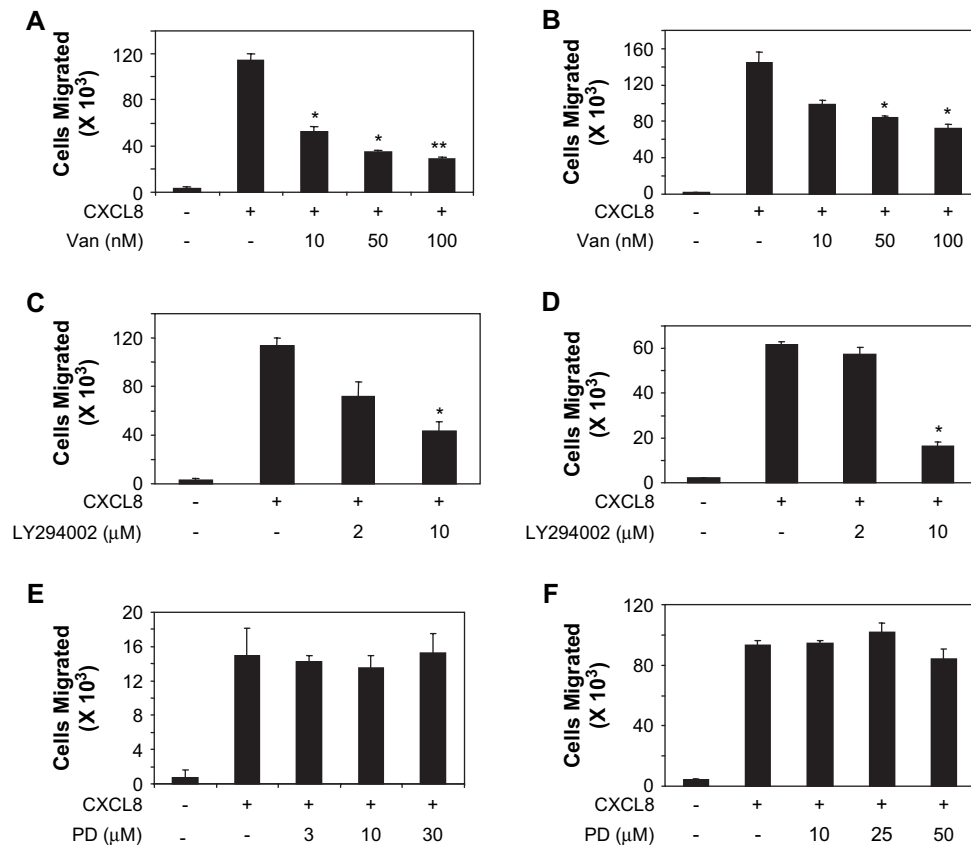
Fig. 3. Effect of phosphatase, MAPK and PI3K inhibitors on CXCL8-induced chemotaxis. CXCR1-L1.2 (A, C and E) or CXCR2-L1.2 (B, D and F) cells were pre-incubated for 1 h with the indicated concentrations of the phosphatase inhibitor Vanadate (Van) (A and B), the PI3K inhibitor LY294002 (C and D) or the MAPK inhibitor PD98059 (PD) (E and F). The cells were then treated with CXCL8 (5 nM) and subjected to chemotaxis assay. The experiments were done in duplicate and are represented as the mean ± SEM; *$P < 0.05$, **$P < 0.005$. The experiments were done three times and representative graphs are shown.

both CXCR1-L1.2 cells (A) and CXCR2-L1.2 cells (B) upon stimulation with CXCL8 (5 nM). This data indicates that a signaling complex comprised of Cbl and the PI3Ks subunit p85 is formed in response to CXCL8.

Cbl modulates CXCL8-induced chemotaxis

We further investigated the role of Cbl in CXCL8-induced, CXCR1- and CXCR2-mediated chemotaxis by transiently transfecting CXCR1-L1.2 and CXCR2-L1.2 cells with wild-type Cbl, the Cbl mutants 70Z-Cbl and G306E-Cbl or with the vector control (pcDNA 3.1). We then examined their effects on the cells in response to CXCL8 treatment (5 nM). We found that wild-type Cbl reduced the CXCR1-L1.2 cell migration to ~50% of the vector control ($P < 0.027$) (Fig. 6A), and decreased the migration of CXCR2-L1.2 cells to ~60% of the vector control ($P < 0.05$) (Fig. 6C). 70Z-Cbl, that lacks the 17 amino acids near the RING finger region and has previously been reported to activate the transforming potential of Cbl, restored the level of the CXCL8-induced migration to that of the vector-transfected control cells in both CXCR1- and CXCR2-L1.2 cells (Fig. 6A and C). The G306E mutant of Cbl (in which the glycine at position 306 in the TKB domain is replaced by glutamic acid) also inhibited cell migration to a similar degree as the wild-type Cbl. The success of the transfection was

confirmed by showing over-expression of Cbl in the lysates of the wild-type- and mutant-transfected cells as compared with those transfected with the vector control (Fig. 6B), or by showing expression of HA in the cells (the wild-type Cbl and Cbl mutants have an HA tag) (Fig. 6D).

