Tumor and Stem Cell Biology

CIP4 Controls CCL19-Driven Cell Steering and Chemotaxis in Chronic Lymphocytic Leukemia

Gema Malet-Engrà1,4,5,7, Julien Vlau2, Loïc Ysebaert2,6, Manon Farcé1,4,5, Fanny Lafouresse1,4,5, Guy Laurent3,6, Frédérique Gaits-Iacovoni2, Giorgio Scita7,8, and Loïc Dupré1,4,5

Abstract

Solid tumor dissemination relies on the reprogramming of molecular pathways controlling chemotaxis. Whether the motility of nonsolid tumors such as leukemia depends on the deregulated expression of molecules decoding chemotactic signals remains an open question. We identify here the membrane remodeling F-BAR adapter protein Cdc42-interacting protein 4 (CIP4) as a key regulator of chemotaxis in chronic lymphocytic leukemia (CLL). CIP4 is expressed at abnormally high levels in CLL cells, where it is required for CCL19-induced chemotaxis. Upon CCL19 stimulation of CLL cells, CIP4 associates with GTP-bound Cdc42 and is recruited to the rear of the lamellipodium and along microspikes radiating through the lamellipodium. Consistent with its cellular distribution, CIP4 removal impairs both the assembly of the polarized lamellipodium and directional migration along a diffusible CCL19 gradient. Furthermore, CIP4 depletion results in decreased activation of WASP, but increased activation of PAK1 and p38 mitogen-activated protein kinase (MAPK). Notably, p38 MAPK inhibition results in impaired lamellipodium assembly and loss of directional migration. This suggests that CIP4 modulates both the WASP and p38 MAPK pathways to promote lamellipodium assembly and chemotaxis. Overall, our study reveals a critical role of CIP4 in mediating chemotaxis of CLL cells by controlling the dynamics of microspike-containing protrusions and cell steering. Cancer Res; 73(11); 3412–24. ©2013 AACR.

Introduction

There is growing evidence that the dissemination of tumor cells is governed by a reprogramming of the molecular machinery controlling motility (1). Solid tumors with high dissemination potential have been shown to upregulate actin cytoskeleton regulators that decode chemotactic cues for the assembly of protrusions supporting migration and invasion (2–4). In particular, high expression of members of the WASP family facilitates the dissemination of several cancers by promoting the assembly of filopodia, lamellipodia, and invadopodia (5, 6). The formation of membrane protrusions involved in migration requires a high degree of coordination between actin cytoskeleton remodeling and induction of membrane curvature. One mechanism to achieve this coordination is mediated by BAR domain-containing proteins, which sense membrane curvature, mold the plasma membrane, and recruit actin nucleators such as WASP- and formin-family proteins (7, 8). The capacity of tumor cells to harness BAR domain-containing proteins is supported by the finding that invasive breast cancer cells overexpress the F-BAR domain-containing Cdc42-interacting protein 4 (CIP4), which regulates invadopodia formation and invasion through the extracellular matrix (9, 10).

The dissemination of hematologic malignancies is thought to be derived from the intrinsic ability of hematopoietic cells and in particular lymphocytes to patrol the body and migrate between distant tissue compartments (11). However, malignant B cells assemble aberrant actin-rich protrusions endowing these cells with high migratory and invasive potential (12). Chronic lymphocytic leukemia (CLL), which is a common lymphoproliferative disorder of mature B lymphocytes (13), is characterized by tumor cells highly responsive to chemotactic cues that circulate at high rate through the blood and accumulate in the bone marrow and lymph nodes (14–18). These aspects of the CLL phenotype may be linked to their propensity to assemble actin-rich structures in response to chemotactic signals, thereby altering normal leukocyte homing and homeostasis (12, 19). Accordingly, the actin regulator HS1 is hyper-phosphorylated in patients with CLL, regulates in vivo migration, and is associated with poor clinical outcome (20, 21). The trafficking and homing of hematopoietic cells to the lymphatic tissues and in particular their migration across the vascular endothelium are controlled in part by CCR7, the receptor for the CCL19 and CCL21 chemokines (22). CCR7 level in CLL cells correlates with lymphadenopathy (15, 23) and is
higher in ZAP-70+/CD38+ CLL cells, whose presence is related to a poor prognosis (24). Therefore, CLL disease progression may correlate with the development of cells that acquire increased potential to invade lymph nodes via the CCL19/21-CCL7 axis.

Our objective was to investigate whether regulators of actin cytoskeleton and membrane curvature are abnormally expressed in CLL to promote assembly of promigratory structures in response to CCL19. We identified the F-BAR domain-containing protein CIP4 as specifically overexpressed in CLL when compared with normal B cells or other subtypes of B-cell malignancies. By combining siRNA/shRNA approaches with real-time tracking of cells migrating in CCL19 gradients, we show that CLL cells depend on CIP4 for the assembly of a highly structured lamellipodium and for directional migration in response to CCL19.

