Cell. Mol. Life Sci. 66 (2009) 884 – 899
1420-682X/09/050884-16
DOI 10.1007/s00018-009-8447-5

Cellular and Molecular Life Sciences

Research Article

Cell migration to the chemokine CXCL8: Paxillin is activated and regulates adhesion and cell motility

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Received 31 July 2008; received after revision 14 December 2008; accepted 16 December 2008
Online First 21 January 2009

Abstract. The chemokine CXCL8 is a powerful inducer of directional cell motility, primarily during inflammation. In this study, we found that CXCL8 stimulation led to paxillin phosphorylation in normal neutrophils, and that both CXCL8 receptors (CXCR1 and CXCR2) mediated CXCL8-induced paxillin phosphorylation. In CXCR2-transfected cells, the process depended on $G_{\alpha i}$ and $G_{\alpha s}$ coupling to CXCR2. Dominant negative (DN) paxillin increased CXCL8-induced adhesion and migration, indicating that endogenous paxillin keeps migration at sub-maximal levels. Furthermore, using activating anti-bodies to β1 integrins, analyses with focal adhesion kinase (FAK) DN variant (FRNK) and co-immuno-precipitations of FAK and paxillin, we found that β1 integrin ligation cooperates with CXCL8-induced stimulation, leading to FAK activation and thereafter to FAK-mediated paxillin phosphorylation. Our findings indicate that paxillin keeps directional motility at a restrained magnitude, and suggest that perturbations in its activation may lead to chemotactic imbalance and to pathological conditions associated with excessive or reduced leukocyte migration.

Keywords. Cell migration, chemokines, CXCL8, CXCR2, paxillin.

Introduction

Cell migration is an essential process in organ homeostasis, inflammation and angiogenesis. Members of the chemokine superfamily are important regulators of leukocyte directional motility to inflammatory sites and to lymphoid organs [1–4]. Of the different chemokines, CXCL8 (Interleukin 8) is a prime inducer of neutrophil directed migration to acute inflammatory sites, and it was also found to promote the motility of other leukocyte sub-types [5–8]. However, the roles of CXCL8 in migration are not limited to leukocytes, as it was found to induce the motility of tumor cells and endothelial cells, thus being a potent angiogenic factor [9–12].

In the inflammatory context, CXCL8-induced migration is essential for the immune integrity of the host. However, since cell motility is a dynamic, sequential, and tightly regulated process, its inappropriate regulation may lead to pathological conditions. For example, intense CXCL8 activities may lead to exacerbated leukocyte migration, resulting in inflammatory diseases such as chronic obstructive pulmonary disease, acute respiratory distress syndrome and others. Alternatively, desensitization of CXCL8-induced migration may form part of the basis of

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pathological conditions in which reduced response to inflammatory signals is observed [13–18].

In view of the important roles played by CXCL8 in inflammatory processes, we wished to provide insights into the regulation of CXCL8-induced directional motility, and to identify the molecular mechanisms controlling adhesion and migration in response to this chemokine. We have previously investigated the involvement of the protein tyrosine kinase focal adhesion kinase (FAK) in CXCL8-induced directed migration, mediated through the two high-affinity CXCL8 receptors, the G protein-coupled receptors CXCR1 and CXCR2 [6–8, 19, 20]. Our findings showed that FAK phosphorylation and activation are essential for CXCL8-induced migration [19]. Also, we discovered that fine-tuning of CXCR1- and CXCR2-mediated signals is important for regulation of migration, and that CXCR2 undergoes more complex regulation than CXCR1 [19, 20].

In the present study we further explored the regulation of CXCL8-induced directed cell motility by focusing on paxillin, a focal adhesion scaffold protein that binds many proteins regulating the actin cytoskeleton, cell adhesion and cell migration. Paxillin is stimulated by integrin ligation induced by adhesion to extracellular matrix (ECM) proteins, and by signals delivered by activated G protein-coupled receptors and growth factor receptors [21–25]. The activation of paxillin is mediated mainly by phosphorylation of four tyrosine residues located at its N-terminal domain. Thus far, three tyrosine kinases were found to bind paxillin and to induce its phosphorylation on tyrosine residues, including FAK, proline-rich tyrosine kinase 2 (Pyk2) and Src family members. Importantly, of the four tyrosine phosphorylation sites of paxillin, the Y118 and Y31 are those having major importance for regulation of cell migration [21, 22, 24, 26–28].

By controlling the organization and function of focal contact areas and of the cytoskeleton, paxillin controls processes of adhesion and motility. While many studies indicated that paxillin is a positive regulator of cell migration, others have suggested that paxillin activation retards cell motility. Recent studies suggested that the opposing effects of paxillin on cell migration may depend on the type of migration cues delivered to the cells, and that paxillin controls cellular migration differently in conditions of random motility as compared to directional migration [21–23, 29–33]. To date, many studies have addressed the activation of paxillin in response to integrin engagement with ECM proteins; however, in most cases the analyses were not performed in the presence of chemotactic ligands. Aspects regarding paxillin activation in the context of chemotactic signals are of major interest, because chemoattractants play key roles in integrin activation [1, 23, 34, 35], and because integrin stimulation is a predominant regulator of paxillin activation [21–25]. When paxillin regulation was investigated in the presence of chemokine signals, the majority of studies addressed the homeostatic chemokine CXCL12 (SDF-1), showing that it induces paxillin phosphorylation. However, only very few of these studies analyzed the direct roles played by paxillin in chemokine-induced directional migration, and the mechanisms regulating its activation [29, 36–40].

To better understand the involvement of paxillin in motility induced by chemotactic stimuli, we focused in this study on CXCL8, because of its prime activities in the inflammatory process. The findings obtained in our study indicate that, following CXCL8-induced activation, endogenous paxillin keeps migration and adhesion at sub-maximal levels; therefore, paxillin acts as a negative regulator of the directional motility induced by this chemokine. Furthermore, our findings indicate that CXCL8-induced paxillin phosphorylation depends on an initial step of integrin activation, and that this step cooperates with CXCL8-induced signals, leading to stimulation of FAK, and eventually to FAK-mediated paxillin phosphorylation and activation.

Overall, this study provides novel insights into the roles of paxillin in directional migration induced by an inflammatory chemokine, and indicate that paxillin acts as a negative regulator of motility in response to directional cues provided by CXCL8. Our observations indicate that paxillin is tightly controlled, and suggest that inappropriate regulation of paxillin upon CXCL8 stimulation may lead to chemotactic imbalance in pathological conditions.

Materials and methods

Transfected cells. Constructs expressing wild-type (WT) human CXCR1 and CXCR2 were generated, followed by full-length sequencing that verified the desired sequences. Stable polyclonal CXCR1 and/or CXCR2 transfectants of rat basophilic leukemia (RBL) 2H3 cells and of human embryonic kidney (HEK) 293 cells were established with these constructs. CXCR1 or CXCR2 were expressed by the transfected cells at similar levels in over 85% of transfected RBL and HEK cells. All cell types responded potently to CXCL8, as determined by migration and internalization assays ([19, 20, 41–54] and figures presented in the current paper). Untransfected and/or empty vector-transfected RBL and HEK cells did not express CXCL8 receptors and did not bind CXCL8 ([42–44, 52, 53] and data not shown).

For experiments with WT paxillin or with dominant-negative (DN) paxillin, CXCR2-RBL cells were transiently transfected with pEGFP-C3 vector (Clontech, Palo Alto, CA) or with the same vector expressing WT paxillin or DN paxillin (both generated in our laboratory). Based on published studies on paxillin [26–28], DN paxillin was created by mutating Y118 and Y31 to phenylalanine. In all three transfection types, FACS analyses showed that 50–60 % of the cells expressed the transfected GFP vectors 24 h after the transfection.

For experiments with FRNK (FAK-related non-kinase domain), CXCR2-HEK cells were transiently transfected with pEGFP-C1 vector (Clontech) or with the same vector containing FRNK (a kind gift from M. A. Schwartz, Robert M. Berne Cardiovascular Research Center, University of Virginia, VA). In both transfection types, FACS analyses showed that 40–60 % of the cells expressed the transfected GFP vectors 48 h after the transfection.

The transfections of WT paxillin, DN paxillin or FRNK cDNAs did not affect the expression levels of CXCR2, and the receptor expression levels remained similar to those of untransfected cells (data not shown).