Cbl has no effect on the phosphorylation of Erk 1/2

Because the MAPK pathway is known to be involved in chemokine signaling and migration (35–39), we analyzed by western blotting whether stimulation of transfected CXCR1-L1.2 and CXCR2-L1.2 cells with 5 nM of CXCL8 showed any effect on the phosphorylation of Erk 1/2 MAPK. While CXCL8 did induce phosphorylation of Erk 1/2 in both the CXCR1-L1.2 (Fig. 7A, top panel) and CXCR2-L1.2 (Fig. 7B, top panel) cells, there was no difference in phosphorylation between the L1.2 cells when transfected with wild-type Cbl, 70Z-Cbl, G306E-Cbl or the vector control. Equal amounts of protein were present in each lane (Fig. 7A and B, bottom panels). These data suggest that Cbl has no effect on CXCL8-induced MAPK activation.

Mutant Akt blocks migration

Since we found that Akt is phosphorylated upon stimulation with CXCL8, we next examined cells over-expressing either
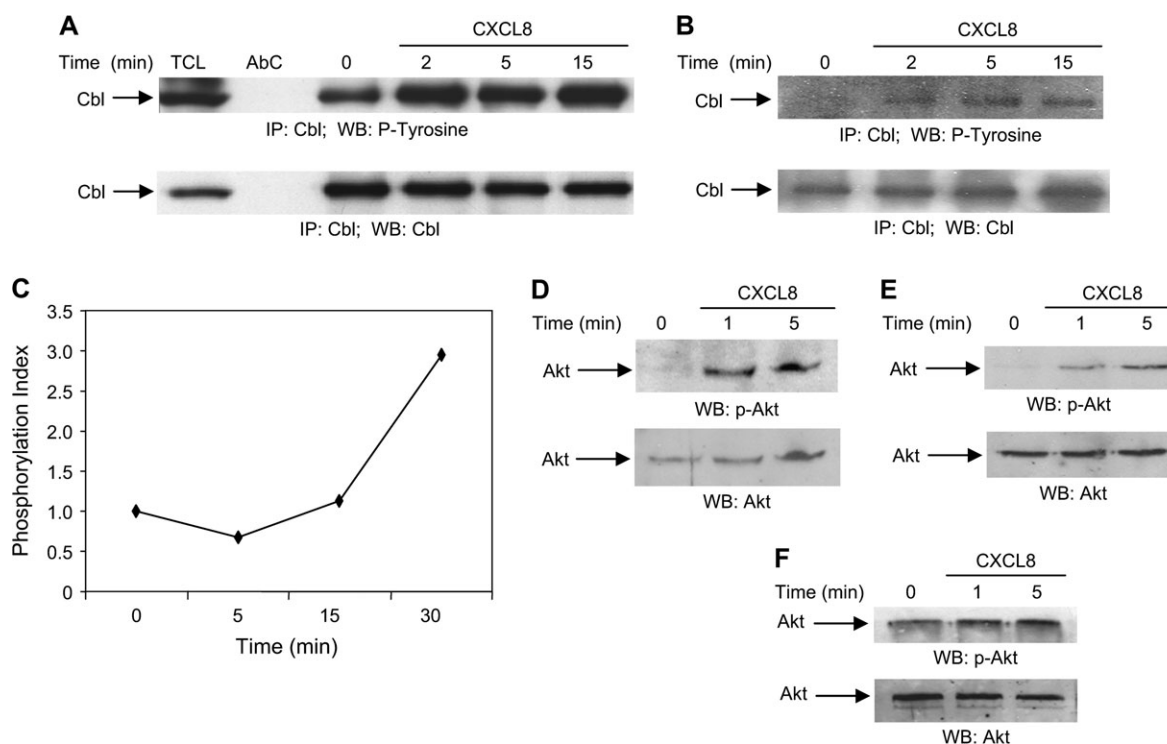
Fig. 4. CXCL8 induces the phosphorylation of Cbl and Akt. CXCR1-L1.2 cells (A and D), CXCR2-L1.2 cells (B and E) or human neutrophils (C and F) were unstimulated (0) or stimulated with CXCL8 (5 nM) and then lysed at the indicated time points. Cell lysates (1 mg) were immunoprecipitated (IP) with anti-Cbl antibody and the complexes resolved on SDS–PAGE, then western blotted (WB) with anti-phosphotyrosine antibodies (PY99, 4G10) (A and B, top panels). Total protein was detected by probing the blots with anti-Cbl antibody (A and B, bottom panels). Cell lysates (50 µg) were also run on SDS–PAGE and immunoblotted with anti-phosphorylated Akt (p-Akt) (D–F, top panels). Total protein was detected by probing the blots with anti-Akt antibody (D–F, bottom panels). The phosphorylation of Cbl was determined by performing flow cytometry analysis in whole cells after stimulation with CXCL8 (5 nM), using staining for Cbl Tyr 700. For the quantitative analysis of protein phosphorylation, the ratio of phosphorylation versus total protein in each sample was obtained by flow cytometry. The phosphorylation index for Cbl, as shown in the graph (Fig. 4C), was determined by calculating the value of this ratio in each sample and presenting the ratio as the fold increase over the control sample. Each experiment was performed three times and representative ones are shown. AbC = antibody control, TCL = total cell lysate.

wild-type Akt or a kinase-dead mutant of Akt for any effects on migration. CXCR1-L1.2 and CXCR2-L1.2 cells were transiently transfected with pcDNA3.1, wild-type Akt or mutant Akt (both types of Akt DNA had HA tags) and, 2 days after transfection, were exposed to CXCL8 in a migration assay. Two concentrations of CXCL8 were used to rule out the possibility that the transfection of the cells merely modified the efficacy of the chemotactic gradient. As shown in Fig. 8, CXCR1-L1.2 and CXCR2-L1.2 cells over-expressing wild-type Akt showed no difference in migration as compared with the vector control. The kinase-dead mutant of Akt, however, blocked CXCR1-L1.2 cell migration by ~60–70% when using either a 5 nM (Fig. 8A) or a 1 nM (data not shown) concentration of CXCL8. CXCR2-L1.2 cell migration was reduced by only 25% when using 5 nM of CXCL8 (Fig. 8C) and by 40% with 1 nM of CXCL8 (data not shown). These results indicate that Akt is important for CXCL8-induced chemotaxis. CXCR1-L1.2 (Fig. 8B) and CXCR2-L1.2 (Fig. 8D) cells were checked for successful transfection by using immunoprecipitation with Akt antibody and western blotting with HA antibody.