Materials and Methods

Patients and cell lines

Peripheral blood samples were collected from patients with CLL after obtaining informed consent and approval from the Toulouse University Medical Center Ethic Committee. After separation by Ficoll centrifugation, cells were used freshly or stored as frozen samples (HIMIP collection). According to the French law, HIMIP collection has been declared to the Ministry of Higher Education and Research (DC 2008-307 collection 1) and obtained a transfer agreement (AC 2008-129) after approval by the local ethical committee (Comité de Protection des Personnes Sud-Ouest et Outremer II). Clinical and biologic annotations of the samples have been declared to the national committee on data processing (Comité National Informatique et Libertés). For some experiments, B-leukemic cells were purified by magnetic separation according to manufacturer’s instructions (EasySep Human B Cell Enrichment Kit w/o CD43 depletion; Stem cell Technologies). Control B cells from healthy donors were isolated from buffy coats (Etablissement du Sang) using a negative selection kit (EasySep Human B Cell Enrichment Kit; Stem Cell Technologies). B-cell purity was assessed by flow cytometry. The CLL-derived cell lines JVM3 and MEC2 were purchased from DSMZ (n° ACC18 and ACC500, respectively) authenticated by Mycoplasma infection.

Quantitative reverse transcription PCR

Total RNA was isolated from purified B cells using the TRIzol reagent (Invitrogen). Two microgram of RNA was used, with 100 ng random hexamers, in a reverse transcription reaction (SUPERSCRIPT II; Invitrogen). One-tenth nanogram of cDNA was amplified, in triplicate, in a reaction volume of 25 μL with 10 pmol/L of each gene-specific primer and the SYBR-green PCR MasterMix (Applied Biosystems). Real-time PCR was conducted on the ABI/Prism 7700 Sequence Detector System (PerkinElmer/Applied Biosystems), using a pre-PCR step of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Specificity of the amplified products was confirmed by melting curve analysis (Dissociation Curve; PerkinElmer/Applied Biosystems) and by 6% PAGE. Prepara-

tions with RNA template without reverse transcriptase were used as negative controls. Samples were amplified with primers for each gene [for details, see quantitative PCR (qPCR) primer list in Supplementary Table S1] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene (other housekeeping genes, including rRNA 18S and β-actin were also tested with comparable results). The C_{T} values were normalized to the GAPDH curve and the relative expression of each gene was expressed as the ratio relative to control B cells isolated from the blood of 3 healthy donors.

Western blot analysis and GST pull-down

Cells were lysed with Cytobuster Protein Extraction Reagent (Novagen) containing NaF, Na_{3}VO_{4} (1 mmol/L each), and protease inhibitor cocktail (Sigma). Proteins from total cell lysates were resolved by gel SDS-PAGE and analyzed by Western blotting. The following primary antibodies were used to reveal the membranes: anti-CIP4 (clone 21; BD Transduction Laboratories), anti-GAPDH (clone 374; Chemicon), anti-Cdc42 (sc-87; Santa Cruz Biotechnology), anti-total P38 (9212; Cell Signaling), anti-pP38 (clone 3D7; Cell Signaling), anti-pErk1/2 (E10; Cell Signaling), anti-pAKT1/2/3 (2604; Cell Signaling), anti-pPAK1 (Ser199/204)/PAK2 (Ser192/197); 2605; Cell Signaling), anti-WASP (H-250; Santa Cruz Biotechnology), anti-pWASP (phospho Y-290; Abcam), anti-FBP17 (gift from Dr. P. De Camilli, Yale University School of Medicine, New Haven, CT; ref. 25) and anti-Toca-1 (developed in Dr. G. Scita’s laboratory). Horseradish peroxidase-conjugated secondary antibodies were from Promega. Where indicated, JVM3 cells were pre-treated for 10 minutes with either dimethyl sulfoxide (DMSO) or 50 μmol/L IPA-3 (Sigma). Cells were then stimulated for 5 minutes with 100 ng/mL CCL19 before lysis. Recombinant glutathione S-transferase (GST) and GST-Cdc42 were purified from Escherichia coli and the immobilized GST-Cdc42 protein was preloaded with GDP or GTPγS as described previously (26). Cell lysates were prepared for GST pull-down experiments as described earlier and incubated with 20 μg of each fusion protein bound to glutathione-sepharose beads for 2 hours at 4°C. The beads were washed 4 times with PBST (1% Tween 20) and boiled at 95°C for 5 minutes. Samples were subjected to SDS-PAGE and analyzed by Western blotting with CIP4 monoclonal antibody (mAb). Active Cdc42 pull-down was conducted as previously described (27).

siRNA and shRNA-mediated CIP4 knockdown

Transient CIP4 knockdown was obtained by nucleofection of JVM3 cells and patient CLL cells with 500 nmol/L CIP4-specific siRNA (GGCGAAACGUAGCUGUAA) or scrambled control siRNA (GGGAGAGUGAGUGAGUGUA) purchased from Dharmacon. Cells were nucleofected with the Amaxa system (Amaxa Biosystems/Lonza) using Nucleofector Solution V and Nucleofector program X-001 for JVM3 cells and U-013 for patient CLL cells. Stable CIP4 knockdown was obtained by transduction of JVM3 cells with MISSION lentiviral transduction particles (Sigma) encoding 2 short hairpin RNAs (shRNA) targeting CIP4 (TRCN063186 and TRCN299602). As negative controls, MISSION nontarget shRNA control transduction particles (SHC002V and SHC202V) were used. Stable
knockdown cell lines were generated by selecting transduced cells with 1 μg/mL puromycin. Silencing efficiency was controlled by Western blotting.

