IL-8-Induced Migratory Responses through CXCR1 and CXCR2: Association with Phosphorylation and Cellular Redistribution of Focal Adhesion Kinase†

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ABSTRACT: CXCR1 and CXCR2 mediate migratory activities in response to IL-8 and other ELR$^+$-CXC chemokines (e.g., GCP-2 and NAP-2). In vitro, activation of migration is induced by low IL-8 concentrations (10−50 ng/mL), whereas migratory shut-off is induced by high IL-8 concentrations (1000 ng/mL). The stimulation of CXCR1 and CXCR2 by IL-8 concentrations that result in migratory activation induced focal adhesion kinase (FAK) phosphorylation in a $G_{\alpha i}$-dependent manner. The expression of FRNK, a dominant negative mutant of FAK, perturbed migratory responses to the activating dose of 50 ng/mL IL-8. The migration-activating concentrations of 50 ng/mL GCP-2 and NAP-2 induced less potent migratory responses and FAK phosphorylation in CXCR2-expressing cells as compared with IL-8. These results indicate that FAK is phosphorylated, and required, for the chemotactic response under conditions of migratory activation by ELR$^+$-CXC chemokines. In addition, FAK phosphorylation was determined following exposure to migration-attenuating concentrations of IL-8. In CXCR1-RBL cells this treatment resulted in FAK phosphorylation, in similar levels to those induced by activating concentrations of IL-8. In contrast, in CXCR2-RBL cells the migration-attenuating concentrations of IL-8 induced promoted levels of FAK phosphorylation and different patterns of FAK phosphorylation on its six potential tyrosine phosphorylation sites, as compared to activating concentrations of the chemokine. Exposure to IL-8 resulted not only in FAK phosphorylation but also in its cellular redistribution, indicated by the formation of defined contact regions with the substratum, enriched in phosphorylated FAK and vinculin. Overall, FAK phosphorylation was associated with, and found to be differently regulated upon, ELR$^+$-CXC chemokine-induced migration.

The migration of neutrophils to sites of acute inflammation is induced by several chemotactic factors, including members of the ELR$^+$-CXC subclass of chemokines (chemokines expressing the ELR motif) (*1*−*6*). Interleukin 8 (IL-8, CXCL8)[1] is the most potent chemoattractant in this group, which includes also other members that diverge in their ability to induce migration, such as granulocyte chemotactic protein 2 (GCP-2, CXCL6), neutrophil activating protein 2 (NAP-2, CXCL7), and melanocyte growth stimulatory activity (MGSA, CXCL1) (*1*, *7*−*13*). Several of these chemokines (e.g., IL-8 and MGSA) may play a key role not only in physiological responses but also in pathological situations in which migration of endothelial cells or tumor cells may contribute to metastatic processes (*14*−*16*).

The first membranous components that interact with ELR$^+$-CXC chemokines are the CXCR1 and CXCR2 receptors. CXCR1 and CXCR2 are G protein-coupled receptors (GPCR) (*17*, *18*) whose activities are controlled by the type of chemokine that they encounter (*7*−*13*, *19*, *20*). Both CXCR1 and CXCR2 are expressed by neutrophils and were shown to bind IL-8 with high affinity. CXCR2 is a highly promiscuous receptor, for which all of the ELR$^+$-CXC chemokines are functional ligands. In contrast, only IL-8 and GCP-2 are considered as agonists for CXCR1 (*19*−*21*). The various chemokines induce, through the two receptors, different signaling pathways that result in highly complex responses. In addition, the two receptors may cross-regulate each other's activities intracellularly, as suggested by observations on interactions between CXCR1 and CXCR2 (*22*−*27*).

In similarity to many other GPCR, the responses mediated by CXCR1 and CXCR2 are regulated by processes of receptor activation and desensitization. Studies of CXCR1 and CXCR2 have indicated that in vitro the migratory responses to IL-8 that are mediated by these two receptors are activated by low chemokine concentrations (e.g., for IL-8: 10−50 ng/mL) (*8*, *9*, *11*, *28*−*32*). In contrast, exposure to high IL-8 concentrations (such as 1000 ng/mL) results in attenuation of migratory responses (*8*, *28*−*30*). The shift from activation to desensitization of migration is dose-dependent and gradual (*28*, *29*, *32*), providing evidence for the delicate

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[1] Abbreviations: ELR$^+$-CXC chemokines, ELR-expressing CXC chemokines; FAK, focal adhesion kinase; FRNK, FAK-related nonkinase domain; GCP-2, granulocyte chemotactic protein 2; GPCR, G protein-coupled receptors; HEK, human embryonal kidney; IL-8, interleukin 8; NAP-2, neutrophil activating protein 2; PTx, pertussis toxin; PY, phosphorylated tyrosine; RBL, rat basophilic leukemia; RT, room temperature.

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interplay between many cellular factors that tightly regulate the migratory process.

The observations on the in vitro dose-dependent nature of the migratory process may have high relevance to the regulation of inflammatory processes in vivo. Several publications have indicated that low concentrations of ELR$^+$-CXC chemokines (diverging from pmol/site to 1−100 ng/mL) induce potent migratory responses. Under these conditions, the chemokines may induce neutrophil accumulation at inflammatory sites if injected locally, or increase the number of circulating neutrophils upon intravenous injection (*3*−*6*). In contrast, it was demonstrated by several studies that the presence of ELR$^+$-CXC chemokines in the circulation of IL-8-transgenic mice, or high local concentrations of these chemokines, prevent specific neutrophil migration into inflamed tissues (*33*−*37*).

The desensitization of migratory responses may be of physiological relevance in limiting the flux of neutrophils under specific conditions of continuous inflammatory stimuli, which may result in damage to neighboring, healthy tissues. Such a migratory attenuation may be mediated by several mechanisms that may be related to the extremely high local and serum chemokine concentrations that possibly accompany prolonged inflammatory conditions: First, the high serum chemokine concentration may perturb the existence of a chemotactic gradient that is required for neutrophil migration to the inflamed site. Second, migratory shut-off may be mediated by desensitizing mechanisms that cause CXCR1 and CXCR2 attenuation. Indeed, it was shown that high ELR$^+$-CXC chemokine serum levels are accompanied by attenuation of neutrophil migration to inflammatory sites (*33*−*35*). Evidence was provided to the functional desensitization of CXCR2 under these conditions, possibly contributing to migratory shut-off (*35*).

Therefore, it is possible that in vivo and in vitro, chemokine concentrations play a key role in the regulation of the migratory process, whether activated or desensitized. The process of CXCR1 and CXCR2 activation, which gives rise to multiple responses including migration, requires the coupling of the appropriate G proteins to the receptors, followed by a complex cascade of signaling events that give rise to migration (*24*, *31*, *38*−*41*). On the other hand, the attenuation of CXCR1 and CXCR2 is mechanistically different from that of migratory activation. The desensitization of these receptors is mediated by phosphorylation on carboxyl terminus serine and threonine residues (*28*, *42*, *43*). Such phosphorylation events are assumed to result in uncoupling of G proteins from the receptors and therefore in attenuation of migration. It is of importance to note that events of signaling shut-off, which are mediated by G-protein uncoupling, may be accompanied by complementary cellular processes that stimulate other and not necessarily related signaling pathways, as was indicated by the studies of Lefkowitz et al. (*44*). Similarly, it is possible that the process of migratory-attenuation, which is mediated by G-protein uncoupling, is accompanied by the induction of complementary signaling pathways and that these alternative pathways contribute to the migration shut-off by exerting functions that are required for effective attenuation.

The important role played by IL-8 and other ELR$^+$-CXC chemokines in chemotaxis emphasizes the requirement for better understanding of the down stream events that mediate their ability to induce migration. Migratory responses of leukocytes, as well as of endothelial and tumor cells, are mediated by integrins and are the outcome of coordinated events that tightly regulate cellular adhesion and rearrangement of the actin cytoskeleton (*45*). One of the key players in this process is the protein tyrosine kinase focal adhesion kinase (FAK). FAK is involved in signal transduction pathways that regulate cell adhesion and motility, as well as cell growth and survival. With respect to cell mobilization, FAK plays a role in formation of focal contacts, spreading, and induction of signaling pathways that are required for cellular motility. In the course of the migratory response, a FAK-regulated dynamic turnover in focal contact formation controls processes of attachment and detachment, which are required for cell movement. Upon stimulation that is mediated through integrins, growth factor receptors, and other receptor types, FAK is phosphorylated on tyrosine residues and activated. Six potential tyrosine phosphorylation sites were characterized on FAK; however, specific roles in FAK-mediated activities were assigned to only four of these sites (*46*−*50*).

It has been suggested previously that FAK may play a role in the control of migratory responses that are induced by chemoattractants (*46*). However, its regulation following stimulation with ELR$^+$-CXC chemokines, as well as its regulation under activating versus desensitizing conditions, were not determined as yet. The fine-tuning of ELR$^+$-CXC chemokine-mediated chemotaxis, which is a key process in the regulation of the migration of neutrophils in the course of acute inflammation, may be the result of the coordinated activity of multiple cellular regulators and effectors, including FAK.