Cell preparation for phosphorylation assays. The phosphorylation of paxillin, Pyk2 and/or FAK was determined in adherent and/or suspended CXCR1-RBL, CXCR2-RBL or CXCR2-HEK cells, as well as in neutrophils, following the preparation of the cells, as follows:

Adherent CXR1-RBL, CXCR2-RBL and CXCR2-HEK cells: The cells were grown as previously described [19]. Briefly, prior to phosphorylation assays, the cells were transferred to starvation medium containing 0.5 % FCS. While adherent, the cells were stimulated with CXCL8 (PeproTech, Rocky Hill, NJ) diluted in BSA medium (RPMI containing 1 % BSA and 25 mM HEPES) or with BSA medium alone. The reaction was stopped by addition of ice-cold PBS. Thereafter, the cells were lysed and paxillin phosphorylation was determined as indicated below. In specific experiments, prior to stimulation by CXCL8, the cells were pre-exposed to pertussis toxin (PTx; 100 ng/ml; List Biological Laboratories, Campbell, CA) or cholera toxin (CTx; 100 ng/ml; List Biological Laboratories) for 2 h at 37°C. Also, in specific experiments, prior to stimulation by CXCL8 the cells were pre-exposed to tyrphostin A9 (AG17; 3 or 10 μM; Sigma, St. Louis, MO) or PP2 (10 μM; Calbiochem, La Jolla, CA) for 1 h and 1.5 h at 37°C, respectively. When dimethyl sulfoxide (DMSO) was used to solubilize the drugs, similar dilutions of DMSO were used as controls. The drugs did not affect the expression levels of CXCR2 by the cells, nor did they affect cell viability.

Suspended RBL cells, with or without exposure to fibronectin: The cells were grown overnight in non-tissue culture plates coated by 1 % ultrapure agarose in starvation medium containing 0.4 % methyl cellulose (4000 centipoises; Sigma). Under these conditions, the cells were viable, receptor expression was similar to that of adherent cells, and the cells responded intactly to CXCL8-induced signals (*e.g.*, CXCR2 internalization; data not shown). In complementary experiments, cells that were grown in suspension were plated on fibronectin-coated plates (40 μg/ml; Biological Industries, Beit Ha'amek, Israel) for 45 min at 37°C. Following these procedures, the cells were stimulated with CXCL8 diluted in BSA medium or with BSA medium alone, lysed and paxillin phosphorylation was determined as described in "Phosphorylation assays" below.

HEK cells in studies with antibodies to β1 integrins: The cells were plated on tissue culture plates with growth medium, then with starvation medium for another 24 h. Thereafter, the cells were trypsinized, treated with trypsin inhibitor (Sigma), and exposed to three treatments: (1) Cells exposed to fibronectin: The cells were resuspended in BSA medium, and plated on non-tissue culture plates that were coated by 40 μg/ml fibronectin for 2 h at 37°C and blocked by 1 % heat-inactivated BSA. Following plate washings with PBS, the cells were added to the plates for 2 h at 37°C in the presence of 5 mM Mg^{2+} and 0.5 mM Mn^{2+}. (2) Cells in suspension: The cells were cultured for 2 h at 37°C on plates coated by 1 % heat-inactivated BSA to determine their adhesion phenotype, or placed in test tubes for 20 min at 37°C to reduce basal phosphorylation prior to CXCL8 stimulation and determination of paxillin phosphorylation. (3) Cells stimulated by activating antibodies to β1 integrins: The cells were stimulated by the TS2/16 antibodies against β1 integrins (a kind gift from M. J. Humphries, Welcome Trust Center for Cell-Matrix Research, UK). Non-tissue culture plates were coated by 30 μg/ml $F(ab')_2$ fragment of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), blocked by 1 % heat-inactivated BSA and then coated with 50 μg/ml TS2/16 antibodies. The cells were incubated on the plates for 2 h at 37°C in the presence of Mg^{2+} and Mn^{2+} (as above). Following these three treatments, the cells were stimulated by CXCL8 diluted in BSA medium or by BSA medium alone, at 37°C. The reaction was stopped by the addition of ice-cold PBS. The cells were then lysed and paxillin phosphorylation was determined as described below. No adhesion was observed in cells that were plated on BSA-coated plates or on

plates coated by $F(ab')_2$ fragment of goat anti-mouse IgG only.

Human neutrophils: Neutrophils were derived from heparinized blood of healthy individuals by separation on Polymorphprep (Axis-Shield, Oslo, Norway) to over 90% purity (approved by the Helsinki Committee of Schneider Children's Medical Center). The neutrophils were then plated for 2 h at 37°C, in the presence of Mg^{2+}, Mn^{2+} (as above) and 1.2 mM Ca^{2+} on non-tissue culture plates that were coated by 250 μg/ml fibrinogen, with or without CXCL8, and were blocked by 1% heat-inactivated BSA. The reaction was stopped by the addition of ice-cold PBS, the cells were scraped and lysed, and paxillin phosphorylation was determined as described below.

Phosphorylation assays. To determine phosphorylation, the transfected RBL and HEK cells were lysed in RIPA lysis buffer. Neutrophils were lysed in buffer containing 50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 3 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 50 mM NAF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 mM PMSF. Lysis was followed by immunoprecipitation and Western blotting as described previously [19]. Briefly, immunoprecipitation was performed with mouse anti-paxillin antibodies (BD Bioscience, San Jose, CA; cat. no. 610052), goat anti-Pyk2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. SC-1515), or rabbit anti-FAK antibodies (Santa Cruz Biotechnology; cat. no. SC-558). Protein loading on the gel was determined by antibodies against each of the specific proteins (same as above), and phosphorylation was determined by monoclonal murine antibodies against phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) or by rabbit polyclonal antibodies against Y118 of paxillin in its phosphorylated form (kindly donated by B. Geiger, Weizmann Institute, Israel; Biosource International, Camarillo, CA). Following incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, the membrane was subjected to enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK) and bands on immunoblots were quantitated by densitometry. In all assays, statistical analyses assuming Gaussian distributions were performed by Two-Sample Assuming Unequal Variances Student's *t*-tests.

In co-immunoprecipitation analyses, adherent CXCR2-RBL cells were non-stimulated or stimulated by CXCL8 diluted in BSA medium. Following preparation of cell lysates, co-immunoprecipitations were performed in the presence of 0.01% Tween and high NaCl molarity. The antibodies for FAK and paxillin were as above.

Migration assays. Following transfection with vectors expressing WT paxillin, DN paxillin, or an empty vector only, CXCR2-RBL cells were grown in adhesion in starvation medium, and then loaded into 48-well microchemotaxis chambers. Migration in response to 50 ng/ml CXCL8 was performed for 2.5 h with a 8-μm pore membrane coated by 20 μg/ml fibronectin (Biological Industries), as previously described [43]. The cells were stained and counted in five high-power fields by light microscopy, in triplicates. Statistical analysis assuming Gaussian distributions was performed by Two-Sample Assuming Unequal Variances Student's *t*-tests.

Adhesion assays. Adhesion assays were performed as previously described [55]. Briefly, following transfection with vectors expressing WT paxillin, DN paxillin or an empty vector only, CXCR2-RBL cells were grown in adhesion in starvation medium, then resuspended in BSA medium. The cells were plated into a 96-well non-tissue culture plates coated with 15 μg/ml fibronectin, with or without CXCL8 and blocked by 1% heat-inactivated BSA. The plates were centrifuged for 5–7 min at room temperature, non-adherent cells were removed by washings, and the extent of cell adhesion was evaluated by an alkaline phosphatase (ALP) assay at 405 nm. Preliminary analysis assured that the ALP intensity was proportional to cell numbers in the wells. Statistical analysis assuming Gaussian distributions was performed by Two-Sample Assuming Unequal Variances Student's *t*-tests.

Results

Research outline. The major aim of this study was to determine the regulation of paxillin in response to CXCL8, the most potent of all ELR^+-CXC chemokines in induction of neutrophil migration to inflammatory sites. Accordingly, this study was initiated by determining the ability of CXCL8 to induce paxillin phosphorylation in neutrophils, the prototype of cells migrating in response to CXCL8. Neutrophils express both CXCR1 and CXCR2, the two high-affinity receptors for CXCL8 [5–8]. Using transfected cells that expressed only CXCR1 or only CXCR2, we first examined whether each of the receptors is functional in this respect. We then performed more detailed analyses in the CXCR2 context only, to provide comprehensive information on the roles of paxillin in CXCL8-induced migration and adhesion, and on the regulation of paxillin activation. The reason for focusing on CXCR2 was that this receptor was found to undergo more intricate modes of regulation than

CXCR1 (e.g. [5, 19, 20, 46, 48, 56, 57]). Moreover, CXCR2 has been the subject of intense and published research on CXCL8-induced migratory responses. Therefore, a study performed in a CXCR2 system could provide data that can be viewed in concert with additional findings in other CXCR2-based cell systems, enabling a more comprehensive view of the regulation of CXCL8-induced migration.