Transwell chemotaxis assay

Cells (JVM3 or purified leukemic cells from patients with CLL) were resuspended in RPMI, 0.5% bovine serum albumin (BSA). A total of 300 μL containing 0.5 × 10⁵ patient cells or 0.1 × 10⁵ JVM3 cells was loaded on the top chamber of a Transwell culture PET (polyethylene terephthalate) membrane insert (BD Falcon) with a pore size of 3 μm. Filters were transferred into wells containing 1,000 μL RPMI, 0.5% BSA with varying concentrations of CCL19 (Peprotech). Transwell plates were incubated at 37°C in 5% CO₂ for 6 hours for patient cells and overnight for JVM3 cells. Inserts were then removed and cells that transmigrated in the lower wells were resuspended in 250 μL fluorescence-activated cell sorting (FACS) buffer plus 50 μL calibration beads (BD Biosciences). The same number of beads was acquired for each sample and cells were counted using a FACS-Calibur flow cytometer (BD Biosciences). The migration index was calculated as the number of cells transmigrating toward CCL19 divided by the number of cells transmigrating spontaneously in the absence of CCL19.

Real-time chemotaxis

Chemotaxis of JVM3 cells was analyzed in real-time using 2-dimensional (2D) chemotaxis μ-slides coated with Collagen IV (Ibidi). Cells (0.3 × 10⁶ cells in 10 μL) were loaded into the central transversal chamber and incubated at 37°C for 50 minutes to allow cell attachment. Fresh RPMI was loaded into adjacent reservoirs and a CCL19 gradient was created following the manufacturer’s instructions. Cell migration was recorded at 37°C, 5% CO₂ by taking a picture every 2 minutes using an inverted wide-field microscope (Zeiss Axiovert) with a 2.5× objective and MetaMorph32 software. Images were analyzed with the ImageJ 1.42q software (W.S. Rasband, National Institute of Health, Bethesda, MD), using the Manual Tracking plugin (F. Cordelières, Institut Curie, Orsay, France). Chemotaxis plots and migration parameters were obtained with the Chemotaxis and Migration tool from Ibidi.

Confocal laser scanning microscopy

To visualize and measure lamellipodium formation, JVM3 cells or primary CLL cells were seeded for 5 minutes on glass slides precoated with 4 μg/mL ICAM-1, in the presence of 100 ng/mL soluble CCL19. Cells were fixed with 3% paraformaldehyde, permeabilized in 3% BSA/0.1% saponin, and stained in 3% BSA/0.1% saponin with either anti-α-tubulin rabbit pAb (Abcam) followed by goat anti-rabbit mAb labeled with Alexa Fluor 488 or phalloidin labeled with Alexa Fluor 633. They were additionally stained with 4,6-diamidino-2-phenylindole (DAPI) before mounting. Pictures of individual JVM3 cells collected with a ×63 objective mounted on a Confocal Zeiss 710 microscope were analyzed on ImageJ by delineating manually the borders of the lamellipodium to calculate its surface. To investigate the effect of p38 inhibition on CCL19-induced lamellipodium assembly, lamellipodium surface was measured as above in JVM3 cells that were treated for 1 hour with 10 μmol/L SB203580 (Calbiochem) or DMSO, before CCL19 exposure. To study the subcellular localization of CIP4 and Cdc42, JVM3 cells activated for 5 minutes with immobilized ICAM-1 and soluble CCL19 were fixed, permeabilized (as above), and stained with anti-Cdc42 rabbit pAb (Santa Cruz Biotecnology) and anti-CIP4 mAb (clone 21; BD Transduction Laboratories), followed by goat anti-rabbit mAb labeled with Alexa Fluor 488 and goat anti-mouse mAb labeled with Alexa Fluor 546. The relative localization of Cdc42 and CIP4 was analyzed using the Linescan function of ImageJ. To study the subcellular localization of CIP4 in migrating cells, 3 × 10⁵ JVM3 cells were transiently nucleofected with 1 to 3 μg of a plasmid encoding GFP–CIP4. After 24 hours, dead cells were removed using a Dead Cell Removal Kit (Miltenyi). For time-lapse video recording, cells were placed in Ibidi chemotaxis chambers and a Confocal Zeiss 710 microscope was used to track cells every 8 seconds using a ×63 objective.

Results

CIP4 is expressed at high levels in CLL cell lines and is required for chemotaxis toward CCL19