In the present study, we investigated the regulation and the role of FAK in the migratory responses that are induced by IL-8, analyzing its control following chemokine stimulations that give rise to either activation or attenuation of migratory responses. To this end, we have determined FAK phosphorylation and localization under the two extreme migratory conditions: migratory activation that is induced by low IL-8 concentrations (50 ng/mL) and of migratory attenuation, induced by high IL-8 doses of 1000 ng/mL. In addition, to gain insight into events that may be involved in the tight regulation of CXCR2 by different ELR$^+$-CXC chemokines, FAK phosphorylation was evaluated upon exposure to the two other less potent ELR$^+$-CXC chemokines, GCP-2 and NAP-2.

The results of our study revealed that under conditions of migratory activation, the stimulation of CXCR1 and CXCR2 results in FAK phosphorylation. FAK activity was shown to be directly required for chemotaxis, as demonstrated by the use of a dominant negative mutant of FAK. However, the stimulation of CXCR2 in conditions of migration shut-off gave rise to further promoted FAK phosphorylation levels, indicating that FAK is differently regulated under migratory-attenuating versus migratory-activating conditions. Of interest was the fact that under conditions of migratory attenuation, differences were observed between CXCR1 and CXCR2, providing evidence to the unique role of each receptor in the regulation of migration. In addition, our study has indicated that IL-8 stimulation results not only in FAK phosphorylation but also in its redistribution to membrane regions that form definite contact areas with the substratum.

The different modes of FAK regulation may shed light on its role in ELR$^+$-CXC chemokine-induced migration, as will be discussed below.

EXPERIMENTAL PROCEDURES

Cell Cultures, Transfections, and Characterization of Receptor Expression by Transfected Cells. Human embryonal kidney (HEK) 293 cells and rat basophilic leukemia (RBL) 2H3 cells were transfected to stably express the wild type (WT) CXCR1 and WT CXCR2, which were generated by PCR and fully sequenced to ensure the correct sequence. Over 90% of the transfected cells expressed the receptors at significant levels on the cell surface. Vector-transfected cells did not express CXCR1 or CXCR2 and did not migrate to IL-8 (*27*, *29*, *32*, *43*, *51*).

For experiments with FRNK (FAK-related nonkinase domain), CXCR1-HEK cells were transiently transfected with pEGFP-C1 vector (Clontech, Palo Alto, CA) or with the same vector containing FRNK cDNA (a kind gift from Prof. M. A. Schwartz, Department of Vascular Biology, The Scripps Research Institute). FACS analyses showed that over 50% of the transfected cells expressed the transfected GFP vectors 48 h after the transfection, and the transfection did not affect the basal expression level of CXCR1 (data not shown).

Determination of FAK Phosphorylation. HEK 293 or RBL 2H3 cells were plated in 10-cm tissue culture plates with growth medium and were serum-starved in starvation medium (0.5% FCS) for another 24 h. Thereafter, HEK 293 cells were trypsinized, treated with trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), resuspended in BSA medium (RPMI containing 1% BSA and 25 mM HEPES), divided into the different treatment groups, and incubated for 2 h at 37 °C in the absence or in the presence of 100 ng/mL pertussis toxin (PTx; List Biological Laboratories, Campbell, CA). RBL 2H3 cells were washed with PBS at 37 °C, each plate representing a separate treatment group. Following these procedures, both types of cells were incubated with chemokines (PeproTech, Rocky Hill, NJ), diluted in BSA medium, or with BSA medium alone, at 37 °C. The reaction was stopped by the addition of ice-cold PBS.

Cells were then pelleted and lysed with lysis buffer, followed by centrifugation and collection of cleared supernatants. Immunoprecipitation was performed with anti-FAK antibodies (sc-558; Santa Cruz Biotechnology, Santa Cruz, CA). After washings, the final pellet was resuspended in sample buffer and transferred to a nitrocellulose membrane (Schleicher & Schull, Dassel, Germany) that was reacted with the following antibodies (in combinations described in the figure legends): rabbit polyclonal antibodies against FAK (sc-558; Santa Cruz), monoclonal mouse antibodies against phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY), or rabbit polyclonal antibodies against the different phosphorylated tyrosine residues of FAK in their phosphorylated form (pY397, pY407, pY576, pY577, pY861, or pY925; Biosource International, Camarillo, CA). Following washings, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) or with HRP-conjugated sheep anti-mouse antibodies (Amersham Life Sciences, NJ) and subjected to enhanced chemilluminescence (Amersham Pharmacia Biotech, Buckinghamshire, England). Bands on immunoblots were quantitated by densitometry.

The level of phosphorylation is presented as "Fold Induction of Phosphorylation" and was calculated in reference to the total amount of FAK in the gel. The basal level of phosphorylation without stimulation has been given the value of 1.

Migration Assays. Migration was assessed by a 48-well microchemotaxis chamber technique, as previously described (*32*). For migration of cells that express FRNK, CXCR1-HEK cells were transiently transfected with FRNK-expressing vector or with vector only, as described above. Twenty-four hours after transfection, the cells were serum-starved in 0.5% FCS-containing medium for another 24 h. The cells were then trypsinized, treated with trypsin inhibitor (Sigma), resuspended in BSA medium at 5×10^5 cells/mL, and incubated for 2 h at 37 °C. In all the migratory procedures of HEK cells, 5×10^5 cells/mL were loaded in the upper compartment of the chamber. A 10-μm pored membrane was used, following coating with 50 μg/mL rat collagen type 1 (Collaborative Biomedical Products, Bedford, MA). Incubation was performed for 5 h. The migration of RBL 2H3 cells was performed by loading 7.5×10^5 cells/mL in the upper compartment of the chamber. An 8-μm pored uncoated membrane was used. Incubation was performed for 2 h. Statistical analysis was performed using Student's *t* test.

Confocal Analyses. Stable CXCR2-RBL cells were plated on cover glasses in 24-well cell culture clusters. A day later, the medium was exchanged to starvation medium (0.5% FCS) for another 24 h. Following washings in PBS, the cells were treated with IL-8 that was diluted in BSA medium or in medium alone for 5 min at 37 °C. The cells were rinsed in cold PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.5% Triton x-100 at room temperature (RT) for 2 min. Then, blocking buffer was added (3% goat serum, 0.25% gelatin, and 0.15% saponin in PBS) for 1 h at RT. The cell-coated cover glasses were removed from the wells and blocked with human aggregated IgG (IgG from Sigma). This was followed by staining with the following antibodies (in combinations described in the legends to the figures): rabbit polyclonal antibodies against FAK (sc-558; Santa Cruz Biotechnology); monoclonal mouse antibodies against phosphotyrosine (4G10; Upstate Biotechnology); rabbit polyclonal antibodies against site Y576 of FAK, in its phosphorylated form (Biosource International); and monoclonal mouse antibodies against human vinculin (hVIN-1, Sigma). Incubation with the above antibodies was performed for 30 min at 37 °C, followed by rinsing in washing buffer (1% goat serum, 0.25% gelatin, and 0.15% saponin in PBS). The cells were then stained with the corresponding secondary antibodies for 30 min at 37 °C: rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Following additional washings, stained cells were analyzed using a Zeiss confocal laser scanning microscope (Oberkochen, Germany). Zeiss LSM 510 was equipped with 1 mW HeNe laser (543 nM) A63Xna/1.4 C-apochromat oil-immersion lens (Axioplon 2, Zeiss) and long-mass filters (560 nM) were used for all imaging.

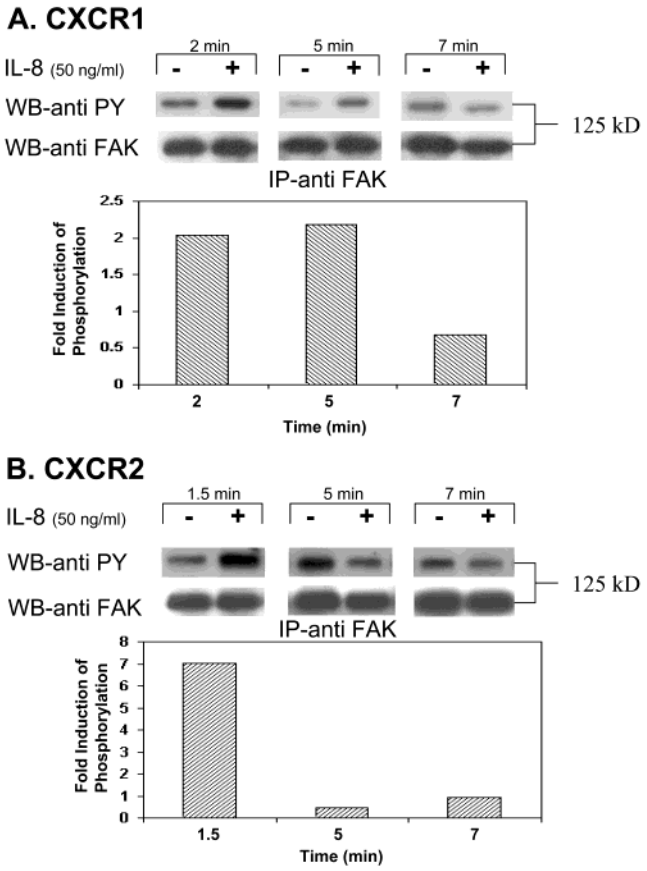

FIGURE 1: IL-8-induced FAK phosphorylation in CXCR1- and CXCR2-expressing cells is a rapid and a time-dependent process. FAK phosphorylation was determined in CXCR1-HEK cells or CXCR2-HEK cells in response to 50 ng/mL IL-8 for the indicated time points at 37 °C. FAK phosphorylation was analyzed by immunoprecipitation with anti-FAK antibodies, followed by Western blotting with antibodies against phosphorylated tyrosine or against FAK (following stripping and reprobing of the same membrane). (A) CXCR1-HEK cells. (B) CXCR2-HEK cells. IP, immunoprecipitation and WB, Western blotting. Fold induction of phosphorylation are phosphorylation levels calculated in reference to the total amount of FAK protein in the gel, where the basal level of phosphorylation without stimulation has been given the value of 1. A representative experiment of at least three independent experiments performed is presented.