Akt regulates Cbl phosphorylation

In order to further study the effects of Akt on the regulation of chemotaxis by Cbl, we transiently transfected CXCR1-L1.2 and CXCR2-L1.2 cells with pcDNA3.1 (vector control), wild-type Akt or mutant (kinase-dead) Akt. We found that Akt modulates the phosphorylation of Cbl. While over-expression of wild-type Akt had little effect on Cbl phosphorylation (data not shown), over-expression of the kinase-dead Akt mutant, which impedes CXCL8-induced chemotaxis, significantly diminished Cbl phosphorylation in CXCR1-L1.2 (Fig. 9A, upper panel) and CXCR2-L1.2 (Fig. 9B, upper panel) cells as compared with cells transfected with the vector control. Equal amounts of protein were present in each lane (Fig. 9A and B, lower panels). Densitometric imaging analysis showed that the intensity of phosphorylation decreased 3- to 4-fold from the vector to the Akt mutant in a comparison by time point. Furthermore, pre-treatment of untransfected CXCR1-L1.2 cells with the PI3K inhibitor LY294002 also significantly reduced CXCL8-induced Cbl phosphorylation as compared with the DMSO solvent control-treated cells (Fig. 9C, upper panel). The protein levels were similar in all lanes (Fig. 9C, lower panel). This suggests that the PI3K/Akt pathway regulates the CXCL8-induced phosphorylation of Cbl.

Proteasome inhibitor blocks receptor internalization

To determine whether the proteasomal activity had any effect on receptor internalization, we pre-treated CXCR1- and
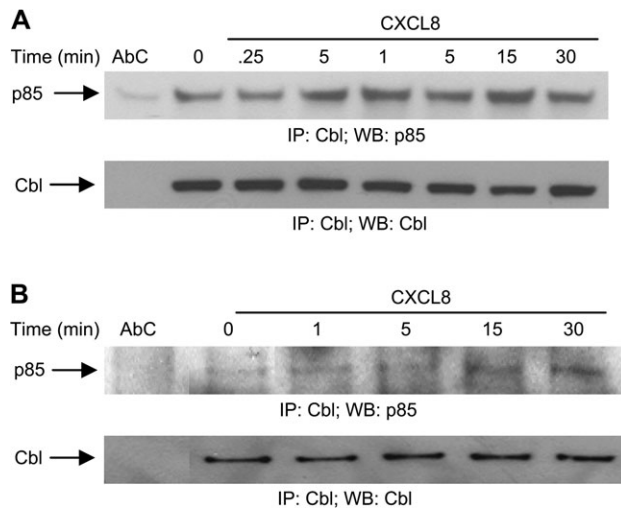
Fig. 5. CXCL8 enhances the association of Cbl with p85. Lysates of CXCR1-L1.2 cells (A) and CXCR2-L1.2 cells (B), prepared from un-stimulated (0) or CXCL8-stimulated (5 nM) cells for the indicated time points, were immunoprecipitated (IP) with anti-Cbl antibody. The complexes were resolved on SDS-PAGE and western blotted (WB) with anti-p85 antibody to analyze the association of p85 with Cbl in response to CXCL8 (A and B, upper panels). The blots were stripped and re-probed for total protein with anti-Cbl antibody (A and B, lower panels). The experiments were done in duplicate. Representative blots are shown. AbC = antibody control.

CXCR2-L1.2 cells with the proteasome inhibitor Lactacystin for 1 h and then stimulated the cells with CXCL8 (1 μg ml^{-1}) at 37°C. Cells were stained for the CXCR1 and CXCR2 receptors and their expression was analyzed by flow cytometry in order to determine receptor internalization. We found that Lactacystin treatment inhibited ligand-induced internalization in the CXCR1-L1.2 (Fig. 10A) and CXCR2-L1.2 (Fig. 10B) cells. This indicates that proteasomal activity is important for both cell migration and receptor internalization and that proteasome inhibitors may inhibit cell migration by blocking this internalization.