As indicated above, in this study we have used cells that we transfected to express only CXCR1 or only CXCR2. In most of our experiments we used rat basophilic leukemia 2H3 cells (RBL) that were transfected with vector expressing CXCR1 or CXCR2. We chose RBL cells because, similar to neutrophils, they are derived from the myeloid lineage. In addition, CXCR2-transfected human embryonal kidney 293 (HEK) cells were used on specific occasions, when we found that they were advantageous over the RBL cells (please see below). Chemokine receptor-transfected RBL and HEK cells have been extensively used in studies of chemokines, and following transfection with CXCL8 receptors they have shown high resemblance to neutrophils in their ability to respond to CXCL8 [19, 20, 41–54, 58]. Also, in both cell systems, untransfected/empty vector-transfected cells did not bind CXCL8 and did not respond to CXCL8 stimulation ([42–44, 52, 53] and data not shown).

Throughout our study, we have used CXCL8 at 50–100 ng/ml, concentrations that were shown in dose-dependent analyses to potently and optimally induce the migration to CXCL8 of neutrophils, as well as of RBL and HEK cells expressing CXCL8 receptors ([19, 20, 43, 48, 59–61] and data not shown).

Endogenous paxillin is phosphorylated upon CXCL8 stimulation and down-regulates CXCL8-induced migration and adhesion. First, neutrophils from healthy donors were stimulated by CXCL8 at a concentration that induces potent migration of these cells [59–61]. This treatment led to phosphorylation of paxillin on tyrosine residues (Fig. 1A), indicating that the chemokine induces paxillin phosphorylation under the same conditions that induce migration. Thereafter, we stimulated CXCR1-RBL cells or CXCR2-RBL cells by CXCL8, also at a concentration that induces optimal migration of these cells ([19, 20, 43, 48] and data not shown). The results shown in Figure 1B and Table 1 indicate that both CXCR1 and CXCR2 responded to CXCL8 stimulation by inducing phosphorylation of paxillin. Therefore, as was the case in neutrophils, paxillin phosphorylation in the transfected cells was associated with activation of migration. Furthermore, when we focused on CXCR2, we found that CXCL8-induced paxillin phosphorylation was a rapid, time-dependent and transient process (Fig. 1C).

The Y31 and Y118 sites of paxillin were found to be important for the regulation of migratory processes [21, 22, 24, 26–28]. Of these two sites, only the Y118 site was found to undergo reproducible phosphorylation following CXCL8 stimulation (Fig. 2A and Table 2). Further analyses indicated that both $G_{\alpha i}$ and $G_{\alpha s}$ proteins were involved in CXCL8-induced paxillin phosphorylation (Fig. 2B). CXCL8-induced paxillin phosphorylation was inhibited by 53.5±6.5 % ($p<0.05$) when the cells were pre-treated by pertussis

Table 1. Both CXCR1 and CXCR2 mediate responses of CXCL8-induced paxillin phosphorylation.[a]

Experiment no.	Fold induction of paxillin phosphorylation upon CXCL8 stimulation	
	CXCR1-RBL	CXCR2-RBL
Exp. 1	2.38	2.66
Exp. 2	3.02	4.95
Exp. 3	1.51	1.75
Exp. 4		6.05

[a] Summary of the findings of the experiments performed with CXCR1-RBL cells (three experiments) and with CXCR2-RBL cells (four experiments), whose results are averaged in Fig. 1B. The cells were stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation.

Table 2. CXCL8-induced phosphorylation of paxillin occurs on the Y118 site.[a]

Treatment	Value of py118 / paxillin			
	Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 4
–CXCL8	0.17	0.31	0.68	0.52
+CXCL8	0.73	1.01	1.83	2.1
	Fold induction of paxillin phosphorylation			
+CXCL8/–CXCL8	4.29	3.26	2.69	4.04

[a] Summary of the findings of four experiments indicating that CXCL8 induces phosphorylation of the Y118 site of paxillin, as demonstrated in the Western blot results of Fig. 2A. In these experiments, CXCR2-RBL cells were stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation and determination of phosphorylation by antibodies specific to Y118 of paxillin in its phosphorylated form.

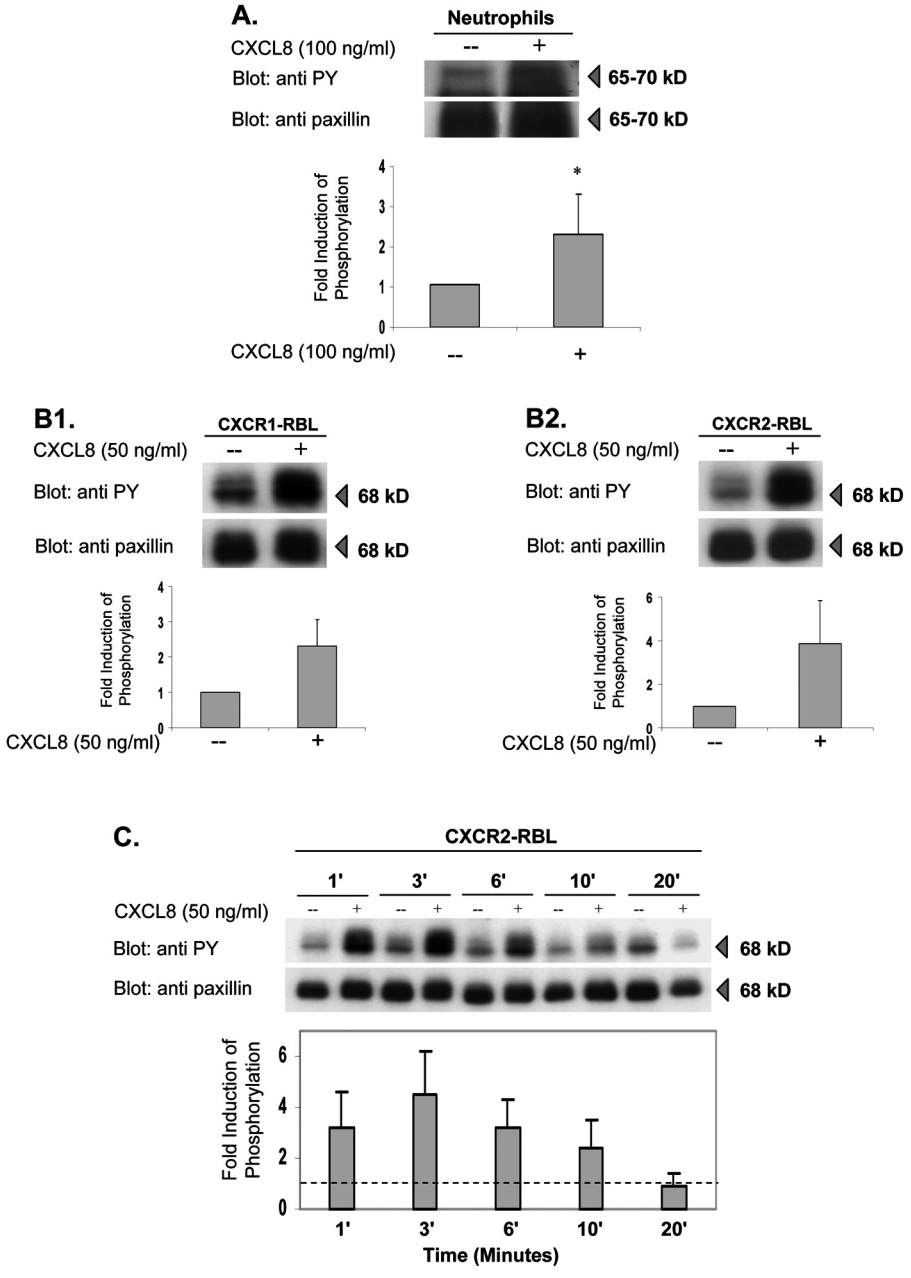


Figure 1. CXCL8 induces paxillin phosphorylation in neutrophils, CXCR1-RBL cells and CXCR2-RBL cells. Paxillin phosphorylation in human neutrophils (*A*), CXCR1-RBL cells (*B*) and CXCR2-RBL cells (*B, C*) was induced by CXCL8 in concentrations that were shown in dose-dependent analyses to potently induce the migration of these cell types [19, 20, 59–61]. (*A*) Neutrophils were cultured for 2 h on dishes coated by fibrinogen, with or without 100 ng/ml CXCL8, followed by paxillin immunoprecipitation. The Western blot is a representative of n=5, and the densitometry graph is the mean ± SD of n=5. The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. * $p<0.05$ for the difference in paxillin phosphorylation between CXCL8-stimulated neutrophils and non-stimulated neutrophils (as determined by densitometry values). (*B*) Adherent CXCR1-RBL cells and CXCR2-RBL cells were stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation. (*B1*) CXCR1-RBL cells. (*B2*) CXCR2-RBL cells. The Western blots are representatives and the densitometry graph is the mean ± SD of n=3 for CXCR1-RBL cells and n>3 for CXCR2-RBL cells, described in detail in Table 1. In the figure, the basal level of phosphorylation without CXCL8 stimulation was given the value of 1. (*C*) Kinetics of CXCL8-induced paxillin phosphorylation in adherent CXCR2-RBL cells. The cells were stimulated by 50 ng/ml CXCL8 for the indicated time points at 37°C, followed by paxillin immunoprecipitation. The Western blot is a representative of n>3, and the densitometry graph is the mean ± SD of n>3. The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. PY, Phosphorylated tyrosine.