RESULTS

FAK Is Phosphorylated and Regulates Migration Under Migratory-Activating Conditions. Because of the differential control of CXCR1 as compared to CXCR2, and possible cross-regulation between the two receptors in neutrophils (*22*−*27*), this overall study was performed in cell systems in which each of the receptors was expressed independently of the other. The cell systems were based on CXCR1- or CXCR2-transfected hematopoietic rat basophilic leukemia (RBL) 2H3 cells or human embryonal kidney (HEK) 293 cells. In both systems, the general characteristics of CXCR1 and CXCR2 activities are similar to those in neutrophils, as they can mediate migration and undergo ligand-induced desensitization and internalization (*27*−*29*, *32*, *43*, *51*−*55*).

In general, the two cell systems of HEK 293 cells and of RBL 2H3 cells were used alternatively throughout the study. HEK 293 cells were used mainly for initial characterization and for determination of mechanistic issues related to FAK phosphorylation (Figures 1 and 4). These cells were used also to analyze aspects that necessitated the overexpression of FRNK (Figure 2) since transfections are more easily performed on HEK 293 cells than on hematopoietic RBL 2H3 cells. The RBL 2H3 cells were used mainly for the analysis of aspects that are of physiological relevance (Figures 3 and 5−9). This system is of a hematopoietic origin and therefore coincides more with the native hematopoietic cells that express CXCR1 and CXCR2 (e.g., neutrophils). The aspects that were analyzed in the RBL 2H3 system included the regulation of FAK by different ELR$^+$-CXC chemokines, FAK phosphorylation following exposure to activating and desensitizing concentrations of IL-8, and determination of FAK localization following IL-8 stimulation.

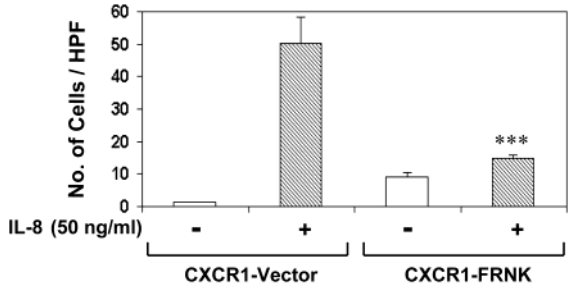

FIGURE 2: FAK is directly involved in cell migration in response to IL-8, under conditions of migratory activation. The migration of CXCR1-HEK cells in response to 50 ng/mL IL-8 following transient transfection with FRNK-expressing vector as compared to transfection with vector alone is shown. CXCR1-Vector, cells transfected with vector alone; CXCR1-FRNK, cells transfected with FRNK-expressing vector; HPF, high power field and ***$p < 0.0001$ for migration of CXCR1-FRNK cells vs CXCR1-Vector cells. A representative experiment of four independent experiments performed is presented.

To determine whether FAK may have a direct role in migratory responses that are induced by ELR$^+$-CXC chemokines, we first analyzed the regulation of FAK in chemokine concentrations that induce migratory activity, namely 50 ng/mL. Most of the experiments presented were performed in response to IL-8, the most potent chemoattractant of all ELR$^+$-CXC chemokines. We first analyzed the ability of IL-8 to induce FAK phosphorylation on tyrosine residues in HEK 293 cells that express CXCR1 (CXCR1-HEK cells) or CXCR2 (CXCR2-HEK cells). The results of Figure 1 indicate that in both cell types the exposure to 50 ng/mL IL-8 resulted in FAK phosphorylation, which is a key event in FAK activation (*46*−*50*). In CXCR1-expressing cells, the values of fold induction of FAK phosphorylation following IL-8 stimulations for 2, 5, and 7 min were 1.43 ± 0.3, 2.28 ± 0.4, and 1.08 ± 0.5, respectively. In CXCR2-expressing cells, IL-8 stimulation for 1.5, 5, and 7 min gave rise to FAK phosphorylation for which the values of fold induction of FAK phosphorylation were 4.6 ± 2.8, 0.69 ± 0.2, and 0.66 ± 0.3, respectively. FAK phosphorylation in response to IL-8-induced stimulation was a time-dependent process and was rapidly up-regulated upon exposure to IL-8. This was indicated by high levels of FAK phosphorylation that were induced at time points as early as 2 or 1.5 min for CXCR1- or CXCR2-expressing cells, respectively. FAK phosphorylation following CXCR1 stimulation peaked at 5 min. In contrast, the phosphorylation of FAK upon stimulation of CXCR2 has declined at this time point. Therefore, under migratory-activating conditions, differences were observed

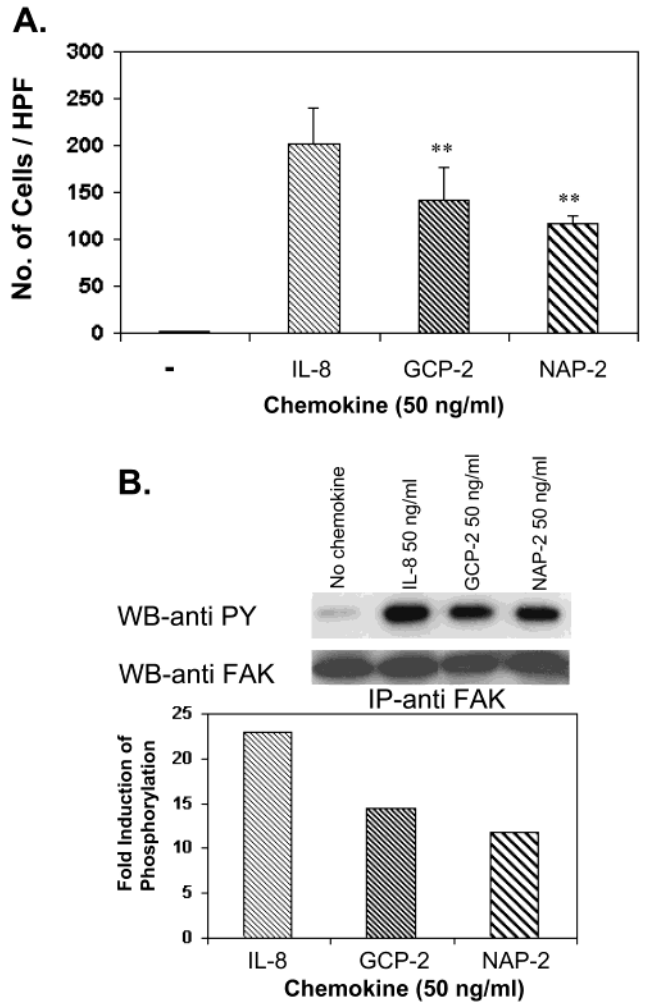

FIGURE 3: Lower ability of GCP-2 and NAP-2 to activate the migratory process is associated with reduced levels of FAK phosphorylation. Determination of the abilities of IL-8, GCP-2, and NAP-2 to induce migration and FAK phosphorylation in CXCR2-RBL cells is shown. (A) Migration of CXCR2-RBL cells in response to 50 ng/mL of each of the chemokines. HPF, high power field and $^{**}p = 0.005$ for migration in response to GCP-2 and NAP-2 vs migration to IL-8. (B) FAK phosphorylation in CXCR2-RBL cells, stimulated by 50 ng/mL of each of the chemokines for 5 min at 37 °C. FAK phosphorylation was analyzed as in Figure 1. IP, immunoprecipitation and WB, Western blotting. Fold induction of phosphorylation are phosphorylation levels calculated in reference to the total amount of FAK protein in the gel, where the basal level of phosphorylation without stimulation has been given the value of 1. A representative experiment of three independent experiments performed is presented.

in the kinetics of FAK phosphorylation upon stimulation of CXCR1 as compared to CXCR2.