Discussion

CXCL8 acts as a potent chemoattractant for neutrophils. Moreover, CXCL8-induced and CXCR1/CXCR2-mediated chemotaxis has been reported to play a key role in inflammatory responses and in the defense against infectious agents (1–7). Recently, CXCL8 was also shown to increase the motility of cancer cells and to enhance tumor angiogenesis and metastasis (10, 11, 15, 16). However, the signaling pathways that regulate CXCL8-induced chemotaxis have not been fully elucidated. In the present study, we have further explored CXCR1/CXCR2-mediated chemotactic signaling mechanisms and have shown that Cbl and Akt are key components of this pathway. Previous studies have found that Cbl can recruit ubiquitin-carrier enzymes or ubiquitin-conjugating enzymes as well as direct the multi-ubiquitination and degradation of various proteins (40, 41). Cbl also contains multiple protein interaction motifs that form complexes with several signal transducers and regulators (42–44). It has been suggested that the tyrosine phosphorylation of Cbl regulates its ubiquitin
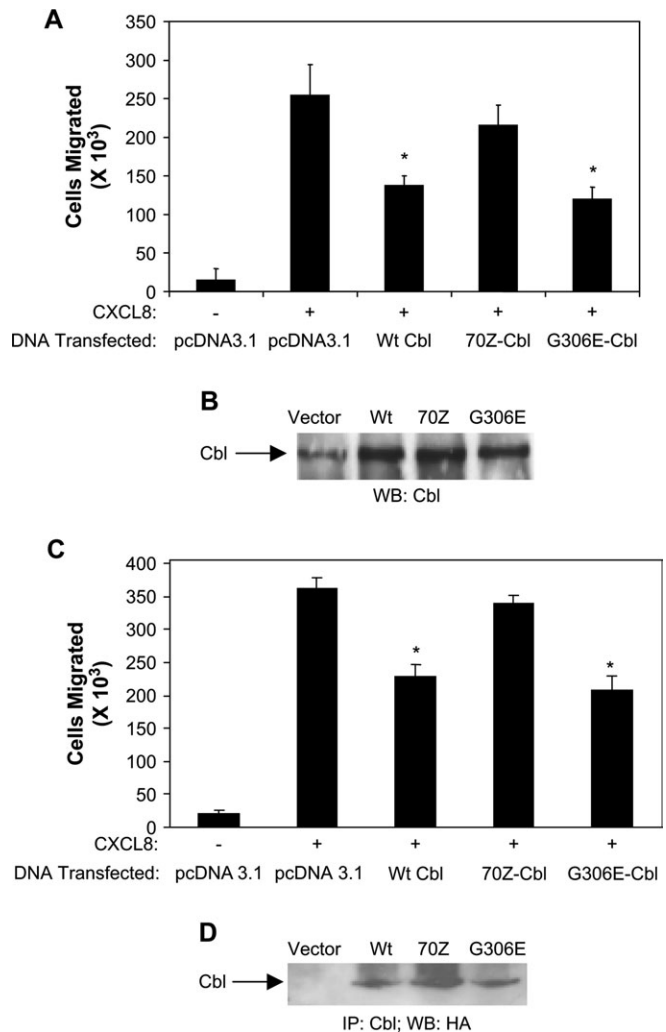
Fig. 6. Cbl regulates CXCL8-induced chemotaxis in CXCR1-L1.2 and CXCR2-L1.2 cells. CXCR1-L1.2 (A and B) and CXCR2-L1.2 (C and D) cells were transfected with pcDNA3.1 (vector control), wild-type (Wt) Cbl, 70Z-Cbl or G306E-Cbl using Metafectene. After 48 h, the cells were treated with CXCL8 (5 nM) and subjected to chemotaxis assay (A and C). Over-expression of Cbl was demonstrated by analyzing lysates of the transfected cells using SDS-PAGE and then western blotting with anti-Cbl antibody (B), or by immunoprecipitation with anti-Cbl antibody and western blotting with anti-HA antibody (D). The experiments were done in duplicate, and are presented as the mean ± SEM. Data are representative of three different experiments. *$P < 0.05$.

ligase function (45, 46). Cbl has been shown to be both a positive and negative regulator, depending on whether it is utilizing its function as an adaptor protein or as a ubiquitin ligase (23, 42, 44, 47, 48). We have shown that over-expression of wild-type Cbl reduces CXCL8-induced chemotaxis in CXCR1- and CXCR2-expressing cells. We have further shown that 70Z/3 Cbl, which lacks E3 ubiquitin ligase activity, has no effect on the migration of these cells. Our studies also demonstrated that the proteasome inhibitors Epoxomicin and Lactacystin inhibit CXCL8-induced chemotaxis. In addition, we observed that Lactacystin blocks the down-modulation of CXCR1 and CXCR2 induced by CXCL8. Although

over-expression of wild-type Cbl partially inhibited the CXCL8-induced CXCR1 down-modulation and 70Z/3 Cbl had no effect (data not shown), it was not possible to precisely interpret the role of Cbl in the receptor internalization process due
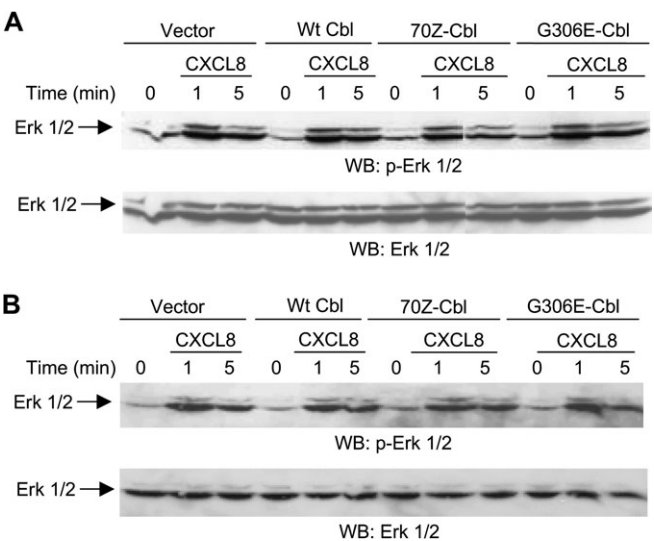