toxin, a specific inhibitor of $G_{\alpha i}$ coupling to G protein-coupled receptors, and by 41.7±16.7 % (p=0.05) upon pre-treatment by cholera toxin that inhibits the coupling of $G_{\alpha s}$ to such receptors. Taken together, these results suggest that in response to CXCL8, the inhibition of $G_{\alpha i}$ and $G_{\alpha s}$ is selective to the Y118 site, because this site constitutes an important target for CXCL8-induced phosphorylation in paxillin.

To determine the roles played by paxillin in CXCL8-induced migration and adhesion, we transiently expressed WT paxillin, DN paxillin [26–28] or an empty control vector, all expressing GFP, in CXCR2-RBL cells. Following transfection, all three cell types expressed comparable high levels of the vectors and of CXCR2; the CXCR2 levels were similar to untransfected cells (data not shown).

The ability of CXCR2-RBL cells expressing WT paxillin or DN paxillin to migrate and adhere following CXCL8 stimulation was compared to cells expressing the empty vector only. The results shown in Figure 3A indicate that WT paxillin did not affect CXCL8-induced cell migration, suggesting that endogenous paxillin has reached saturable levels of activation that were not affected any further by overexpression of WT paxillin. In contrast, expression of DN paxillin led to a significant increase in CXCL8-induced cell migration (p<0.0001). These findings suggest that endogenous paxillin acts as a negative regulator of CXCL8-induced motility, keeping migration to CXCL8 at sub-maximal levels.

Following determination of paxillin roles in CXCL8-induced migration, we tested the effects of WT paxillin and DN paxillin on CXCL8-induced adhesion, as compared to cells expressing empty vector only. While WT paxillin did not affect the adhesion (Fig. 3B1), DN paxillin significantly increased CXCL8-induced cell adhesion (Fig. 3B2; p<0.001). Therefore, it is possible that the restrained migration of the cells in response to CXCL8 was due to sub-maximal levels of cell adhesion induced *via* paxillin activities.

Integrin ligation cooperates with CXCL8 stimulation, together inducing paxillin phosphorylation. The analyses performed so far were generated by stimulating adherent cells by CXCL8, suggesting that signaling through ligated integrins was required for CXCL8-induced paxillin phosphorylation. This possibility was also supported by the fact that in neutrophils, paxillin activation following CXCL8 stimulation was absolutely dependent on divalent cations, which are essential for integrin stimulation, as well as on fibrinogen (an integrin ligand).

To determine the roles of integrins in CXCL8-induced paxillin activation, we analyzed the ability of CXCL8

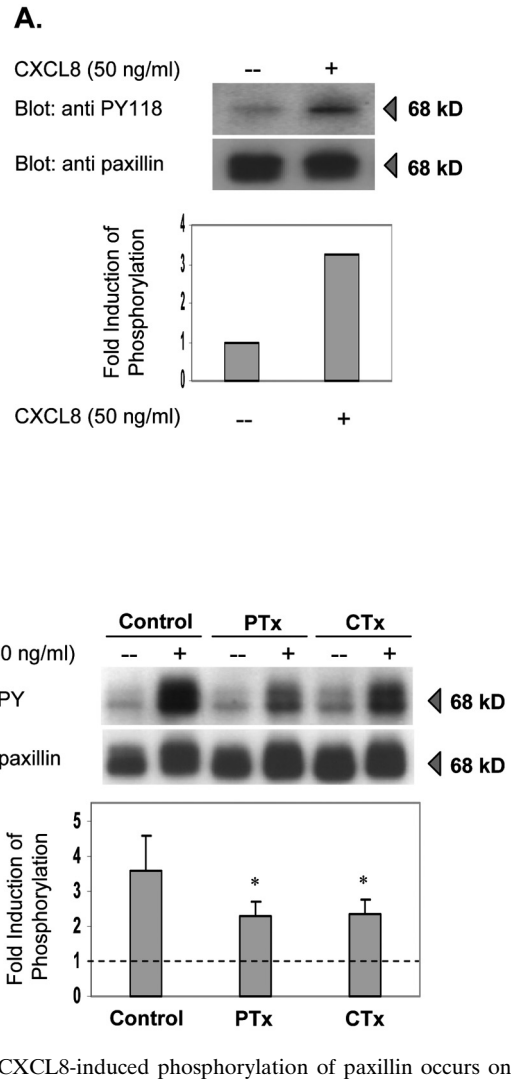


Figure 2. CXCL8-induced phosphorylation of paxillin occurs on the Y118 site, and requires coupling of $G_{\alpha i}$ and $G_{\alpha s}$ to CXCR2. (*A*) CXCL8-induced paxillin phosphorylation on Y118 in adherent CXCR2-RBL cells. The cells were stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation and determination of phosphorylation by antibodies specific to Y118 of paxillin in its phosphorylated form. The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. The results of the Western blot and the densitometry analysis are representatives of n=4, described in detail in Table 2. (*B*) CXCL8-induced paxillin phosphorylation depends on $G_{\alpha i}$ and $G_{\alpha s}$ coupling to CXCR2. Adherent CXCR2-RBL cells were pre-exposed to the $G_{\alpha i}$ inhibitor pertussis toxin (PTx, 100 ng/ml; The drug was resuspended in water) or the $G_{\alpha s}$ inhibitor cholera toxin (CTx, 100 ng/ml; the drug was resuspended in water) for 2 h at 37°C. The cells were then stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation. Control cells were incubated without the drug. PTx and CTx did not affect the expression levels of CXCR2 by the cells, nor did they affect cell viability (data not shown). * $p \leq 0.05$ for the difference between PTx/CTx-treated cells and untreated cells. The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. The Western blot is a representative of n=3, and the densitometry graph is the mean ± SD of n=3. PY, Phosphorylated tyrosine.