In addition to determination of FAK phosphorylation in response to IL-8-induced stimulation in conditions of migratory activation, we analyzed the direct involvement of FAK in the migratory process by using its dominant negative mutant, namely FRNK (FAK-related nonkinase domain) (*46*, *48*, *49*, *56*) in HEK 293 cells. To this end, CXCR1-HEK cells were transiently transfected by a GFP-expressing vector that either expressed or did not express FRNK. The resulting cells were shown to highly express the transfected vectors, as well as the CXCR1 receptor. To determine the role of FAK under conditions in which the migratory response is most efficiently activated and optimal, this analysis was

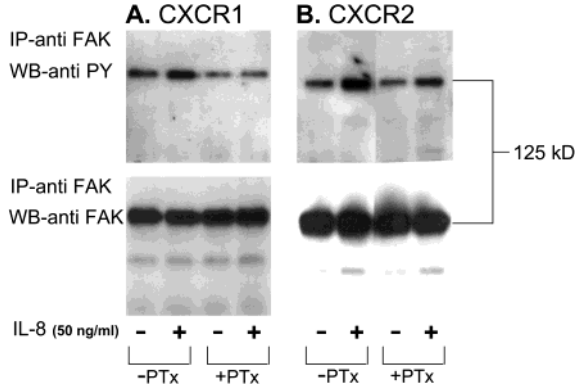

FIGURE 4: FAK phosphorylation requires $G_{\alpha i}$ coupling. The levels of FAK phosphorylation in CXCR1-HEK or CXCR2-HEK cells in response to stimulation with 50 ng/mL IL-8 were determined in cells untreated or treated by 100 ng/mL pertussis toxin (PTx) for 2 h at 37 °C. (A) CXCR1-HEK cells. Stimulation with IL-8 for 2 min. (B) CXCR2-HEK cells. Stimulation with IL-8 for 1.5 min. FAK phosphorylation was analyzed as in Figure 1. IP, immunoprecipitation and WB, Western blotting. A representative experiment of at least four independent experiments performed is presented.

performed in response to an IL-8 concentration that induces a highly potent migration, namely 50 ng/mL (*28*, *29*, *32*). These experiments were not performed with sub-optimal IL-8 concentrations (lower or higher than 50 ng/mL) or desensitizing IL-8 concentrations (1000 ng/mL) since these conditions do not yield optimal and highly potent migratory responses (*28*, *29*, *32*).

As shown in Figure 2, the migration of CXCR1-HEK cells was largely perturbed by the expression of FRNK. In all the experiments performed, the migration of vector-transfected cells was significantly higher ($p < 0.0001$) than the migration of FRNK-transfected cells. Migration indices were calculated for the migration of vector-transfected cells and FRNK-transfected cells. These indices represent, for each cell type, the value of "no. of cells migrating to IL-8/no. of cells migrating to medium". In all the experiments, the index of migration of vector-transfected cells was markedly higher than that of FRNK-transfected cells. In these experiments, the indices of migration of vector-transfected cells versus FRNK-transfected cells were as follows: expt 1−115.7 versus 11.3; expt 2−39.6 versus 1.6; expt 3−11.5 versus 1.2; and expt 4−8.4 versus 1.0. Overall, these results indicate that FAK is required for the regulation of migration under migration-activating conditions.

To gain insight into the tight regulation of migratory responses and to study whether there is a direct association between migratory activation and FAK phosphorylation levels, we have performed experiments in which FAK phosphorylation was determined following exposure of CXCR2-expressing cells to GCP-2 and NAP-2, as compared to IL-8. Previous studies on neutrophils and on transfected cells have shown that GCP-2 and NAP-2 are less potent inducers of migration than IL-8, as was observed by the use of chemokine concentrations that activate the migratory responses (*8*, *9*, *11*, *13*, *28*, *29*). This fact motivated us to analyze the ability of IL-8, GCP-2, and NAP-2 to induce FAK phosphorylation and to correlate it with the potency of migration. To this end, hematopoietic RBL cells that express CXCR2 (CXCR2-RBL cells) were exposed to 50 ng/mL of each of the chemokines. As shown in Figure 3A,

IL-8 potently induced the migration of CXCR2-RBL cells, whereas GCP-2 and NAP-2 had lower abilities to induce migration. In all the experiments performed, the migration to IL-8 was significantly higher ($p = 0.005$ to $p < 0.0001$) than the migration to GCP-2 and NAP-2. The results of the migration assays were analyzed in terms of migration indices, where the index of migration represents the number of cells migrating to chemokine/number of cells migrating to medium. In these experiments, the indices of migration to IL-8 were considerably higher than the indices of migration to GCP-2 and NAP-2. The indices of migration to IL-8 versus GCP-2 versus NAP-2 were as follows: expt 1−201.1 versus 140.9 versus 111.4; expt 2−69.5 versus 47.3 versus 5.3; expt 3−5.6 versus 2.3 versus 3.

Following determination of migration, the ability of the three chemokines at 50 ng/mL to induce FAK phosphorylation was analyzed in the CXCR2-RBL cells. As demonstrated in Figure 3B, the exposure of the cells to each of the three chemokines for 5 min resulted in FAK phosphorylation (note that the kinetics of FAK phosphorylation in these cells is different than in CXCR2-HEK cells, Figure 1B). Of interest was the fact that IL-8 induced higher levels of FAK phosphorylation than GCP-2 and NAP-2, being in direct association with the higher ability of IL-8 to induce migration of CXCR2-expressing cells, as compared to GCP-2 and NAP-2. These results, combined with the FRNK experiments, indicate that FAK is required and regulates the fine-tuning of the migratory response to ELR^+-CXC chemokines under conditions of migratory activation.

To provide further insight into the regulation of IL-8-induced FAK phosphorylation, we determined whether $G_{\alpha i}$ coupling is a prerequisite for FAK phosphorylation. The activation of cellular responses by IL-8 in neutrophils and in CXCR1- or CXCR2-transfected cells is mediated by the coupling of $G_{\alpha i}$ proteins, although other G proteins (e.g., $G_{\alpha 16}$) may also be involved (*24*, *31*, *38*−*41*, *51*). The classical method used for determination of the role of $G_{\alpha i}$ proteins in IL-8-induced migratory activation relies on the use of pertussis toxin (PTx), a specific inhibitor of $G_{\alpha i}$ coupling to GPCR (*57*). Therefore, to determine the requirement for $G_{\alpha i}$ protein-coupling for IL-8-induced FAK phosphorylation, we evaluated the ability of PTx to affect IL-8-induced FAK phosphorylation in HEK 293 cells. PTx was used in concentration of 100 ng/mL, shown in our previous study (*31*) to potently inhibit IL-8-induced migratory responses of CXCR1-HEK and CXCR2-HEK cells.

The results of Figure 4A show that FAK phosphorylation in response to 50 ng/mL IL-8 was inhibited in CXCR1-HEK cells by exposure to PTx. In all of these experiments, the values of fold induction of phosphorylation were higher for cells not treated by PTx (mean: 2.42 ± 1.3) than for cells treated by PTx (mean: 1.08 ± 0.4). Moreover, complete abrogation of phosphorylation was noted in four out of six experiments. Similar analysis was performed on cells that express the more promiscuous receptor CXCR2. Inhibition of FAK phosphorylation was observed in CXCR2-HEK cells following PTx treatment (Figure 4B) in all the experiments that were performed, with fold induction of phosphorylation values higher for cells not treated by PTx (mean: 3.73 ± 2.3) than for cells treated by PTx (mean: 1.22 ± 0.8). Complete abrogation of phosphorylation was noted only in two out of six experiments. The above results indicate that FAK phosphorylation following IL-8 stimulation of CXCR1 and CXCR2 is dependent on the coupling of $G_{\alpha i}$ proteins. However, the possibility that other G proteins are involved in this process cannot be excluded, especially for CXCR2 stimulation.

FAK Phosphorylation Under Conditions of Migratory Shut-Off. Studies on neutrophil infiltration in the course of inflammation suggest that under specific conditions of continuous inflammatory stimuli, the migratory response should be shut-off to prevent the continuous and deleterious flux of leukocyte infiltration to the inflammatory site. It is possible that under these conditions, chemokines are highly released at the inflammatory site, resulting in receptor desensitization and attenuation of neutrophil migration. Such a regulatory mechanism is supported by in vitro findings, demonstrating that the migration of neutrophils and of CXCR1- and CXCR2-transfected HEK 293 cells is attenuated in vitro by exposure to high IL-8 concentrations (1000 ng/mL) (*28*, *29*, *35*, *36*). Under these conditions, the regulation of FAK may be different than under conditions of migratory activation.

To determine the regulation of FAK in the course of migratory attenuation and to compare it with its phosphorylation during migratory activation, we determined the level of FAK phosphorylation in RBL cells expressing either CXCR1 (CXCR1-RBL cells) or CXCR2 (CXCR2-RBL cells) following exposure to 1000 ng/mL IL-8 (attenuating) versus 50 ng/mL IL-8 (activating). The analysis of FAK phosphorylation under these conditions was performed in parallel to evaluation of the ability of the two IL-8 concentrations to induce migratory responses.

First, the migration of CXCR1-RBL and CXCR2-RBL cells in response to 50 and 1000 ng/mL IL-8 was analyzed. In similarity to neutrophils and to HEK 293 cells that express CXCR1 or CXCR2 (*8*, *28*−*30*, *35*, *36*), the migration of CXCR1-RBL cells and CXCR2-RBL cells was attenuated upon exposure to 1000 ng/mL IL-8. In all the experiments, CXCR1-RBL cells migrated significantly less ($p < 0.0008$ to $p < 0.0001$) to 1000 ng/mL IL-8 as compared to 50 ng/mL IL-8 (as illustrated in a representative experiment in Figure 5A). The indices of migration in response to 50 or 1000 ng/mL were calculated by using the following equation: no. of cells migrating to IL-8/no. of cells migrating to medium. In all of these experiments, the index of migration to 50 ng/mL was considerably higher than the index of migration to 1000 ng/mL. The indices for migration to 50 ng/mL IL-8 versus 1000 ng/mL were as follows: expt 1−7.1 versus 1.5; expt 2−3.0 versus 1.9; expt 3−2.2 versus 1.1.