Fig. 7. Cbl regulates chemotaxis via a MAPK-independent pathway. CXCR1-L1.2 (A) and CXCR2-L1.2 (B) cells were transfected with pcDNA3.1 (vector control), wild-type (Wt) Cbl, 70Z-Cbl or G306E-Cbl using Metafectene. After 48 h, the cells were treated with CXCL8 (5 nM) for the indicated time points. The cell lysates were analyzed using SDS-PAGE and western blotting (WB) with anti-phospho-Erk 1/2 (p-Erk 1/2) antibody (upper panels). The blots were then stripped and re-probed for total protein with anti-Erk 1/2 antibody (lower panels). The experiment was done multiple times and representative blots are shown.

to the variable inhibition observed between experiments. These results suggest that Cbl and the proteasome pathway may regulate CXCL8-induced chemotaxis and receptor trafficking. The trafficking properties of the CXCR1/CXCR2 receptors have been shown to be important for CXCL8-induced chemotaxis (49, 50). It was demonstrated that CXCR2 mutants that do not undergo internalization exhibit a reduced chemotactic response toward their ligand, CXCL8 (51–53). Internalization and recycling of other chemokine receptors, such as CXCR4 and CCR5, have also been reported to be important for chemotaxis (30).

Additionally, Cbl may function by regulating the activity of downstream signaling mediators. We found that over-expression of wild-type Cbl or Cbl mutants has no effect on CXCL8-induced MAP (Erk 1/2) kinase activation and that the MAPK inhibitor PD98059 does not block CXCL8-induced chemotaxis. This suggests that Cbl does not mediate MAPK activation. MAPK activation has previously been shown to have no effect on CXCL8-induced chemotaxis (38), but has been reported to regulate chemotaxis mediated by other chemokine receptors (36).

We have demonstrated that Akt regulates CXCL8-induced chemotaxis, as over-expression of a dominant negative Akt mutant inhibited this chemotaxis. The mechanism through which Akt regulates chemotaxis is not well known. In neutrophils, Akt has been found to be recruited to the leading edge of cells during chemotaxis. Furthermore, Akt has been observed to modulate cell motility through PAK (54). PAK phosphorylates myosin light chain kinase, which in turn regulates cell spreading (55).