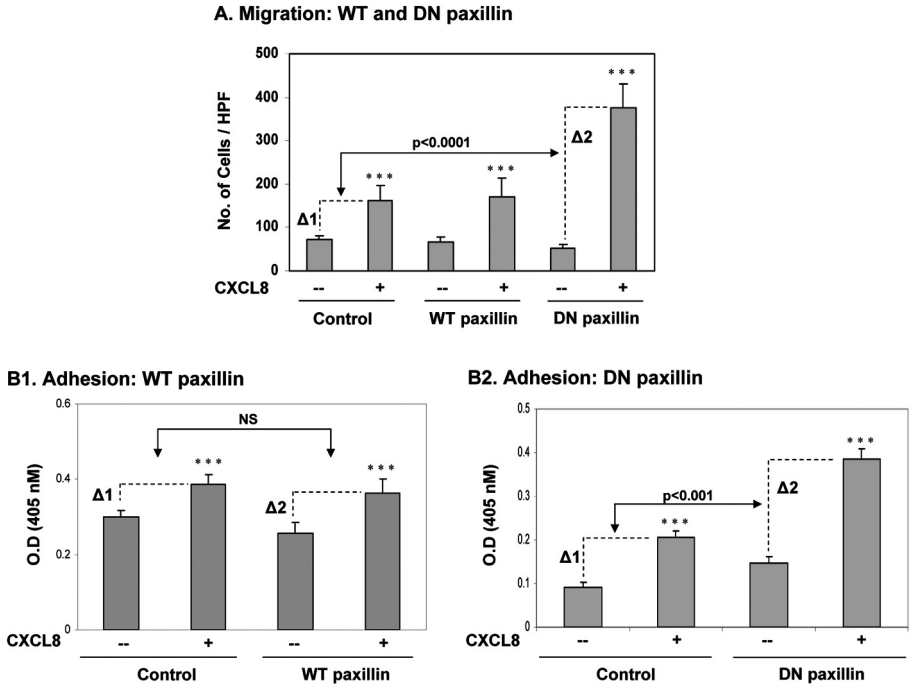
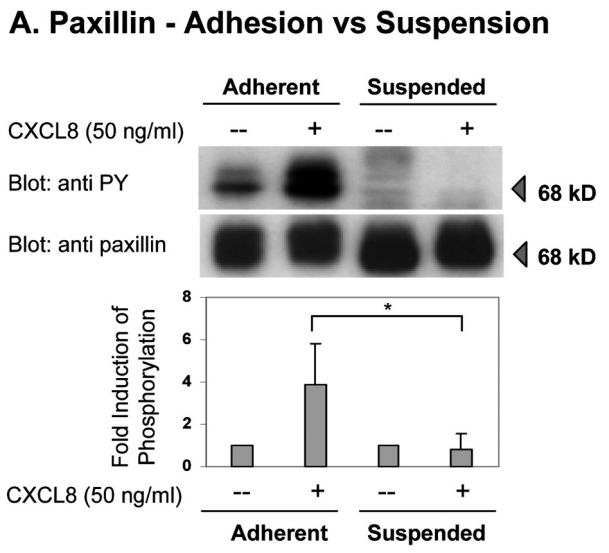


Figure 3. Endogenous paxillin negatively regulates CXCL8-induced migration and adhesion. The migration (*A*) and adhesion (*B*) of CXCR2-RBL cells in response to CXCL8 following transient transfection with wild-type (WT) paxillin or dominant negative (DN) paxillin was determined in comparison to cells transfected by an empty vector alone (control). For all three transfections, FACS analyses demonstrated that 50–60 % of the cells expressed the transfected vectors, and that the transfections did not affect CXCR2 expression by the cells or cell viability (data not shown). (*A*) CXCL8-induced migration of CXCR2-RBL cells grown in adherent conditions. Following removal of the cells from their growth plates, migration was determined in response to 50 ng/ml CXCL8 or to BSA medium in modified Boyden chambers. Each value represents three replicates. *** $p<0.0001$ for migration in response to CXCL8 *versus* migration in response to BSA medium. Δ1: The migration induced by CXCL8 of cells transfected by vector only; Δ2: The migration induced by CXCL8 of cells transfected by DN paxillin. $p<0.0001$ for the difference between Δ1 and Δ2. The results are representatives of $n=2$ for WT paxillin and $n=3$ for DN paxillin. HPF: High-power field. (*B*) CXCL8-induced adhesion of CXCR2-RBL cells grown in adhesion. Following removal of the cells from their growth plates, their adhesion to wells coated by fibronectin, with or without CXCL8, was determined. Following this procedure, cells that did not adhere to the plates were removed by washing several times, and the extent of cell adhesion was determined by alkaline phosphatase (ALP) analysis at 405 nm. (*B1*) Adhesion of cells transfected by WT paxillin as compared to control cells transfected by vector only. *** $p<0.0001$ for cell adhesion to fibronectin with CXCL8 *versus* adhesion to fibronectin alone. Δ1: The increase in adhesion induced by CXCL8, of cells transfected by vector only; Δ2: The increase in adhesion induced by CXCL8, of cells transfected by WT paxillin. NS: Not significant for the difference between Δ1 and Δ2. The results are representatives of $n=4$. (*B2*) Adhesion of cells transfected by DN paxillin as compared to control cells transfected by vector only. *** $p\leq0.0001$ for cell adhesion to fibronectin with CXCL8 *versus* adhesion to fibronectin alone. Δ1: The increase in adhesion induced by CXCL8, of cells transfected by vector only; Δ2: The increase in adhesion induced by CXCL8, of cells transfected by DN paxillin. *** $p<0.001$ for the difference between Δ1 and Δ2. The results are representatives of $n=4$.

to induce paxillin phosphorylation in cells that were grown in suspension and, therefore, deprived of adhesion-dependent signals mediated *via* integrins. Of note, the suspended cells were viable, expressed normal levels of CXCR2 and responded well to CXCL8 (*e.g.*, in CXCL8-induced internalization; data not shown).

The results shown in Figure 4A show that, in contrast to the adherent cells, no phosphorylation of paxillin was observed in suspended cells stimulated by CXCL8. This finding indicates that paxillin phosphorylation in response to CXCL8 stimulation is adhesion dependent, possibly requiring integrin pre-stimulation. To test this possibility further, the suspended cells were exposed to fibronectin, an ECM protein that preferentially stimulates the β1 integrin chain in many of the integrin heterodimers, enabling us to avoid restriction to a limited type of integrin heterodimers. The results shown in Figure 4B indicate that paxillin phosphorylation was completely recovered upon CXCL8 stimulation in suspended cells that were cultured on fibronectin, reaching levels of phosphorylation comparable to those observed in adherent cells.

The above findings raised questions regarding the type of integrin required for CXCL8-induced paxillin activation. Since cell adhesion to fibronectin is highly dependent on β1 integrins and paxillin binds to β1 integrins [22, 62], we asked if the activation of β1 integrins is required for CXCL8-induced paxillin activation. To determine this issue, we exposed the cells to β1 integrin activating antibodies, followed by



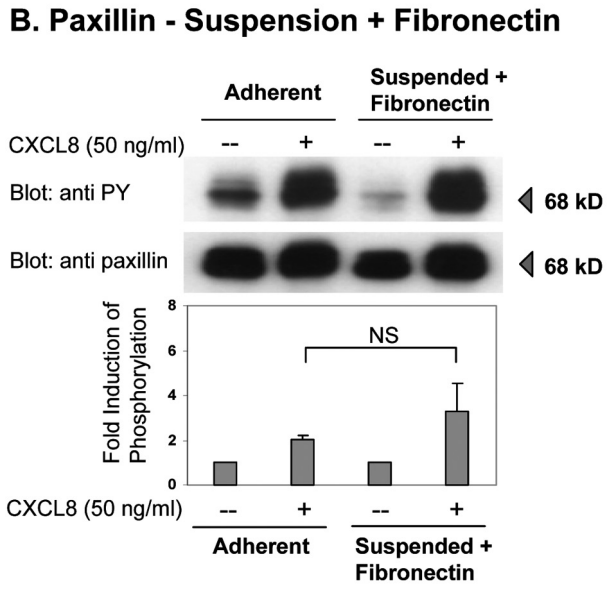


Figure 4. Pre-stimulation by adhesion-dependent integrin ligation is required for CXCL8-induced paxillin phosphorylation. (*A*) CXCL8-induced paxillin phosphorylation in CXCR2-RBL adherent cells *versus* cells grown in suspension. The cells were stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation. * $p=0.05$ for the difference between adherent cells and cells grown in suspension. The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. The Western blots are representatives of $n=4$, and the densitometry graphs are the mean ± SD of $n=4$. (*B*) CXCL8-induced paxillin phosphorylation in CXCR2-RBL adherent cells *versus* cells grown in suspension that were thereafter exposed to fibronectin-coated dishes for 45 min at 37°C. Then, the cells were stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation. NS: Not significant. The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. The Western blots are representatives of $n=4$, and the densitometry graphs are the mean ± SD of $n=4$. PY, Phosphorylated tyrosine.

determination of cell adhesion and of CXCL8-induced paxillin phosphorylation. Because of relative high spontaneous adhesion of RBL cells, this part of the research was performed in CXCR2-HEK cells, which have weaker spontaneous adhesion to the substratum, therefore preventing background adhesion that is not mediated by the antibodies to integrins. CXCR2-HEK cells were appropriate for this analysis also because they migrate well to CXCL8 (as indicated in our previous studies [42, 44, 54]). The experiment included three treatments:

1. Cells exposed to fibronectin – In this positive control, CXCR2-HEK cells were exposed for 2 h to fibronectin. The cells attached firmly and spread, forming tight contacts with the substratum at specific and definite regions of the cells (Fig. 5A1). Furthermore, as expected, CXCL8 induced paxillin phosphorylation in these cells (Fig. 5B and Table 3).
2. Cells in suspension – Following incubation in suspension, the cells did not attach to the substratum, but rather remained as floating aggregates in the medium (Fig. 5A2). In line with previous findings on CXCR2-RBL cells, no significant paxillin phosphorylation was observed in the suspended CXCR2-HEK cells upon CXCL8 stimulation (Fig. 5B and Table 3). These results substantiate the need for cell adhesion for CXCL8-induced paxillin phosphorylation, suggesting a key role for integrins in this process.
3. Cells stimulated by the TS2/16 activating antibodies to β1 integrins – These cells were exposed for 2 h to the β1-activating antibody TS2/16 [63, 64]. The cells attached to the substratum (Fig 5A3), and their stimulation by CXCL8 gave rise to paxillin phosphorylation in levels similar to those induced by exposure to fibronectin (Fig. 5B and Table 3).