Similarly, the migration of CXCR2-RBL cells to 50 and to 1000 ng/mL IL-8 was determined, indicating that the cells migrate more potently to 50 ng/mL IL-8 (Figure 6A). In all the experiments performed, the migration to 50 ng/mL was significantly higher ($p < 0.0001$) than the migration to 1000 ng/mL. The indices of migration to 50 ng/mL were markedly higher than those to 1000 ng/mL in all of the experiments. The indices of migration to 50 ng/mL versus 1000 ng/mL were as follows: expt 1−201.1 versus 34.6; expt 2−88.3 versus 48.1; expt 3−69.5 versus 29.6; expt 4−6.3 versus 2.1; expt 5−5.5 versus 2.6; and expt 6−5.5 versus 1.3.

Overall, the results of the migration assays indicate that the migratory responses are indeed attenuated in CXCR1-RBL and CXCR2-RBL cells by exposure to high concentra-

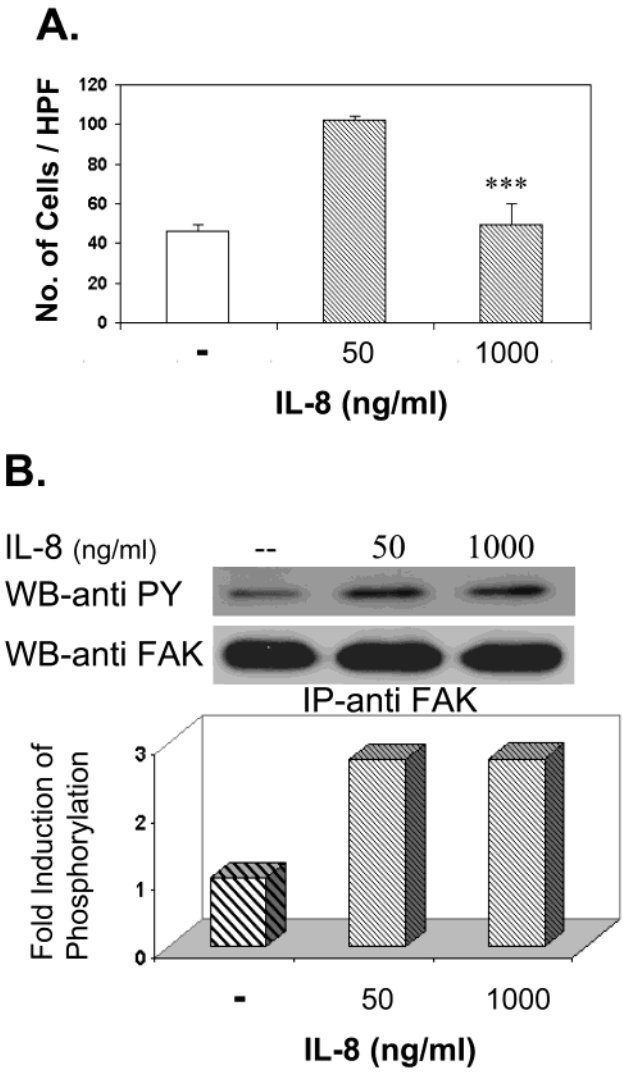

FIGURE 5: FAK is phosphorylated in conditions of IL-8-induced attenuation of CXCR1-RBL migration. The migration of CXCR1-RBL cells was determined in response to 50 ng/mL vs 1000 ng/mL IL-8. In conjunction, FAK phosphorylation in response to 50 ng/mL vs 1000 ng/mL IL-8 was determined. (A) Migration. HPF, high power field and $***p < 0.0001$ for the difference between migration to 50 ng/mL vs 1000 ng/mL IL-8. (B) Determination of FAK phosphorylation, performed at 5 min stimulation at 37 °C. FAK phosphorylation was analyzed as in Figure 1. IP, immunoprecipitation and WB, Western blotting. Fold induction of phosphorylation are phosphorylation levels calculated in reference to the total amount of FAK protein in the gel, where the basal level of phosphorylation without stimulation has been given the value of 1. A representative experiment is presented for migration and for FAK phosphorylation (for each: out of three independent experiments).

tions of IL-8. Then, FAK phosphorylation levels were determined under conditions of migration shut-off (induced by 1000 ng/mL IL-8) as compared to potent activation of migration (upon exposure to 50 ng/mL IL-8). First, FAK phosphorylation was determined in CXCR1-RBL cells, indicating that FAK is phosphorylated not only upon exposure to 50 ng/mL IL-8 but also upon stimulation with 1000 ng/mL (Figure 5B). In general, similar levels of FAK phosphorylation were induced in these cells by the two different IL-8 concentrations. When a similar analysis was performed on CXCR1-HEK cells, FAK was phosphorylated by 50 and 1000 ng/mL; however, a variation was observed between the experiments, therefore no definite conclusion could be made regarding the comparison between the relative abilities of the two IL-8 concentrations to induce FAK phosphorylation (data not shown).

When CXCR2 was stimulated by 50 and 1000 ng/mL IL-8 in RBL 2H3 cells, a different pattern of FAK phosphorylation was observed than upon stimulation of CXCR1 in these cells (in which the 1000 ng/mL IL-8 resulted in similar levels of FAK phosphorylation to those induced by 50 ng/mL IL-8). The stimulation of CXCR2 in RBL 2H3 cells by activating and attenuating concentrations of IL-8 indicated that FAK is differently regulated under these two extreme exposures to IL-8: higher levels of FAK phosphorylation were detected following stimulation with IL-8 concentrations in which migration is shut off (1000 ng/mL) than with concentrations that induce potent activation of migration (50 ng/mL IL-8) (Figure 6B). It is important to indicate that a similar increase in FAK phosphorylation under conditions of migration shut-off was observed also in CXCR2-HEK cells (data not shown).

These results indicate that upon stimulation of CXCR2, migration shut-off is accompanied with higher levels of FAK phosphorylation as compared to those associated with activation of migration and point to a modified regulation of FAK phosphorylation under the attenuating conditions. Such differential regulation of FAK may contribute to the attenuation of the migratory response, possibly through formation of more stable focal contacts that eventually perturb the process of cell migration, as will be discussed below (see Discussion). In addition, our results indicate that in RBL 2H3 cells CXCR1 and CXCR2 differ in their ability to promote FAK phosphorylation upon exposure to migration-activating versus migration-attenuating IL-8 concentrations. Such differences between the two receptors may contribute to their specific role in the regulation of migratory responses.

Identification of FAK Tyrosine Residues That Are Phosphorylated Following Exposure to IL-8. To further characterize the events associated with FAK phosphorylation following the stimulation of CXCR2 by activating versus desensitizing IL-8 concentrations in RBL 2H3 cells, we determined which of the tyrosine residues of FAK are phosphorylated following exposure to IL-8. FAK was shown to express six potential tyrosine phosphorylation sites, including Y397, Y407, Y576, Y577, Y861, and Y925 (*48*, *49*). The prime event in FAK activation is autophosphorylation at site Y397, resulting in recruitment of Src kinases and the consecutive phosphorylation of the other sites (*48*, *49*, *58*). Some of the additional phosphorylation sites (pY576, pY577, and pY925) were shown to regulate the kinase activity of FAK or to account for its ability to stimulate down stream mediators (*47*−*49*).

We have analyzed the phosphorylation of FAK at these six different phosphorylation sites in RBL 2H3 cells expressing CXCR2. The analysis was performed by using antibodies recognizing specifically each of the sites of FAK in its phosphorylated form (*59*). The results (Figure 7) indicate that upon exposure of the cells to 50 ng/mL IL-8, in conditions of migratory activation, only five of the sites were phosphorylated, including Y397, Y576, Y577, Y861, and Y925. The Y407 site was not reproducibly phosphorylated in response to this IL-8 treatment. In contrast, the exposure