Given the involvement of WASP-family proteins in the regulation of tumor cell migration, expression of a selected set of WASP-family proteins (WASP, N-WASP, and WAVE1-3) and direct interactors (WIP, FBP17, and Toca-1, CIP4) was analyzed by quantitative reverse transcription PCR (qRT-PCR) in JVM3 and MEC2 CLL cell lines. Levels of mRNA in the CLL cell lines were normalized to those measured in normal peripheral blood B cells. This analysis revealed that the mRNAs encoding the F-BAR domain-containing CIP4 were expressed at elevated levels in both CLL cell lines (Fig. 1A). The other mRNA analyzed in the CLL cell lines was either mildly overexpressed (WASP, WIP, WAVE1-2, and FBP17), expressed at levels comparable with those of control B cells (N-WASP, Toca-1), or undetected (WAVE3). Accordingly, the CIP4 protein was also overexpressed in JVM3 and MEC2 when compared with normal peripheral blood B cells (Fig. 1B). CIP4 overexpression seemed to be restricted to the CLL cell lines as CIP4 expression in cell lines corresponding to different B-cell malignancies was comparable or even lower than that in normal B cells (Fig. 1B). Further analysis of the levels of the CIP4-related proteins Toca-1 and FBP17 showed that CIP4 overexpression is a specific feature of this protein as there no concomitant increase nor compensation by the other members of the CIP4 family, Toca-1 and FBP17 (Fig. 1B). Because of its high expression in CLL cell lines and its membrane–actin interface regulatory role, we formulated the hypothesis that CIP4 might regulate CLL cell migration. The contribution of CIP4 to CLL cell migration was tested by knocking down CIP4 expression in JVM3 cells and measuring the ability of those cells to migrate toward CCL19. Nucleofection with CIP4 siRNA efficiently and reproducibly depleted JVM3 cells in CIP4 (Fig. 1C). Transwell assays showed that the migration of cells knocked down for CIP4 toward a CCL19 gradient was significantly reduced (Fig. 1D). To test whether this decrease in chemotactic response could be the result of either defective motility or defective orientation within the chemokine gradient, single cell tracks were recorded in Ibidi chemotaxis μ-slides. Most CIP4-expressing JVM3 cells
(scrambled siRNA) followed migratory paths directed toward the source of CCL19 (Fig. 1E). A similar pattern of migration was obtained for mock-nucleofected JVM3 cells (data not shown). Conversely, JVM3 cells knocked down for CIP4 moved along tracks that followed random directions, similarly to cells not exposed to any CCL19 gradient (Fig. 1E). Measurement of the forward migration index along the y-axis (coincident with the direction of the CCL19 gradient) confirmed that CIP4-expressing JVM3 cells migrated directionally toward CCL19 (Fig. 1F). In contrast, CIP4 knocked down cells displayed no forward migration, supporting the finding that cells lacking CIP4 displayed a random migration pattern (Fig. 1F). CIP4 knocked down cells remained motile both in basal conditions and in response to CCL19, with migration speed comparable to control JVM3 cells.
with that of scrambled siRNA-treated cells (Fig. 1F). Therefore, those results show that CIP4 is dispensable for JVM3 cell motility, but is required for CCL19-evoked cell steering and chemotaxis.

**CIP4 is required for CCL19-driven lamellipodium assembly in JVM3 cells**

Because the best-characterized function of CIP4 is to regulate receptor endocytosis, the abrogation of chemotaxis consecutive to CIP4 knockdown might be linked to an abnormal control of CCL19-induced CCR7 internalization. However, JVM3 cells depleted for CIP4 by shRNA-encoding lentiviral vectors expressed normal basal levels of surface CCR7, which was equally internalized in CIP4 knocked down and control cells (Supplementary Fig. S1). This result indicates that CIP4 control of CCL19-driven chemotaxis does not result from altered CCR7 endocytosis. Through its F-BAR domain, CIP4 might also associate to membrane protrusions in migrating cells. We tested this possibility by comparing the morphologies of JVM3 cells depleted or not for CIP4. Independently of CIP4 expression, JVM3 cells plated on ICAM-1 displayed various morphologies and emitted numerous types of membrane protrusions. A hallmark of CCL19 stimulation in CIP4-expressing JVM3 cells was the assembly of a lamellipodium, which consisted of a wide flat protrusion made of a veil embedded with microspikes. This structure was observed both in fixed cells following short exposure to ICAM-1 plus CCL19 (Fig. 2A) and in live cells exposed to a CCL19 gradient (Supplementary Fig. S2A). Real-time imaging revealed that this lamellipodium is a dynamic structure leading the cells toward the source of CCL19 (Supplementary Movie S1). At the opposite side of the lamellipodium, JVM3 cells emitted a thinner protrusion resembling a uropod. Furthermore, the microtubule organizing center (MTOC) was usually localized along the lamellipodium–uropod axis, mostly between the lamellipodium and the nucleus. In contrast, cells depleted for CIP4 (Fig. 2B) failed

**Figure 2.** CIP4 is required for CCL19-driven lamellipodium assembly in JVM3 cells. A, representative confocal images of JVM3 cells either untransduced or transduced with a control vector or 2 CIP4 shRNA-encoding vectors that were plated on ICAM-1 in the absence or presence of 100 ng/mL soluble CCL19. Cells were stained with an antitubulin antibody and DAPI. To visualize the microtubule network and the MTOC, maximum intensity projections of z-stacks were built with the Zen software (Carl Zeiss). B, CIP4 expression level analyzed by Western blotting in untransduced JVM3 cells or in JVM3 cells transduced with lentiviral particles encoding the indicated shRNA sequences (1 control sequence and 2 sequences targeting CIP4 mRNA). C, lamellipodium surface of the indicated JVM3 cells measured following incubation on ICAM-1 in the absence or presence of CCL19. The mean ± SEM of the lamellipodium surface within each group of cells is represented (n = 19–62 cells/group). An unpaired Student t test was applied to compare the groups, ****, P < 0.001; ns, nonsignificant.
to assemble a polarized lamellipodium upon CCL19 exposure and lost the MTOC axial positioning. These cells formed multiple filopodia and flat protrusions devoid of microspikes, which extended in different directions (Fig. 2A). The morphologic alteration induced by CIP4 depletion was further documented by a loss in lamellipodium surface in CCL19-stimulated JVM3 cells (Fig. 2C). The role of CIP4 in controlling JVM3 cell lamellipodium assembly was confirmed in live cells exposed to a CCL19 gradient. Most cells treated with a CIP4 siRNA failed to assemble the microspike-containing lamellipodia predominantly observed in control cells (Supplementary Fig. S2A). This failure was associated with frequent changes in directions of migration and reduced directional persistence (Supplementary Fig. S2B). As observed by time-lapse video recording, instead of assembling a lamellipodium, CIP4 knocked down JVM3 cells emitted transiently and sequentially multiple types of protrusions (Supplementary Movie S2). In agreement with the observation in fixed cells, these cells emitted flat protrusions and short filopodia, as well as narrow lamellipodia containing 1 to 2 microspikes only, or blebs (Supplementary Fig. S2C). Clearly, these cells failed to orientate along the chemokine gradient (Supplementary Fig. S2D). Taken together, these findings show that CIP4 is crucial for the polarized assembly of the lamellipodium in response to CCL19 in JVM3 cells.