Taken together, the above results indicate that β1 integrin ligation is a pre-requisite for CXCL8-induced paxillin phosphorylation, and suggest that β1 integrin activation cooperates with CXCL8-induced signals in stimulating paxillin phosphorylation.

CXCL8-induced paxillin phosphorylation does not depend on Pyk2 and Src activation but rather on FAK activation. To provide further insights into the intracellular mechanisms leading to CXCL8-induced paxillin activation, and to identify the tyrosine kinases that phosphorylate paxillin following CXCL8 stimulation, we focused on the three tyrosine kinases that have so far been shown to bind paxillin upon integrin

Table 3. CXCL8-induced paxillin phosphorylation requires pre-ligation of β1 integrins.[a]

Experiment no.	Fold induction of paxillin phosphorylation upon CXCL8 stimulation		
	Fibronectin	Suspension	β1-activating antibodies
Exp. 1	1.47	1.16	1.67
Exp. 2	2.58	1.22	2.1
Exp. 3	1.6	1.21	1.41

[a] Summary of the findings of three experiments indicating that the phosphorylation level of paxillin induced by CXCL8 under conditions of β1 integrin stimulation is similar to that of cells adhering on fibronectin, and is higher than of cells grown in suspension, as demonstrated in the Western blot results of Fig. 5B. In these experiments, CXCR2-HEK cells underwent the following three treatments: (1) fibronectin exposure: cells were plated on fibronectin-coated dishes for 2 h at 37°C; (2) in suspension: cells were placed in test tubes for 20 min at 37°C to reduce basal phosphorylation prior to CXCL8 stimulation; or (3) stimulation by the TS2/16 activating antibodies to β1 integrins: cells were plated on plates coated by F(ab')2 fragment of goat anti-mouse IgG, followed by the TS2/16 β1-activating antibody for 2 h at 37°C. The cells of the three treatments were then stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation.

stimulation, and to phosphorylate it on tyrosine residues: Pyk2, Src and FAK [21, 24].

Addressing Pyk2, we first asked whether CXCL8 stimulation acts on Pyk2, as indicated by its phosphorylation on tyrosine residues. The results shown in Figure 6A indicate that CXCL8 indeed induced potent Pyk2 activation. Furthermore, similar to CXCL8-induced paxillin phosphorylation, the CXCL8-induced phosphorylation of Pyk2 was dependent on adhesion and integrin pre-stimulation (Fig. 6B, C). The similar characteristics of paxillin and Pyk2 stimulation by CXCL8 suggested that these two mediators may cooperate. However, experiments of co-immunoprecipitation indicated that Pyk2 did not bind paxillin (data not shown) and suggested that Pyk2 was not involved in paxillin activation. This possibility was substantiated by the fact that the Pyk2 inhibitor tyrphostin A9 did not reduce paxillin phosphorylation upon CXCL8 stimulation (Fig. 7A). This result was evident despite the fact that tyrophostin A9 was functional, as indicated by reduced constitutive level of paxillin phosphorylation (when the cells were not stimulated by CXCL8) following pre-treatment with this drug (Fig. 7A).

In addition, we explored the roles played by members of the Src family of kinases in CXCL8-induced paxillin phosphorylation, primarily in view of their roles in CXCL8-induced migration [65, 66]. Src kinases were promising candidates because they were found to be involved in FAK phosphorylation following the stimulation of CXCR2-RBL cells by CXCL8 (Fig. 7B1). However, the findings of Figure 7B2 indicate that Src kinases probably did not phosphorylate paxillin directly in cells stimulated by CXCL8, because their inhibition by PP2 did not lead to reduction in paxillin phosphorylation.

These analyses were followed by determination of the roles of FAK in CXCL8-induced paxillin phosphorylation. Our past studies indicated that FAK is essential for CXCL8-induced migration, and that its activation following CXCL8 stimulation *via* CXCR2 was dependent on adhesion and integrin pre-stimulation [19, 20]. These findings motivated us to determine whether FAK regulates paxillin phosphorylation upon CXCL8 stimulation. Using co-immunoprecipitation assays, we found that CXCL8 stimulation led to a substantial increase in physical associations between FAK and paxillin (Fig. 8A). This association was time dependent, and was minimally observed in the absence of CXCL8 activation. Furthermore, inhibition of FAK activities by its dominant negative variant FRNK gave rise to a significant reduction in paxillin phosphorylation upon stimulation by CXCL8 (Fig. 8B). These findings indicate that upon CXCL8 stimulation, FAK binds paxillin and induces its activation.

Taken together, the results presented above indicate that Pyk2 and Src are not involved in CXCL8-induced paxillin phosphorylation, but rather that FAK is one of the kinases that induce paxillin phosphorylation upon exposure to this chemokine.

Discussion

To provide insights to the molecular mechanisms involved in directional migration induced by CXCL8, we focused on paxillin, as this was rarely been investigated in chemokine-induced migration, and only little evidence is available on its direct roles in controlling directional motility induced by such factors. To date, paxillin was found to act as a positive regulator of cell migration is many studies, but also as a negative regulator in others. The basis for the opposing roles played by paxillin in migration is not fully elucidated, and there have been several explanations. Among others, it was proposed that the roles of paxillin depend on the stimuli provided to the cells (*e.g.*, the type of ECM protein inducing cell adhesion), and that paxillin may have differential activities in