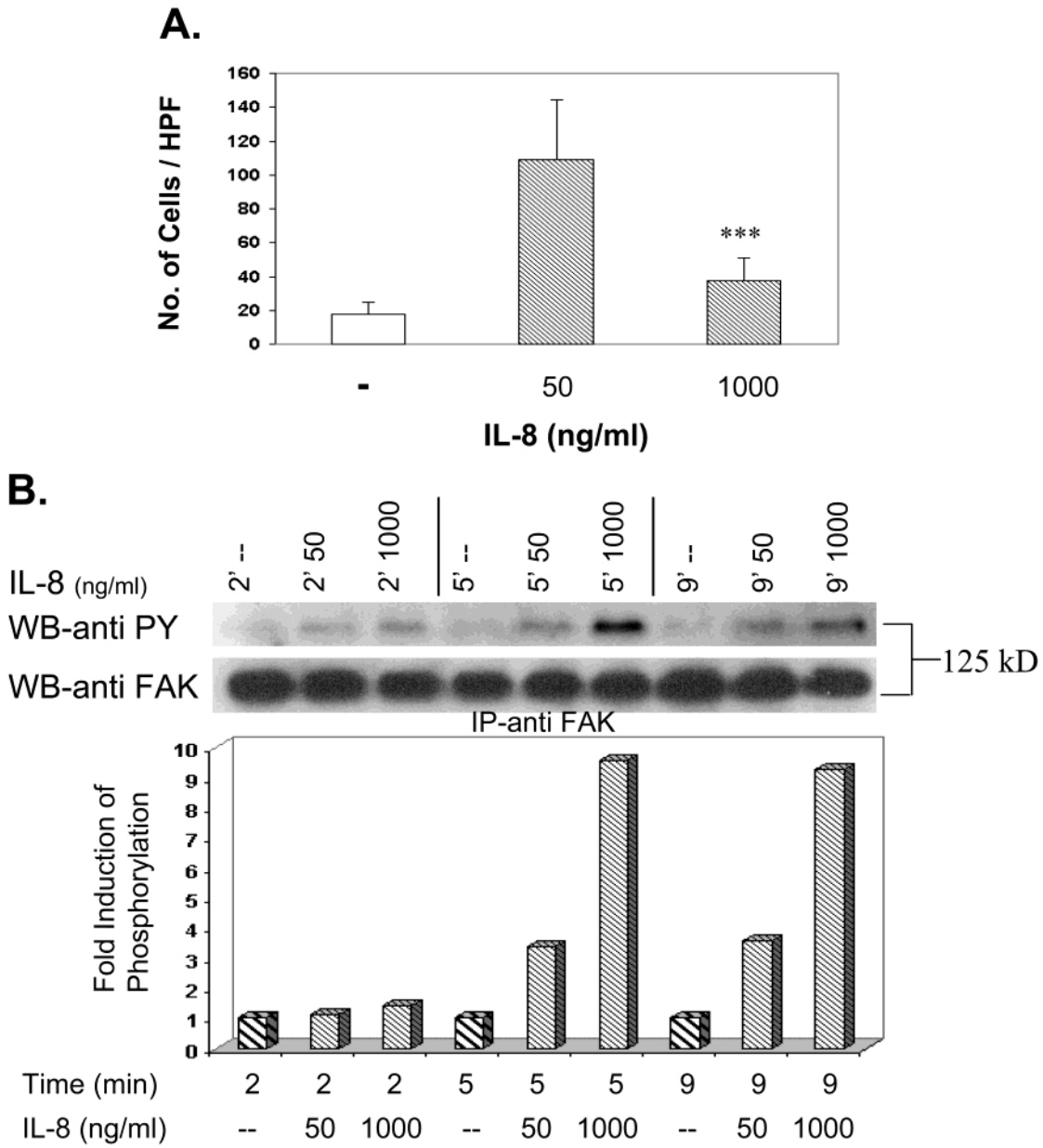

FIGURE 6: IL-8-induced attenuation of CXCR2-RBL migration is accompanied by further increased levels of FAK phosphorylation. The migration of CXCR2-RBL cells was determined in response to 50 ng/mL vs 1000 ng/mL IL-8. In conjunction, FAK phosphorylation in response to 50 ng/mL vs 1000 ng/mL IL-8 was determined. (A) Migration. HPF, high power field and ***$p < 0.0001$ for the difference between migration to 50 ng/mL vs 1000 ng/mL IL-8. (B) Determination of FAK phosphorylation, performed at the indicated time points, at 37 °C. FAK phosphorylation was analyzed as in Figure 1. IP, immunoprecipitation and WB, Western blotting. Fold induction of phosphorylation are phosphorylation levels calculated in reference to the total amount of FAK protein in the gel, where the basal level of phosphorylation without stimulation has been given the value of 1. A representative experiment is presented for migration (out of six independent experiments) and for FAK phosphorylation (out of at least three independent experiments).

of the cells to IL-8 concentrations that result in migratory-shut-off (1000 ng/mL) induced FAK phosphorylation on all six sites: Y397, Y407, Y576, Y577, Y861, and Y925.

These experiments also enabled the comparison between the phosphorylation levels of the six FAK phosphorylation sites under the migration-activating versus the migration-attenuating conditions. It was found that the phosphorylation of four of these sites was elevated upon treatment with the shut-off concentration of 1000 ng/mL as compared to the activating dose of 50 ng/mL: Y397, Y407, Y576, and Y861. For sites Y577 and Y925, although increase in FAK phosphorylation was observed in several of the experiments, no definite conclusions could be made regarding their phosphorylation by 50 ng/mL IL-8 versus 1000 ng/mL IL-8.

The results on Y397, Y407, Y576, and Y861 provide another illustration for the differential regulation of FAK that is induced by the exposure of the cells to the two extreme concentrations of IL-8, namely the 50 ng/mL that induce migration and the 1000 ng/mL in which migration is shut off. Therefore, our observations provide novel evidence regarding the differential regulation of the six phosphorylation sites of FAK by two IL-8 treatments that diverge in their migratory effects and may have a highly significant physiological relevance.

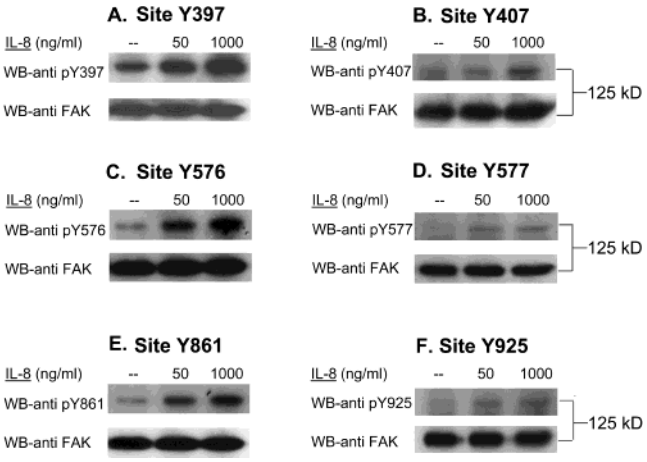

FIGURE 7: IL-8-induced phosphorylation of FAK on each of its six potential tyrosine phosphorylation sites. The phosphorylation of each site of the six tyrosine phosphorylation sites of FAK was determined in CXCR2-RBL cells following stimulation with the migratory-activating (50 ng/mL) or attenuating (1000 ng/mL) concentrations of IL-8 for 5 min at 37 °C. FAK phosphorylation was analyzed by immunoprecipitation with anti-FAK antibodies, followed by Western blotting with antibodies against the specific tyrosines of FAK in their phosphorylated form (pY397, pY407, pY576, pY577, pY861, and pY925), or with antibodies against FAK (following stripping and reprobing of the same membrane). WB, Western blotting. A representative experiment of at least three independent experiments performed is presented.

Contact Formation and FAK Distribution upon Exposure to IL-8. The involvement of FAK in processes that are associated with migration in response to IL-8 and to other ELR$^+$-CXC chemokines may necessitate not only its phosphorylation but also its redistribution to different cellular regions. The localization of FAK following chemokine stimulation was not addressed so far and motivated us to determine this issue. To this end, we analyzed by confocal microscopy the distribution of FAK, and of other components that participate in focal contact formation, following the exposure of the cells to IL-8. This analysis was performed in RBL cells that express CXCR2, and the chemokine was applied in concentrations of 50 and 1000 ng/mL. As shown in Figures 8 and 9, the cellular components that were analyzed (FAK, phosphorylated FAK, and vinculin) were highly expressed in the cells prior to stimulation with IL-8 and were redistributed upon the exposure to the chemokine. Following exposure to both IL-8 concentrations, FAK and the other components were redistributed to defined, but limited, contact areas with the substratum, at cell periphery. The general impression was that the response to 1000 ng/mL was stronger than to 50 ng/mL in terms of relocalization and especially in terms of the relative intensity of expression (mainly with regard to phosphorylated FAK). However, the specific pattern of the relocalization and expression of the tested components did not allow the performance of quan-