Since chemotaxis requires orderly changes in cytoskeletal structure, it is possible that the complex formation of Cbl and
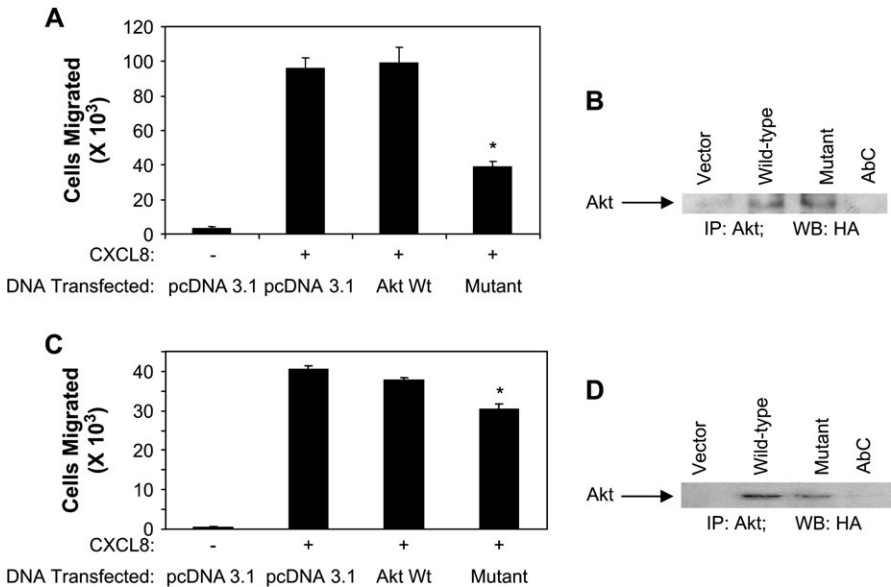
Fig. 8. Akt is involved in CXCL8-induced chemotaxis. CXCR1-L1.2 (A and B) and CXCR2-L1.2 (C and D) cells were transfected with pcDNA3.1 (vector control), HA-tagged wild-type Akt (Wt) or an HA-tagged kinase-dead Akt mutant (mutant). Forty-eight hours after the start of transfection, the cells were treated with 5 nM CXCL8 (A and C) and subjected to a chemotaxis assay. This experiment was done in duplicate, and is presented as the mean ± SEM. The success of the transfection was confirmed by immunoprecipitating (IP) cell lysates with anti-Akt antibody, and then performing western blotting (WB) with anti-HA antibody (B and D). Data shown are representative of three experiments. AbC = antibody control.
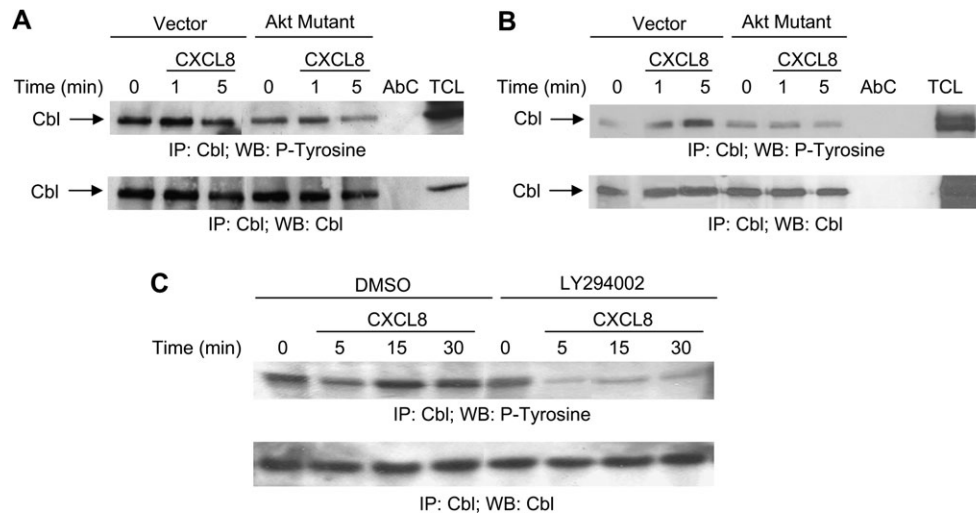
Fig. 9. Akt regulates Cbl phosphorylation. CXCR1-L1.2 (A and C) and CXCR2-L1.2 (B) cells were either untransfected or were transfected with pcDNA3.1 (vector) or with a kinase-dead Akt mutant (Akt mutant), as shown. Forty-eight hours after the start of transfection, the cells were stimulated with CXCL8 (5 nM) for the indicated time points. One set of cells was treated with either DMSO (solvent control) or LY294002 for 1 h before the CXCL8 stimulation (C). The cell lysates (1 mg) were next immunoprecipitated (IP) with anti-Cbl antibody, then analyzed by using SDS-PAGE and western blotting (WB) with anti-phosphotyrosine (P-Tyrosine) antibodies (PY99 and 4G10, upper panels). The blots were stripped and re-probed for total protein with anti-Cbl antibody as indicated (A–C, lower panels). The experiments were done three times and representative blots are shown.
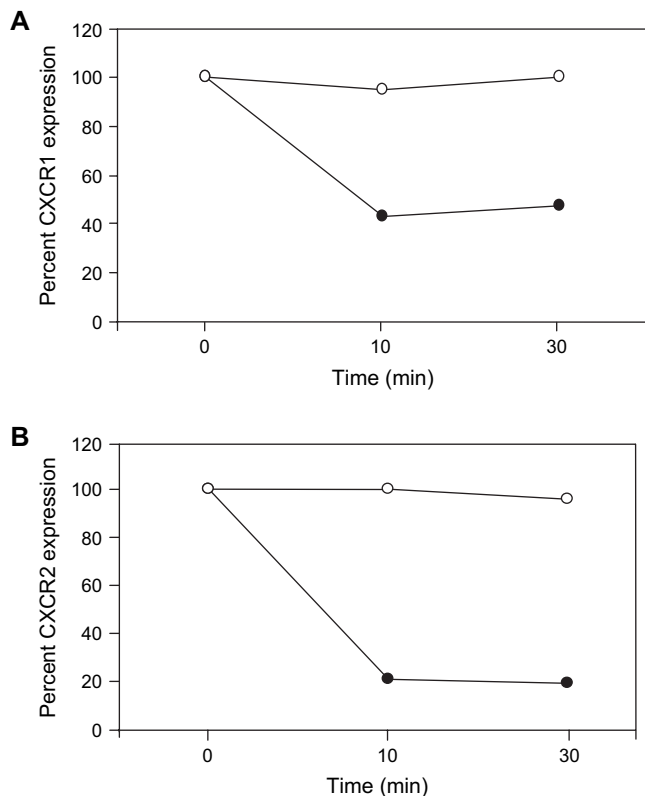
Fig. 10. Internalization of the CXCR1 and CXCR2 receptors is dependent on the proteasome pathway. Internalization of CXCR1 (A) and CXCR2 (B) L1.2 cells after 1 h starvation in the absence (filled circles) or presence (open circles) of the proteasome inhibitor Lactacystin at 37°C. Cells were stimulated at 37°C with 1 µg ml^{-1} of CXCL8 to induce receptor internalization. One hundred percent receptor expression was taken as the mean fluorescence at time point '0'. Experiments were done three times, and representative graphs are shown.

p85 leads to changes in phosphorylation resulting in alterations to the cytoskeleton and focal contacts. Cbl has also been reported to interact with proteins that co-localize to actin and modulate cytoskeletal responses (56). In addition, several cytoskeletal proteins such as dynamin and the p130^{cas}–Crk II complex associate with Cbl (57, 58). Thus, our study demonstrates that CXCR1- and CXCR2-mediated signal transduction involves the participation of Cbl and Akt during chemotaxis. Further analysis of these processes should significantly increase our understanding of CXCL8-induced, CXCR1- and CXCR2-mediated chemotactic signaling pathways.

Abbreviations

ECL	enhanced chemiluminescence system
PI3K	phosphatidylinositol-3 kinase
TKB	tyrosine kinase binding

References

1 Premack, B. A. and Schall, T. J. 1996. Chemokine receptors: gateways to inflammation and infection. *Nat. Med.* 2:1174.
2 McKay, C. R. 1996. Chemokine receptors and T cell chemotaxis. *J. Exp. Med.* 84:799.

3 Youngs, S. J., Ali, S. A., Taub, D. D. and Rees, R. C. 1997. Chemokines induce migrational responses in human breast carcinoma cell lines. *Int. J. Cancer* 71:257.