**CIP4 dynamically distributes to the lamellipodium**

To further investigate the mechanism by which CIP4 controls lamellipodium dynamics, JVM3 cells expressing a CIP4–GFP fusion protein were observed by time-lapse microscopy during directional migration toward CCL19. JVM3 cells placed in media without chemokine were either round or displayed narrow cytoplasmic extensions emitting short filopodia. These cells displayed a cytoplasmic distribution of CIP4 with local enrichment in discrete patches (Fig. 3A). Exposure to a CCL19 gradient led to lamellipodium assembly and to the polarization of CIP4 toward the lamellipodium rear and along microspikes (Fig. 3B and Supplementary Movie S3). GFP–CIP4 intensity profile along microspikes revealed that CIP4 was enriched either at the rear of microspikes or along these structures (Fig. 3C–E). Parallel analysis of GFP–actin and GFP–CCR7 in migrating JVM3 cells indicated that microspikes were enriched in actin and contained CCR7 (Supplementary Movies S4 and S5). Higher magnification examination showed that the microspikes extended in length up to 6 μm from the lamella until the tip of the lamellipodium and harbored pendular movements at an angular speed of 1 to 6.5 rad/s. Notably, CIP4 accumulated along microspikes as they converged and fused, an event that was associated with microspike shortening and retraction (Fig. 3F and Supplementary Movies S6–S8). The study of CIP4 localization during microspike dynamics revealed that coincidently with microspike contact (interdistance < 2 μm), CIP4 was recruited at the rear and then along the contacting microspikes. As the microspikes started to retract (within 0–30 seconds following maximal CIP4 accumulation), CIP4 intensity decreased in a parallel manner (Fig. 3G–I and Supplementary Movies S6–S8). The dynamic patterns of CIP4 distribution suggest that this molecule is associated with the assembly of lamellipodia and with the turnover of radial microspikes.

**CCL19-driven activation of Cdc42, WASP, and MAPK in CIP4-depleted JVM3 cells**

CIP4 was originally described as an effector of Cdc42 (28), a Rho GTase that plays a key role in polarized migration (28, 29). We therefore analyzed whether CIP4 interacted with Cdc42 in JVM3 cells and whether CIP4 depletion might affect Cdc42 activation in these cells. To test directly whether active Cdc42 and CIP4 interacted in JVM3 cells exposed to CCL19, cell lysates were subjected to pull-down experiments with Cdc42-coated beads. Active GST-Cdc42 (GTP form) bound CIP4 very efficiently, whereas no binding was detected with either inactive GST-Cdc42 (GDP form) or GST alone, indicating that CIP4 interacted with active Cdc42 in our model (Fig. 4A). The association of CIP4 to Cdc42 in our model raised the possibility that CIP4 might influence Cdc42 activation or stability. However, CIP4-depleted JVM3 cells displayed both normal total Cdc42 levels and normal CCL19-evoked Cdc42 activation (Fig. 4B). Next, we tested whether CIP4, as a bona fide effector of Cdc42 (28), was required to mediate signaling downstream of this GTPase. Most notably, biochemical reconstitution experiments showed that TOCA-1, and presumably also CIP4 and FBP17, were critical to promote optimal activation of the otherwise inhibited actin nucleation-promoting factors WASP and N-WASP (30). Hence, we tested the activation status of WASP, which is specific of the hematopoietic system. Loss of CIP4 significantly impaired WASP activation in response to CCL19 stimulation as indicated by its tyrosine phosphorylation status (Fig. 4C; ref. 31), suggesting that one mechanism underlying protrusion deficiency consecutive to CIP4 knockdown might be impaired WASP activation and subsequent WASP-dependent actin polymerization. The mitogen-activated protein kinase (MAPK) p38 and Erk1/2 are additional, important regulators of cell migration (32) and have been shown to act downstream of Cdc42 (33). We first assessed the involvement of the p38 pathway on chemotaxis of primary CLL cells by using the p38 pharmacologic inhibitor SB203580. Leukemic cells isolated from the blood of a patient with CLL displayed a reduced migration toward CCL19 upon SB203580 treatment (Fig. 4D). We then investigated whether CIP4 depletion had an impact on p38 and Erk1/2 phosphorylation. As expected from the reported constitutive expression of phosphorylated p38 in CLL (34), nonstimulated control JVM3 cells displayed a high p38 phosphorylation status. Interestingly, in these cells, CCL19 treatment did not modify p38 phosphorylation. CIP4-depleted JVM3 cells also displayed a high basal phosphorylation of p38, which, however, displayed a 2-fold increase upon CCL19 treatment (Fig. 4E and F). p38 phosphorylation has been linked to the activation of PAK1, a known effector of Cdc42 (35). Therefore, we assessed PAK1 phosphorylation status in control and CIP4-depleted JVM3 cells. Group 1 PAKs (PAK1/2/3) were not activated in non-stimulated cells and were activated upon CCL19 treatment (Fig. 4E). As observed for p38, depletion of CIP4 in JVM3 cells markedly increased PAK1 phosphorylation as compared with control cells. To evaluate the role of PAK in p38 activation...
following CIP4 depletion, cells were treated with IPA-3, a selective PAK1/2/3 inhibitor that binds covalently to the PAK1 regulatory domain and prevents binding to the upstream activator Cdc42 (26). As expected, IPA-3 treatment before CCL19 stimulation inhibited PAK phosphorylation and reduced p38 phosphorylation back to control levels, indicating...
that PAK is responsible for p38 phosphorylation in CLL19-stimulated JVM3 cells. IPA-3 treatment also inhibited Erk activation in control and CIP4-depleted JVM3 cells suggesting that PAK is also regulating Erk activation in this model. Altogether, these data suggest that in our CLL model, CIP4 regulates CLL19-directed cell migration by modulating the activation of WASP and PAK/p38 as central motility-related pathways.