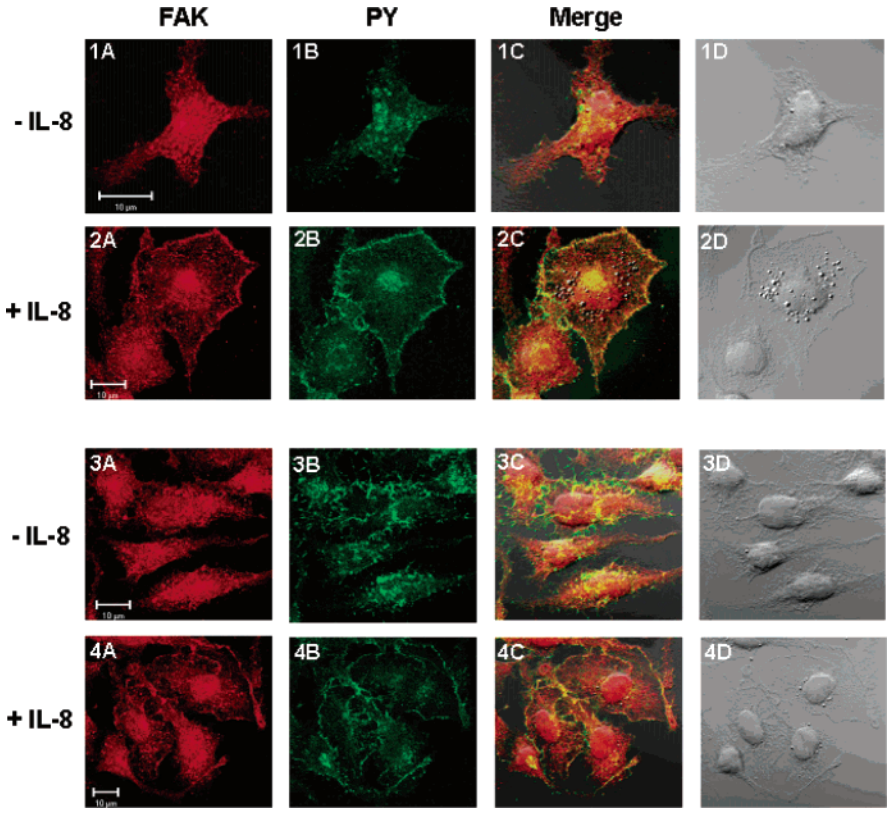

FIGURE 8: IL-8-induced contact formation: distribution of FAK and proteins phosphorylated on tyrosine residues. The localization of FAK and proteins phosphorylated on tyrosine residues following stimulation of CXCR2-RBL cells with IL-8 was determined by confocal analysis. The expression of FAK was determined by rabbit polyclonal antibodies against FAK, followed by staining with rhodamine-conjugated goat anti-rabbit IgG. The expression of proteins phosphorylated on tyrosine residues (PY) was determined by monoclonal mouse antibodies against phosphotyrosine, followed by staining with FITC-conjugated goat anti-mouse IgG. Panels 1 and 3: Untreated cells. Panels 2 and 4: Cells treated by 1000 ng/mL IL-8 for 5 min at 37 °C. 1A, 2A, 3A, and 4A: expression of FAK. 1B, 2B, 3B, and 4B: expression of proteins phosphorylated on tyrosines residues (PY). 1C, 2C, 3C, and 4C: merge of FAK and proteins phosphorylated on tyrosine residues. 1D, 2D, 3D, and 4D: Nomarsky contrast demonstration of cell morphology. Panels 1 and 2: High magnification of cells. Panels 3 and 4: Low magnification of cells, showing a group of cells. The reference bar in the lower left corner represents 10 μm. A representative experiment of at least three independent experiments performed is presented.

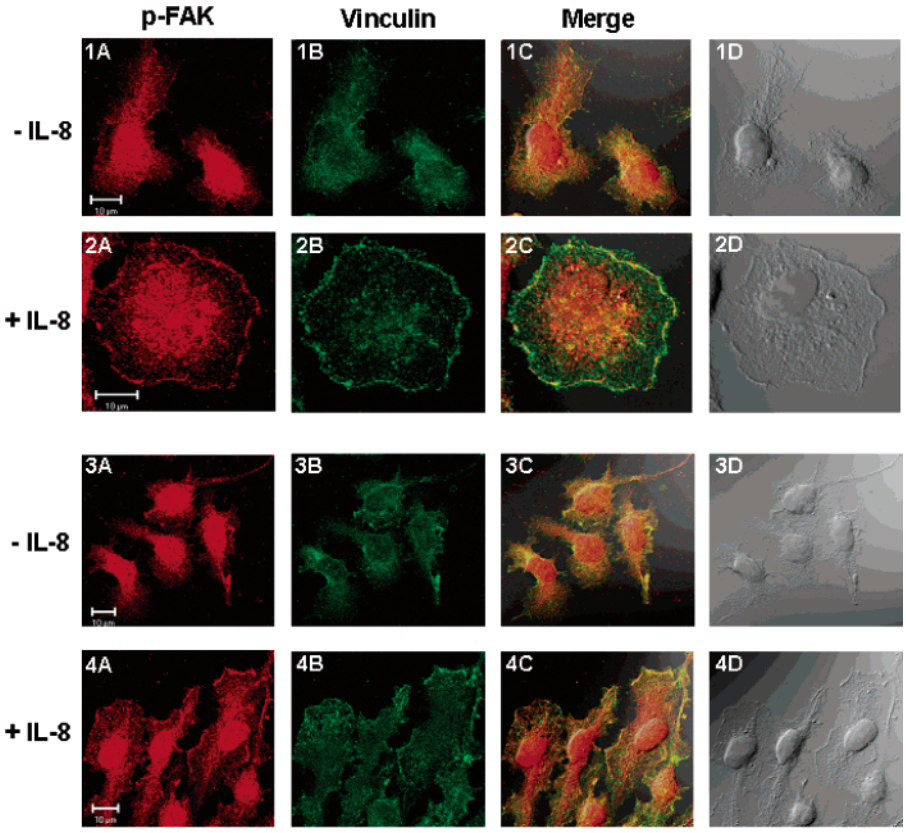

FIGURE 9: IL-8-induced contact formation: distribution of phosphorylated FAK and vinculin. The localization of phosphorylated FAK and vinculin following stimulation of CXCR2-RBL cells with IL-8 was determined by confocal analysis. The expression of phosphorylated FAK (p-FAK) was determined by rabbit polyclonal antibodies against site pY576 of FAK, followed by staining with rhodamine-conjugated goat anti-rabbit IgG. The expression of vinculin was determined by monoclonal mouse antibodies against vinculin, followed by staining with FITC-conjugated goat anti-mouse IgG. Panels 1 and 3: Untreated cells. Panels 2 and 4: Cells treated by 1000 ng/mL for 5 min at 37 °C. 1A, 2A, 3A, and 4A: expression of phosphorylated FAK (p-FAK). 1B, 2B, 3B, and 4B: expression of vinculin. 1C, 2C, 3C, and 4C: Merge of phosphorylated FAK and vinculin expression. 1D, 2D, 3D, and 4D: Nomarsky contrast demonstration of cell morphology. Panels 1 and 2: High magnification of cells. Panels 3 and 4: Low magnification of cells, showing a group of cells. The reference bar in the lower left corner represents 10 μm. A representative experiment of at least three independent experiments performed is presented.

titative analysis of the differences between the two IL-8 concentrations. Therefore, for presentation, the concentration that was the most effective in induction of FAK phosphorylation, namely 1000 ng/mL IL-8, was chosen.

In the first set of experiments performed, we have determined the expression of FAK in conjunction with proteins that are phosphorylated on tyrosine residues (PY proteins). Such proteins should include FAK in its phosphorylated form. IL-8, at 1000 ng/mL, was evenly applied to the cells for 5 min at 37 °C. As shown in Figure 8, this treatment resulted in cell spreading, accompanied by clearly defined areas at cell periphery, of strong attachment to the substratum. Such regions could be hardly observed in cells that were not treated by IL-8. The contact regions in IL-8-treated cells were enriched with FAK, as well as with tyrosine-phosphorylated proteins. Co-localization between FAK and PY proteins was clearly apparent in these contact areas. Altogether, our results indicate that upon stimulation by IL-8, specialized contacts are formed between the cells and the substratum and that FAK, possibly in its phosphorylated form, is highly expressed in these regions.

To directly determine the cellular localization of phosphorylated FAK in contact areas upon stimulation with IL-8, an additional analysis was performed in which the expression and localization of phosphorylated FAK and vinculin were determined (Figure 9). In similarity to FAK, vinculin is a major constituent of focal contacts (*60*, *61*). The expression of phosphorylated FAK was evaluated by the use of antibodies that specifically recognize the phosphorylated 576 site (pY576) of FAK. These antibodies were chosen because the phosphorylation of this site potentiates the catalytic activity of FAK (*62*). Moreover, since the antibodies against pY576 demonstrated high phosphorylation of this site in Western analyses, it was speculated that their use would enable good resolution of FAK phosphorylation in confocal studies.

In agreement with the observations with FAK and PY proteins (Figure 8), following the IL-8 treatment we have observed that phosphorylated FAK (p-FAK) and vinculin were highly expressed and co-localized in regions at the periphery of the cells, where definite contact areas were formed (Figure 9). These results, combined with the observation mentioned above (Figure 8), provide novel evidence regarding the redistribution of phosphorylated FAK, as well as of other components of focal contacts such as vinculin, in response to stimulation with IL-8, and their co-localization at specialized cell regions that form contact with the substratum.

DISCUSSION

The migration of leukocytes in response to chemokines is the hallmark of inflammatory processes (*1*−*6*). This process

may be either potentiated or attenuated, depending on the duration and the potency of the inflammatory process, as well as on chemokine concentration (*3*−*6*, *28*, *29*, *32*−*37*). Migratory attenuation may serve as a key mediator in the limitation of leukocyte infiltration to inflammatory sites under conditions of continuous and prolonged inflammatory stimulation. Migration to chemokines may also serve as a fundamental property of endothelial and tumor cells, as indicated recently by several studies (*14*−*16*).