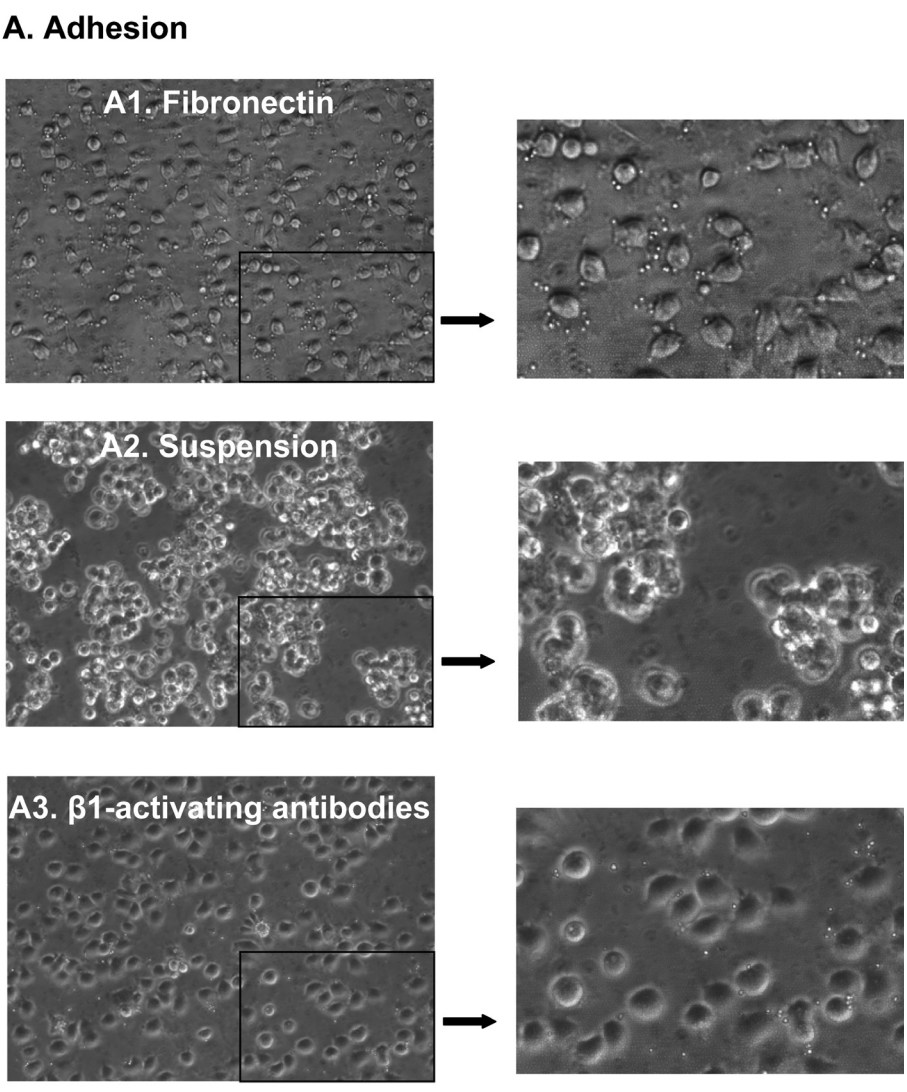


Figure 5. CXCL8-induced paxillin phosphorylation requires preligation of β1 integrins. (*A*) Adhesion prior to CXCL8 stimulation and (*B*) CXCL8-induced phosphorylation of CXCR2-HEK cells. The cells underwent the following three treatments: (1) fibronectin exposure, *i.e.*, cells were plated on fibronectin-coated dishes for 2 h at 37°C; (2) in suspension: in (*A*), cells were plated on BSA-coated plates for 2 h at 37°C; in (*B*), cells were placed in test tubes for 20 min at 37°C to reduce basal phosphorylation prior to CXCL8 stimulation; (3) cells stimulated by the TS2/16 activating antibodies to β1 integrins: cells were plated on plates coated by $F(ab')_2$ fragment of goat anti-mouse IgG, followed by the TS2/16 β1-activating antibody for 2 h at 37°C. (*A*) Light microscopy pictures of the cells in all three conditions, prior to CXCL8 stimulation. No adhesion was observed in cells that were plated on BSA-coated plates or on plates coated by $F(ab')_2$ fragment of goat anti-mouse IgG only. (*B*) For phosphorylation, the cells of the three treatments were stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation. The results are representatives of n=3, described in detail in Table 3. PY, Phosphorylated tyrosine.

random *versus* directional cell motility [21–23, 29–33].

The results of our study provide novel evidence of the roles played by endogenous paxillin in chemokine-induced directional cell motility, showing that it serves as a negative regulator of directed migration in response to CXCL8. This is a unique indication of such roles for paxillin in chemokine-induced motility, standing in line with the study by Yano et al. [32] showing that tyrosine phosphorylation of paxillin reduces haptotactic migration in response to collagen signals. Furthermore, we have shown that when endogenous paxillin is stimulated by CXCL8, adhesion does not reach maximally available levels, suggesting that this sub-maximal adhesion stands at the basis of restrained motility. In past studies, paxillin

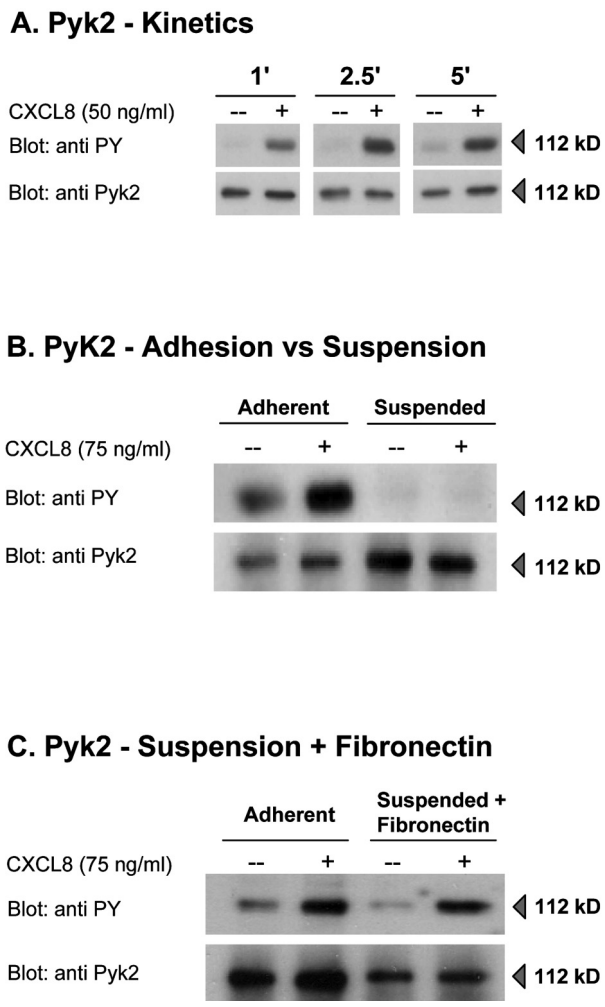


Figure 6. Pyk2 is activated by CXCL8 in an adhesion- and integrin-dependent manner. (*A*) CXCL8-induced Pyk2 phosphorylation in adherent CXCR2-RBL cells was determined in response to 50 ng/ml CXCL8 in the indicated time points at 37°C, followed by Pyk2 immunoprecipitation. The Western blot is a representative of $n=3$ (for 2.5 min time point: $n=2$). (*B*) CXCL8-induced Pyk2 phosphorylation in CXCR2-RBL adherent cells *versus* cells grown in suspension. The cells were stimulated by 75 ng/ml CXCL8 for 1.5 min at 37°C, followed by Pyk2 immunoprecipitation. The Western blots are representatives of $n=3$. (*C*) CXCL8-induced Pyk2 phosphorylation in CXCR2-RBL adherent cells *versus* cells grown in suspension that were thereafter exposed to fibronectin-coated dishes for 45 min at 37°C. Then, the cells were stimulated by 75 ng/ml CXCL8 for 1.5 min at 37°C, followed by paxillin immunoprecipitation. The Western blots are representatives of $n=3$. PY, Phosphorylated tyrosine.

was suggested to be involved in adhesion control, exerting different roles at the leading edge *versus* the lagging edge of the migrating cell [23, 31]. Therefore, it is possible that following CXCL8 stimulation, endogenous paxillin affects focal areas at the leading and lagging edges of the cells in a manner that does not allow for maximal migration to take place.

Since chemokines are prototypical activators of integrins [1, 23, 34, 35], it is reasonable to assume that CXCL8 stimulation induces paxillin phosphorylation by the ability of the chemokine to activate specific integrins [23, 25, 31]. However, it is clear that the signals delivered by CXCL8 stimulation cannot act alone to activate paxillin, because β1 integrin ligation prior to CXCL8 exposure was required. This observation stands in line with previous studies suggesting that paxillin binds to β1 integrins [22, 62], and it indicates that the cells need pre-conditioning by β1 integrin activation to respond to CXCL8. Therefore, the magnitude of paxillin activation and its involvement in CXCL8-induced directional migration are tightly regulated by signals provided jointly by integrins and by the chemokine.

Published studies, including ours on CXCL8 activation, indicate that integrins bind FAK and facilitate its activation [20, 62, 67–69]. It was also shown that FAK controls focal contact turnover by phosphorylating and activating paxillin [21, 22, 24, 68, 69]. Therefore, we suggest that adhesion-induced ligation of β1 integrins leads to signals that cooperate with cues derived by CXCL8 stimulation, together giving rise to FAK clustering and activation. FAK, in turn, stimulates paxillin by inducing its phosphorylation, and probably acts in this respect together with other kinases that are yet to be identified.

Based on our results on Src-mediated FAK activation, we suggest that CXCL8-induced signals lead to formation of FAK-Src complexes, as previously described by other investigators in studies of integrin stimulation [22, 68–70]. This possibility is reinforced by findings showing that Src activation is required for CXCL8-induced migration in neutrophils, as well as in other cell types [65, 66].

Although Src is partly required for FAK activation upon CXCL8 stimulation, it does not play a role in paxillin activation in response to the CXCL8 signals. These findings suggest that the type of FAK that induces paxillin phosphorylation is activated in a Src-independent manner. Therefore, it is possible that other kinases that are independent of Src (or of Pyk2, for that matter) play direct or indirect roles in paxillin activation, which is in line with the fact that FAK inhibition by FRNK does not provide complete reduction in CXCL8-induced paxillin phosphorylation.