4 Cocchi, F., DeVico, A. L., Garzino, D. A., Arya, S. K., Gallo, R. C. and Lusso, P. 1995. Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* 270:1811.

5 Gonzalo, J. A., Lloyd, C. M., Kremer, L. *et al.* 1996. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J. Clin. Invest.* 98:2332.

6 Matsushima, K., Morishita, K., Yoshimura, T. *et al.* 1988. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. *J. Exp. Med.* 167:1883.

7 Strieter, R. M., Koch, A. E., Antony, V. B., Fick, R. B., Jr, Standiford, T. J. and Kunkel, S. L. 1994. The immunopathology of chemotactic cytokines: the role of interleukin-8 and monocyte chemoattractant protein-1. *J. Lab. Clin. Med.* 123:183.

8 Mukaida, N. 2003. Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 284:L566.

9 Shin, W. S., Szuba, A. and Rockson, S. G. 2002. The role of chemokines in human cardiovascular pathology: enhanced biological insights. *Atherosclerosis* 160:91.

10 Xie, K. 2001. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev.* 12:375.

11 Wang, J. F., Liu, Z. Y., Anand, A. R. *et al.* 2004. Alpha-chemokine-mediated signal transduction in human Kaposi's sarcoma spindle cells. *Biochim. Biophys. Acta* 1691:129.

12 Rollins, B. J. 1997. Chemokines. *Blood* 90:909.

13 Chuntharapai, A. and Kim, K. J. 1995. Regulation of the expression of the IL-8 receptor A/B by IL-8: possible functions of each receptor. *J. Immunol.* 155:2587.

14 Li, A., Dubey, S., Varney, M. L., Dave, B. J. and Singh, R. K. 2003. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J. Immunol.* 170:3369.

15 Li, A., Varney, M. L. and Singh, R. K. 2001. Expression of interleukin 8 and its receptors in human colon carcinoma cells with different metastatic potentials. *Clin. Cancer Res.* 10:3298.

16 Hjortoe, G. M., Petersen, L. C., Albrektsen, T. *et al.* 2004. Tissue factor-factor VIIa-specific up-regulation of IL-8 expression in MDA-MB-231 cells is mediated by PAR-2 and results in increased cell migration. *Blood* 103:3029.

17 Richardson, R. M., Ali, H., Pridgen, B. C. *et al.* 1998. Independent regulation by phosphorylation. Multiple signaling pathways of human interleukin-8 receptor A. Independent regulation by phosphorylation. *J. Biol. Chem.* 273:10690.

18 Schraufstatter, I. U., Chung, J. and Burger, M. 2001. IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac pathways. *Am. J. Physiol. Lung Cell Mol. Physiol.* 280:L1094.

19 Suetomi, K., Rojo, D. and Navarro, J. 2002. Identification of a signal transduction switch in the chemokine receptor CXCR1. *J. Biol. Chem.* 277:1526.

20 Richardson, R. M., Marjooram, R. J., Barak, L. S. and Snyderman, R. 2003. Role of the cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation. *J. Immunol.* 170:2904.

21 Thien, C. B. and Langdon, W. Y. 2001. Cbl: many adaptations to regulate protein tyrosine kinases. *Nat. Rev. Mol. Cell Biol.* 2:294.

22 Caveggion, E., Continolo, S., Pixley, F. J. *et al.* 2003. Expression and tyrosine phosphorylation of Cbl regulates macrophage chemokinetic and chemotactic movement. *J. Cell Physiol.* 195:276.

23 Sanjay, A., Houghton, A., Neff, L. *et al.* 2001. Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. *J. Cell Biol.* 152:181.

24 Knall, C., Worthen, G. S. and Johnson, G. L. 1997. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular

25 Sotsios, Y., Whittaker, G. C., Westwick, J. and Ward, S. G. 1999. The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. *J. Immunol.* 163:5954.

26 Tilton, B., Ho, L., Oberlin, E. *et al.* 2000. Signal transduction by CXC chemokine receptor 4. Stromal cell-derived factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. *J. Exp. Med.* 192:313.

27 Curnock, A. P., Logan, M. K. and Ward, S. G. 2002. Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. *Immunology* 105:125.

28 Sun, B. and Firtel, R. A. 2003. A regulator of G protein signaling-containing kinase is important for chemotaxis and multicellular development in dictyostelium. *Mol. Biol. Cell* 14:1727.

29 Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W. and Bourne, H. R. 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287:1037.

30 Fernandis, A. Z., Cherla, R. P., Chernock, R. D. and Ganju, R. K. 2002. CXCR4/CCR5 down-modulation and chemotaxis are regulated by the proteasome pathway. *J. Biol. Chem.* 277:18111.

31 Fuortes, M., Melchior, M., Han, H., Lyon, G. J. and Nathan, C. 1999. Role of the tyrosine kinase pyk2 in the integrin-dependent activation of human neutrophils by TNF. *J. Clin. Invest.* 104:327.

32 Boyum, A., Lovhaug, D., Tresland, L. and Nordlie, E. M. 1991. Separation of leukocytes: improved cell purity by fine adjustments of gradient medium density and osmolality. *Scand. J. Immunol.* 34:697.

33 Marmor, M. D. and Yarden, Y. 2004. Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. *Oncogene* 23:2057.

34 Gilbert, C., Rollet-Labelle, E., Caon, A. C. and Naccache, P. H. 2002. Immunoblotting and sequential lysis protocols for the analysis of tyrosine phosphorylation-dependent signaling. *J. Immunol. Methods* 271:185.