p38 and CIP4 are present within lamellipodia and control their formation

Given the recent identification of p38 as a major driver of chemotaxis in neutrophils (36), we addressed whether the p38 pathway might cooperate with CIP4 in the control of lamellipodium assembly in CLL cells. We first studied the localization of p38 and CIP4 in cells emitting CLL19-evoked lamellipodia. As shown in previous figures, JVM3 cells plated on ICAM-1 and stimulated with CLL19 formed lamellipodia (Fig. 5A). CIP4 displayed a punctate distribution, reminiscent of intracellular vesicles. CIP4 was particularly enriched in large patches at the rear of the lamellipodium. CIP4 was also detected within the lamellipodium itself, albeit at lower levels. The distribution of phospho-p38 within the cell body differed from that of CIP4 and was mainly nuclear, as revealed by the DAPI staining. Differently from CIP4, phospho-p38 could not be detected at the rear of the lamellipodium. However, phospho-p38 was detected within the lamellipodium extension. Along a cross-section of the lamellipodium, CIP4 and phospho-p38 displayed parallel intensity profiles, suggesting that this structure might be a site favoring interaction between these 2 molecules (Fig. 5B). The parallel distribution of CIP4 and p38 over the breadth of the lamellipodium extension was further strengthened by a relatively high Pearson’s coefficient, which applied both for phospho-p38 and total p38 stainings, when calculated for the whole lamellipodium area (Fig. 5C). We then tested whether p38 might control lamellipodium formation in JVM3 cells. Blockade of the p38 pathway with the specific p38 inhibitor SB203580 inhibited lamellipodium extension in control, but not in JVM3 CIP4-depleted JVM3 cells (Fig. 5D). Taken together, our data establish a link between CIP4 and p38 as both molecules are colocalized to the lamellipodium and as they are both necessary to the assembly of this structure promoting directional migration.

Figure 4. CIP4 depletion results in increased p38 activation. A, binding of GST, GST-Cdc42GDP, and GST-Cdc42GTP immobilized on glutathione-sepharose beads to endogenous CIP4 from JVM3 cells. B, Cdc42 activation analyzed by pull-down using GST-PBD from PAK1 and Western blotting in CIP4-expressing and CIP4-depleted JVM3 cells stimulated or not with 100 ng/mL CCL19. C, CIP4-expressing and -depleted JVM3 cells were stimulated for 5 or 20 minutes with 100 ng/mL CCL19. Stimulation was stopped by addition of ice-cold medium and cell lysates were immunoblotted with CIP4, WASP, P-WASP (phosphoY-290), vinculin antibodies. D, primary leukemic cells were pretreated for 1 hour with 2.5 μM/ L SB203580 or DMSO and assessed for CCL19-driven chemotaxis in a Transwell assay with the indicated CCL19 concentrations. The mean ± SD of triplicate values is represented. E, analysis from 5 independent experiments of the fold induction of p38 MAPK phosphorylation following CCL19 stimulation of the indicated cell lines. F, phosphorylation of p38, Erk1/2, and PAK1 analyzed by Western blotting following CCL19 stimulation of CIP4-expressing and -depleted JVM3 cells. Where indicated, the PAK inhibitor IPA-3 was added. Expression of CIP4, total PAK1/2/3, and actin are shown as controls.
CIP4 is highly expressed in primary CLL leukemic cells and regulates CCL19-driven chemotaxis

To investigate the physiopathologic relevance of our findings, the expression of CIP4 and its contribution to CCL19-driven migration were investigated in primary leukemic cells isolated from the peripheral blood of patients with CLL. As shown by the Western blot analysis conducted on a representative patient series, CIP4 was expressed in a majority of samples at higher levels than in control B cells (Fig. 6A). Further analysis of samples from a total of 43 patients with CLL and densitometric quantification indicated that CIP4 was expressed in primary CCL cells in an average of 2.5-fold over the levels of control B cells (range, 0.85–5.45). This analysis reinforced the notion that CLL is associated with high CIP4 expression. The analysis of CIP4 localization in primary CLL cells stimulated by ICAM-1/CCL19 showed that CIP4 was associated with actin-rich protrusions, including lamellipodia (Fig. 6B), thereby confirming our findings from JVM3 cells. To further explore the role of CIP4 in primary CLL cells, its expression was knocked down by siRNA in samples collected from 3 patients (Fig. 6C). CIP4 siRNA-treated leukemic cells from patients 10 and 15 displayed a reduced migration when compared with the corresponding control cells treated with scrambled siRNA. In contrast, the leukemic cells from patient 22, in which levels of CIP4 remained high upon CIP4 siRNA treatment, retained their ability to migrate efficiently toward the CCL19 gradient, reinforcing the idea that CLL cell chemotaxis toward CCL19 is dependent on the presence of a high CIP4 level (Fig. 6C). Given the relative variability of CIP4 expression in primary CLL cells, we investigated whether CIP4 expression levels might correlate with known disease markers by subdividing the 43 patients analyzed into CIP4-high (>2.5-fold increase over control B cells) and CIP4-low (<2.5-fold increase over control B cells) groups of comparable size. As reported in Table 1, CIP4-high patients seemed to be biased toward the IgVH-unmutated status, which is associated with an aggressive form of the disease and a shortened survival (37, 38). Further studies will be necessary to consolidate this association and to investigate how the molecule CIP4 identified here as a key regulator of CLL cell directed migration might be involved in the pathogenesis of the disease.