As a highly coordinated event, cell movement is regulated by numerous factors, including ligand type, receptor specificity, and induction of signaling pathways that induce the activities of specific cellular components. In the present study, we have provided evidence for the regulation of FAK at the levels of phosphorylation and cellular relocalization in response to ELR$^+$-CXC chemokines. FAK was phosphorylated and had a direct role in the regulation of migration under migratory-activating conditions. Of importance is the fact that our results also indicate that FAK was differently regulated upon stimulation of CXCR2 with attenuating concentrations of IL-8. On the whole, our study provides novel findings with regard to the regulation of FAK in ELR$^+$-CXC chemokine-mediated migratory responses, including the following observations:

(1) FAK is directly involved in activation of the migratory process in response to IL-8. This conclusion is based on the observation that FRNK expression resulted in perturbation of migration and on the fact that FAK was phosphorylated following exposure to migratory-activating concentrations of IL-8. In support of the role of FAK in the regulation of migratory responses is the fact that direct relationships existed between the potency of the migratory response that was induced under activating conditions and the level of FAK phosphorylation. This notion was demonstrated by the fact that the levels of FAK phosphorylation were directly associated with the potency of migration induced by IL-8, GCP-2, and NAP-2 under activating conditions.

In general, FAK phosphorylation was rapidly induced upon the exposure to the chemokines. As with many other intracellular signaling events, the outcome of chemokine-induced signaling was manifested as migratory responses only hours later, following the proper organization of cellular components that are necessary for migration.

The ability of IL-8 to induce FAK phosphorylation under conditions of migratory activation was mediated through $G_{\alpha i}$ proteins and may be the result of the capability of the chemokine to activate mediators that are upstream to FAK. Such mediators could include integrins, which are fundamental components for FAK activation (*45*, *46*, *48*−*50*, *60*). The well-described ability of chemokines, including IL-8, to activate integrins (*63*−*67*) proposes the involvement of integrins in IL-8-induced FAK activation.

Our observations on the role of FAK in the regulation of ELR$^+$-CXC chemokine-induced migratory processes pose questions as to the generality of such regulation in the control of chemokine-induced cell motility. This aspect has been touched upon in a relatively small number of studies, demonstrating that chemokines, such as RANTES and SDF-1, induce FAK phosphorylation (or phosphorylation of its homologue, Pyk2) and that FAK is phosphorylated in neutrophils (*68*−*72*). However, our study not only provides evidence for the involvement of FAK in migratory responses that are induced by ELR$^+$-CXC chemokines but also points to the ability of FAK to provide a mean for fine-tuning of such responses in a chemokine-dependent manner and to be differently regulated following exposure to migratory-activating versus migratory-attenuating conditions (see part 3 in the Discussion).

(2) FAK is regulated also at the level of cellular localization. The stimulation with IL-8 resulted not only in upregulation of FAK phosphorylation but also in its redistribution to definite contact areas with the substratum. This is the first demonstration of altered FAK localization upon chemotactic stimulation. The type of contact that was formed following exposure to IL-8 is not clear as yet; nevertheless, it was observed that phosphorylated FAK and other components of focal contacts (vinculin) were co-localized in the contact regions.

(3) In CXCR2-expressing cells, FAK is differently regulated by migratory-activating IL-8 concentrations as compared to chemokine concentrations that induce migratory shut-off. Stimulation by IL-8 is a tightly regulated event, in which migratory activation is promoted in vitro by low IL-8 concentrations (e.g., 10−50 ng/mL) and migration shut-off is induced by exposure to high concentrations of the chemokine (1000 ng/mL) (*3*−*6*, *8*, *9*, *11*, *28*−*33*, *35*, *36*). In CXCR2-expressing cells, the attenuation of the migratory process was accompanied by higher levels of FAK phosphorylation as compared to those observed in activating processes. This was evident in both CXCR2-RBL cells (Figures 6 and 7) and CXCR2-HEK cells (data not shown). In contrast, the stimulation of CXCR1 in RBL 2H3 cells by desensitizing concentrations of IL-8 did not give rise to a similar phenomenon (Figure 5). Although migration was attenuated by stimulation of CXCR1 with high IL-8 concentrations, FAK phosphorylation levels under these conditions were not significantly altered as compared to activating conditions.

On the whole, our results indicate that upon the stimulation of CXCR1 and CXCR2, FAK is phosphorylated. Moreover, FAK phosphorylation is directly required for stimulation of migration under the migration-activating conditions, as indicated by overexpression of FRNK. However, in the case of CXCR2, FAK is differently regulated by activating versus desensitizing exposures to IL-8. The potential contribution of high FAK phosphorylation to migratory attenuation and the possible reasons for differences between CXCR1 and CXCR2 in this respect will be discussed below.

As noted above, in CXCR2-expressing cells FAK phosphorylation was shown to be differently regulated under conditions of migratory shut-off versus conditions of migratory activation. In addition, it was shown that of the six tyrosine phosphorylation sites of FAK, only five were phosphorylated upon treatment with the migration-activating concentration of IL-8, whereas all six sites were phosphorylated by exposure to the migratory shut-off concentration of the chemokine. The differential regulation of FAK under the activating versus attenuating conditions was further observed by the fact that four of six FAK phosphorylation sites (Y397, Y407, Y576, and Y861) were more phosphorylated in response to the shutting-off concentrations of IL-8 as compared to migration-activating conditions. Two of these sites, Y397 and Y576, are directly mediating the kinase activity of FAK (*48*, *49*). No role was assigned as yet to the

two other sites, namely Y407 and Y861. Our results suggest that the differential phosphorylation of these four sites may contribute to divergent activities of FAK under migratory activating versus shutting-off conditions. These results are of major importance since they indicate that the phosphorylation sites of FAK are differently regulated in response to different stimulatory conditions that are of physiological relevance.

The fact that IL-8-induced attenuation of migration in CXCR2-expressing cells is accompanied with highly elevated levels of FAK phosphorylation suggests that the differential levels of FAK phosphorylation may play a role in the shutting-off process of the migratory response. It is possible that the process of migratory shut-off is initiated by G protein uncoupling from the receptors and is assisted by a complementary pathway, such as increased FAK phosphorylation. Increased FAK phosphorylation may directly contribute to the attenuation of migration. The further elevated phosphorylation of FAK in response to migration shutting-off concentrations of IL-8 may disequilibriate the delicate regulation of turnover processes of contact formation, which are regulated by FAK. FAK was shown to control processes of assembly and disassembly of focal contacts. It was postulated that cycles of FAK phosphorylation and dephosphorylation are required for potent migratory processes to take place (*49*, *50*, *73*, *74*). Highly elevated levels of FAK phosphorylation may result in a modified regulation of turnover processes, giving rise to alterations in spreading properties and in ability to migrate. For example, in conditions of highly elevated FAK phosphorylation, the process of FAK dephosphorylation, which is required for detachment, may be inefficient. In such a case, the spreading and attachment of the cells may remain tight, eventually preventing cell movement in response to the chemokine. In accordance with this possibility are several studies indicating that it is not the level of FAK phosphorylation per se but rather the degree of focal contact turnover that dictates the magnitude of cell spreading and eventually affects the migratory process (*74*, *75*).

A complementary explanation for the observation that migratory attenuation is associated with highly elevated levels of FAK phosphorylation is that the over-phosphorylation of FAK results in decay of signaling events that are required for efficient migration. This possibility is supported by a recent study showing that increase in FAK phosphorylation to a steady-state level is accompanied by deactivation of signaling pathways that include ERK2 activity (*76*).

(4) With respect to FAK phosphorylation, the regulation of CXCR1 differs from that of CXCR2. Two major observations suggest that FAK is differently regulated upon stimulation of CXCR1, as compared to CXCR2 stimulation. First, the kinetics of FAK phosphorylation upon stimulation of receptor-transfected HEK 293 cells with activating IL-8 concentrations differed for the two receptors (Figure 1). This was indicated by the fact that FAK phosphorylation in CXCR1-expressing cells peaked at 5 min of agonist stimulation, whereas in CXCR2-expressing cells FAK phosphorylation was more rapidly induced and declined by 5 min. Second, differences in FAK phosphorylation under migratory-activating versus migratory-attenuating concentrations were observed in CXCR2-expressing RBL 2H3 (and also CXCR2-HEK) cells but not in CXCR1-expressing RBL 2H3 cells.

These differences between CXCR1 and CXCR2 provide evidence for the unique and differential regulation of each receptor upon exposure to IL-8, as was already alluded in many different aspects of CXCR1- versus CXCR2-mediated responses (*22*−*26*). Desensitization of migration to IL-8, the most potent ELR$^+$-CXC chemokine, may be important under conditions in which the inflammatory process has occurred for a prolonged time, resulting in exacerbation of chemokine release. Under these conditions, it is important to shut off the infiltration of neutrophils to the inflammatory site. Desensitization of receptor-mediated signaling may be a key event in this process. It is possible that CXCR2 is the key determinant for sensing the conditions that require desensitization. Such a possibility is in agreement with the postulated role of CXCR2 in mediating chemotaxis to IL-8 at distance from the inflammatory site (*22*). As a physiologically relevant control mechanism, the ability of CXCR2 to sense the highly elevated concentrations of IL-8 at a large distance from the target site may result in shutting-off of neutrophil recruitment to the damaged site, and its regulation with this respect is essential for the proper control of the inflammatory process.

In all, our observations provide further evidence for the ability of ELR$^+$-CXC chemokines to regulate FAK and suggest that such regulation may provide an essential mean for fine-tuning of ELR$^+$-CXC chemokine-induced responses.

ACKNOWLEDGMENT

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