Adding to the complexity of FAK-paxillin interactions in CXCL8-induced migration, we observed that, while FAK activation is essential for CXCL8-induced migration, paxillin activities down-regulate this process. These observations are in line with the fact that both FAK and paxillin were found to be positive or negative regulators of migration, depending on many different conditions and mediators [21–23, 29–33, 71, 72]. It is possible that upon CXCL8 stimulation each

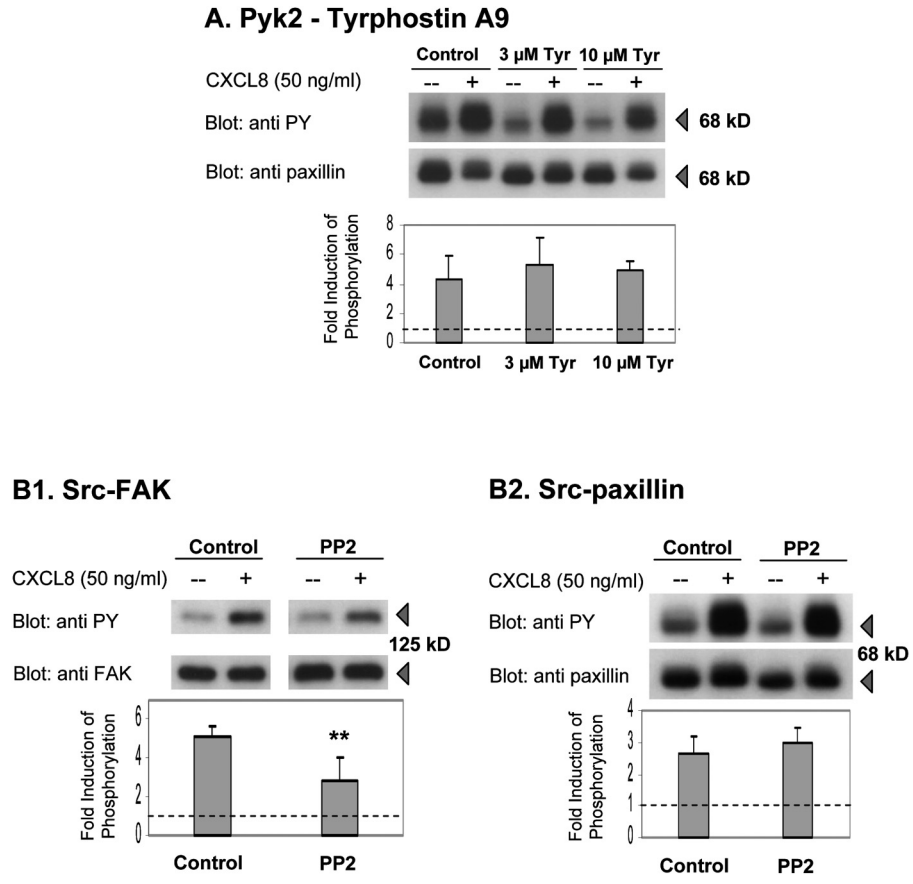


Figure 7. Pyk2 and Src are not involved in CXCL8-induced paxillin phosphorylation. (*A*) The involvement of Pyk2 in CXCL8-induced paxillin phosphorylation. Adherent CXCR2-RBL cells were pre-exposed to the Pyk2 inhibitor tyrphostin A9 (3 and 10 μM) or to its solubilizer (DMSO, control) for 1 h at 37°C. The cells were then stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by paxillin immunoprecipitation. Tyrphostin A9 did not affect the expression levels of CXCR2 by the cells, or cell viability (data not shown). The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. The Western blot is a representative of $n=3$, and the densitometry graph is the mean ± SD of $n=3$. (*B, C*) The involvement of Src in CXCL8-induced FAK phosphorylation (*B1*) and paxillin phosphorylation (*B2*). Adherent CXCR2-RBL cells were pre-exposed to PP2 (10 μM) or to its solubilizer (DMSO, control) for 1.5 h at 37°C. The cells were then stimulated by 50 ng/ml CXCL8 for 3 min at 37°C, followed by FAK or paxillin immunoprecipitation. PP2 did not affect the expression levels of CXCR2 by the cells, or cell viability (data not shown). The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. The Western blots are representatives of $n>3$, and the densitometry graphs are the mean ± SD of $n>3$. ** $p<0.01$ for the difference between PP2-treated cells and cell not treated by the drug. PY, Phosphorylated tyrosine.

of the two proteins is active at a certain manner, and that the combination between their activities dictates the migratory phenotype, leading to sub-maximal levels of motility in response to the chemokine. Therefore, the levels of FAK and paxillin activation, and the way they interact, impose a specific magnitude of migration on the cells and limit it to a sub-maximal extent.

To conclude, we suggest that negative regulation of CXCL8-induced migration that is induced by endogenous paxillin, accompanied by fine tuning of migration by FAK, control the balance of CXCL8-induced migration in inflammatory processes. If paxillin and FAK are inappropriately activated, the balance may be perturbed and this may lead to up-regulation or down-regulation of cell motility. Such unbalanced regulatory processes in response to CXCL8 may lead to excessive or defective migration in inflammation, giving rise to a large variety of pathological conditions that involve unbalanced migratory processes.

Acknowledgments. The authors thank I. Yron (Tel Aviv University, Tel Aviv, Israel) for her constructive assistance and R. Alon (Weizmann Institute, Rehovot, Israel) for providing the protocol for adhesion assays. Grant support: This study was supported by Recannati Foundation and by Federico Foundation.

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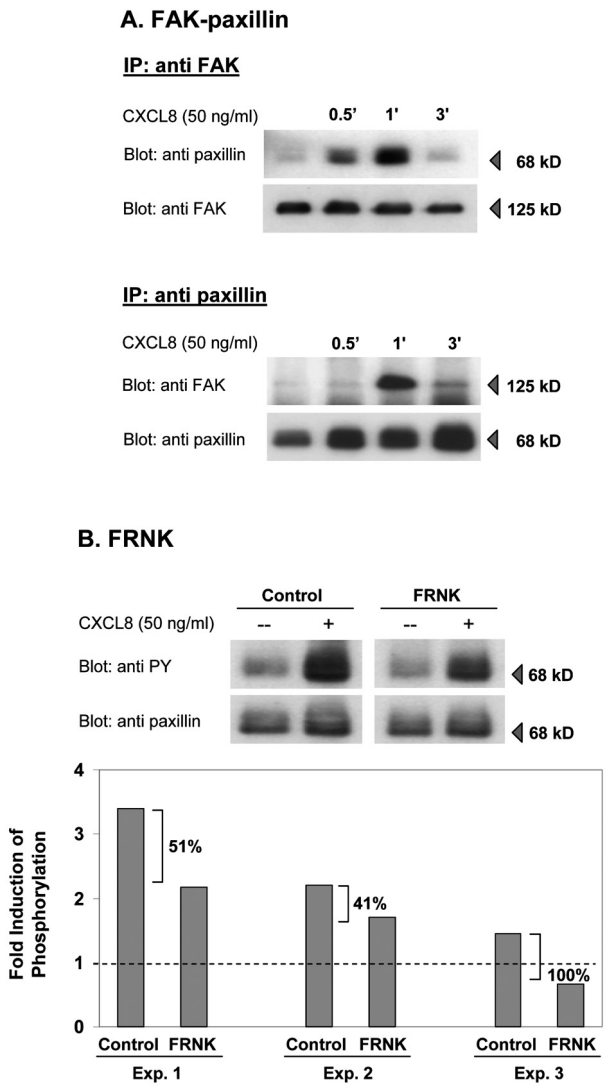

Figure 8. FAK is directly involved in CXCL8-induced paxillin phosphorylation. (*A*) Co-immunoprecipitations of paxillin and FAK. Adherent CXCR2-RBL cells were either non-stimulated or stimulated by 50 ng/ml CXCL8 for the indicated time points at 37°C. Cell lysates were prepared, followed by FAK or paxillin immunoprecipitation. Western blot analyses were performed with antibodies against FAK or paxillin on the same membranes. The Western blots are representatives of $n=3$. (*B*) FRNK, the dominant negative variant of FAK [70, 73], down-regulates CXCL8-induced paxillin phosphorylation. Adherent CXCR2-HEK cells were transiently transfected with FRNK-expressing vector as compared to transfection with vector alone. In both transfections, FACS analyses demonstrated that 40–60% of the cells expressed the transfected vectors, and that the transfections did not affect CXCR2 expression by the cells or cell viability (data not shown). Paxillin phosphorylation was determined in response to 50 ng/ml CXCL8 for 1.5 min (to agree with optimal conditions of associations between FAK and paxillin) at 37°C, followed by paxillin immunoprecipitation. The basal level of phosphorylation without CXCL8 stimulation was given the value of 1. The Western blot is a representative of $n=3$. The bar figure shows the results of the three experiments, including the percentages of paxillin inhibition of paxillin phosphorylation, obtained in each of the experiments. PY, Phosphorylated tyrosine.

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