Discussion

This study provides novel molecular insights into the regulation of the high migratory potency of CLL leukemic cells. Real-time imaging of CLL cell directional migration in a CCL19
gradient revealed that these cells assemble a polarized, particularly large and dynamic actin microspike-containing lamellipodium. More importantly, the assembly and turnover of this protrusive structure that drives the cells toward the source of CCL19 is dependent on CIP4, a F-BAR domain protein known to regulate endocytosis by sensing and modifying membrane curvature (25, 39, 40).

The lamellipodium of CLL cells seems as a broad veil bearing radially arranged microspikes that contain actin bundles. To our knowledge, these structures have never been reported in leukocytes. In addition, very little is known on the dynamics of lamellipodium-associated microspikes as their early description in fibroblasts (41). In CLL leukemic cells, microspike formation and dynamics are dependent on chemokine exposure, suggesting that these structures are involved in chemokine gradient sensing. This possibility is further reinforced by the observation that CCR7 is present along these structures. CIP4-depleted cells display multiple, short-lived, and variably oriented protrusions largely devoid of microspikes. As a consequence of these alterations, cells lose polarity and chemotactic ability. Notably, CIP4 is dynamically and transiently enriched at the rear of microspikes just before these structures fuse and disassemble, highlighting a novel role for CIP4 in regulating microspike turnover in highly migrating tumoral cells.

CIP4 has been shown to control endocytosis of numerous receptors, including receptors triggering cell migration (42). However, in our model CCR7 internalization and cell surface expression are not affected by removal of CIP4, suggesting that its endocytic function might not account for its control over polarized lamellipodia extension, microspike dynamics, and chemotactic cell migration. In the context of prenatal neuronal development, CIP4 has recently been discovered to negatively regulate neurite outgrowth by inducing lamellipodia and veil-like protrusions rich in actin bundles (43). Notably, CIP4 activity in developing neurons was also independent from its role in endocytosis. CIP4 function, instead,
was restricted to the migratory and developmental phase of neuronal cells, and controlled the emission of neuronal protrusion along neurites and axons. Remarkably, this functional role was attributed to the ability of CIP4 to mediate large and extended lamellipodia, a finding similar to what we report in this study in a dramatically different cellular context. Collectively, these results support the notion that one major role of CIP4 is to regulate the dynamics of lamellipodia protrusions, further impacting on cell motility, a central function that might have been hijacked by tumoral cells to promote spreading.

A still unanswered question remains: how does CIP4 promote the membrane and actin dynamics underlying the assembly of the microspike-bearing lamellipodium? Because of their concave membrane-bending activity, F-BAR proteins are usually associated to the regulation of endocytosis. However, some F-BAR proteins including CIP4 have also been associated to the promotion of membrane protrusions such as filopodia and invadopodia (9, 28, 44, 45). Given that microspikes of migrating CLL cells contain actin bundles, it is very likely that additional molecular partners involved in actin polymerization are working together with CIP4. In keeping with this notion, we show that CCL19 stimulates Cdc42 activation and relocalization to the rear of the lamellipodium together with CIP4. CIP4 and Cdc42, in turn, have been shown to interact with WASP/WAVE proteins thereby promoting actin nucleation and filament elongation (7, 9, 46–48). In this context, it is worth noting that Cdc42 is primarily implicated in microspike/filopodia formation, rather than directly in the extension of lamellipodia. However, in several cell types, filopodia and sheet-like lamellipodia rapidly interchange during protrusive activity. On one hand, filopodia are embedded into or arise from preexisting lamellipodia (49). On the other hand, filopodia may become anchoring sites from where lamellipodial veils first form. This latter notion is consistent with our observations and recent data in neurons (43), showing that CIP4 controls microspike dynamics, favoring the assembly of veil-like structures.

Our study highlights a further level of molecular control of lamellipodium assembly in CLL cells by linking for the first time CIP4 to PAK1 and p38 MAPK activation. Our observations strengthen the notion that p38, a known regulator of stress responses, also regulates directed migration (32). Interestingly, p38 MAPK has been shown to be constitutively active in CLL, where it regulates MMP-9 production, suggesting a role in invasion (34). We report here that p38 activation is further increased upon CCL19 exposure in CIP4-depleted CLL cells, suggesting that control of its activation is important in regulating migration. In agreement with our data, it has been reported that over-activation of PAK1 compromised the maintenance of cell polarity in the migrating mesendoderm during Xenopus gastrulation (50). Similarly to CIP4 knockdown, p38 inhibition prevents lamellipodium assembly, an effect that is not additive with CIP4 depletion. The fact that both p38 hyper-activation or inhibition are detrimental to lamellipodium assembly suggests that a balanced p38 activity is required for this process. It therefore indicates that the modulation by CIP4 levels and subsequent PAK activation downstream of Cdc42 can be key regulators of directed migration. Future work will elucidate the mechanism of CIP4 and p38 MAPK collaboration in CLL cells.