35 Hinton, D. R., He, S., Graf, K. *et al.* 1998. Mitogen-activated protein kinase activation mediates PDGF-directed migration of RPE cells. *Exp. Cell Res.* 239:11.

36 Boehme, S. A., Sullivan, S. K., Crowe, P. D. *et al.* 1999. Activation of mitogen-activated protein kinase regulates eotaxin-induced eosinophil migration. *J. Immunol.* 163:1611.

37 Yamboliev, I. A., Wiesmann, K. M., Singer, C. A., Hedges, J. C. and Gerthoffer, W. T. 2000. Phosphatidylinositol 3-kinases regulate ERK and p38 MAP kinases in canine colonic smooth muscle. *Am. J. Physiol. Cell Physiol.* 279:C352.

38 Jones, S. A., Moser, B. and Thelen, M. 1995. A comparison of post-receptor signal transduction events in Jurkat cells transfected with either IL-8R1 or IL-8R2. Chemokine mediated activation of p42/p44 MAP-kinase (ERK-2). *FEBS Lett.* 364:211.

39 Reddy, K. B., Nabha, S. M. and Atanaskova, N. 2003. Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev.* 22:395.

40 Rao, N., Ghosh, A. K., Ota, S. *et al.* 2001. The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. *EMBO J.* 20:7085.

41 Levkowitz, G., Waterman, H., Zamir, E. *et al.* 1998. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* 12:3663.

42 Garcia-Guzman, M., Larsen, E. and Vuori, K. 2000. The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. *Oncogene* 19:4058.

43 Zhang, Z., Elly, C., Qiu, L., Altman, A. and Liu, Y. C. 1999. A direct interaction between the adaptor protein Cbl-b and the kinase zap-70 induces a positive signal in T cells. *Curr. Biol.* 9:203.

44 Miura-Shimura, Y., Duan, L., Rao, N. L. *et al.* 2003. Cbl-mediated ubiquitinylation and negative regulation of Vav. *J. Biol. Chem.* 278:38495.

45 Kassenbrock, C. K. and Anderson, S. M. 2004. Regulation of ubiquitin protein-ligase activity in c-Cbl by phosphorylation-induced

conformational change, and constitutive activation by tyrosine to glutamate point mutations. *J. Biol. Chem.* 279:28017.

46 Melander, F., Andersson, T. and Dib, K. 2004. Engagement of beta2 integrins recruits 14-3-3 proteins to c-Cbl in human neutrophils. *Biochem. Biophys. Res. Commun.* 317:1000.

47 Yokouchi, M., Kondo, T., Houghton, A. *et al.* 1999. Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J. Biol. Chem.* 274:31707.

48 Liu, Y. C. 2004. Ubiquitin ligases and the immune response. *Annu. Rev. Immunol.* 22:81.

49 Yang, W., Wang, D. and Richmond, A. 1999. Role of clathrin-mediated endocytosis in CXCR2 sequestration, resensitization, and signal transduction. *J. Biol. Chem.* 274:11328.

50 Feniger-Barish, R., Belkin, D., Zaslaver, A. *et al.* 2002. GCP-2-induced internalization of IL-8 receptors: hierarchical relationships between GCP-2 and other ELR(+)-CXC chemokines and mechanisms regulating CXCR2 internalization and recycling. *Blood* 95:1551.

51 Fan, G. H., Yang, W., Wang, X. J., Qian, Q. and Richmond, A. 2001. Identification of a motif in the carboxyl terminus of CXCR2 that is involved in adaptin 2 binding and receptor internalization. *Biochemistry* 40:791.

52 Fan, G. H., Yang, W., Sai, J. and Richmond, A. 2002. Hsc/Hsp70 interacting protein (hip) associates with CXCR2 and

regulates the receptor signaling and trafficking. *J. Biol. Chem.* 277:6590.

53 Fan, G. H., Lapierre, L. A., Goldenring, J. R., Sai, J. and Richmond, A. 2004. Rab11-family interacting protein 2 and myosin Vb are required for CXCR2 recycling and receptor-mediated chemotaxis. *Mol. Biol. Cell* 15:2456.

54 Salh, B., Marotta, A., Wagey, R., Sayed, M. and Pelech, S. 2002. Dysregulation of phosphatidylinositol 3-kinase and downstream effectors in human breast cancer. *Int. J. Cancer* 98:148.

55 Kiosses, W. B., Daniels, R. H., Otey, C., Bokoch, G. M. and Schwartz, M. A. 1999. A role for p21-activated kinase in endothelial cell migration. *J. Cell Biol.* 147:831.

56 Scaife, R. M. and Langdon, W. Y. 2000. c-Cbl localizes to actin lamellae and regulates lamellipodia formation and cell morphology. *J. Cell Sci.* 113:215.

57 Bruzzanitim, A., Neff, L., Sanjay, A., Horne, W. C., De Camilli, P. and Baron, R. 2005. Dynamin forms a Src kinase-sensitive complex with Cbl and regulates podosomes and osteoclast activity. *Mol. Biol. Cell* 16:3301.

58 Zhu, T., Goh, E. L., LeRoith, D. and Lobie, P. E. 1998. Growth hormone stimulates the formation of a multiprotein signaling complex involving p130(Cas) and Crk II. Resultant activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). *J. Biol. Chem.* 273:33864.