Our data also suggest that high CIP4 expression is a hallmark of tumoral CLL cells and is associated to the high chemotactic activity of these cells. Breast cancer cell lines also express high levels of CIP4, which regulates invasiveness (9, 10). Very recently, CIP4 expression has also been associated to the invasiveness of osteosarcoma (51). Together, these findings suggest that the tight regulation of CIP4 expression observed during neuronal development might be lost in some tumors, thereby participating to the transformation process. Consistent with this notion, we show that primary leukemic cells from patients with CLL display high expression of CIP4, which is required for CCL19-driven chemotaxis. Analysis of CIP4 protein expression in resting and activated normal B cells and various subtypes of B-cell malignancies suggests that high CIP4 expression may be specific for CLL leukemic cells within the B-cell lineage. In the cohort of 43 patients with CLL analyzed in this study, we noted a bias toward an IgVH-unmutated status in the patient group with the highest CIP4 expression. Further studies on larger cohorts will be necessary to confirm that CIP4-high expression is linked to disease severity. It remains also to be clarified how CIP4 expression and its control over directed cell migration is participating to CLL pathogenesis. Importantly, our data indicate that lowering CIP4 expression to levels comparable with those of normal B cells can impair the chemotactic ability of leukemic cells from patients with CLL. This observation raises the possibility that CLL cells become addicted to CIP4 to migrate toward CCL19. Along that view, CIP4 may have an important therapeutic value and could be considered a molecular target of pharmacologic inactivation to block CLL cells dissemination to lymph nodes, a strategy already pursued with the use of anti-CCR7

### Table 1. CLL patients

<table>
<thead>
<tr>
<th>CLL patient subgroups</th>
<th>CIP4-low</th>
<th>CIP4-high</th>
</tr>
</thead>
<tbody>
<tr>
<td>First line/relapse (n = 21)</td>
<td>95.5%</td>
<td>82.18%</td>
</tr>
<tr>
<td>Gender M/F</td>
<td>62/38%</td>
<td>77/23%</td>
</tr>
<tr>
<td>Age (median)</td>
<td>66y</td>
<td>66y</td>
</tr>
<tr>
<td>Lymphocytosis (median)</td>
<td>68,400</td>
<td>64,000</td>
</tr>
<tr>
<td>Binet stage A/B/C</td>
<td>52/29/19%</td>
<td>64/18/18%</td>
</tr>
<tr>
<td>IgVH unmutated</td>
<td>38%</td>
<td>63.6%</td>
</tr>
<tr>
<td>Deletion 11q</td>
<td>9.5%</td>
<td>18.2%</td>
</tr>
<tr>
<td>Deletion 17p</td>
<td>9.5%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Bulky disease (&gt;5 cm)</td>
<td>23.8%</td>
<td>13.6%</td>
</tr>
<tr>
<td>CD38 &gt; 20%</td>
<td>62.3%</td>
<td>60%</td>
</tr>
<tr>
<td>Indication for therapy</td>
<td>47.6%</td>
<td>59%</td>
</tr>
</tbody>
</table>

Abbreviation: ns, nonsignificant.

*Disease parameters of 43 patients with CLL divided as CIP4-low and CIP4-high (lower/higher than 2.5-fold increase of CIP4 expression in leukemic cells as compared with normal B cells).
antibodies (52). CLL clinical spectrum is wide, ranging from mild lymphocytosis to lymph node enlargement and multi-organ failure. Disease progression may be related to the development of cells acquiring an exacerbated potential to enter and invade lymph nodes via the CCL19/21-CCR7 axis. This is supported by the increased CCR7 expression and CCR7-dependent migration reported in ZAP-70+/CD38+ CLL cells, whose presence is related to a bad prognosis (24, 53). In that context, further studies will be required to elucidate how disease progression may be related to the CIP-driven chemotactic activity characterized here.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were declared.

Authors’ Contributions
Conception and design: G. Malet-Engra, J. Viaud, M. Farcé, F. Lafouresse.
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Malet-Engra, J. Viaud, L. Ysebaert, M. Farcé, F. Lafouresse, L. Dupré.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Malet-Engra, J. Viaud, F. Lafouresse, F. Gaiti-Iacovoni, L. Dupré.
Writing, review, and/or revision of the manuscript: G. Malet-Engra, L. Ysebaert, F. Lafouresse, F. Gaiti-Iacovoni, G. Scita, L. Dupré.

References

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Malet-Engra, L. Ysebaert, M. Farcé, L. Dupré.
Study supervision: L. Dupré.

Acknowledgments
The authors thank Véronique De Mas and Cécile Demur, in charge of the HIMIP cell bank, for preparing and providing primary cells from patients with CLL; Daniel Sapode and Sophie Allart from the Institut National de la Santé et de la Recherche Medicale (INSERM) U1043 imaging facility for assistance in image processing; Daniel Legler for providing the GFP-tagged CCR7 construct; Anne Quillet-Mary, Emile Gross, and Salvatore Valitutti for helpful discussions; and Josipa Spoljaric for article editing.

Grant Support
This work was supported by the European Community (Marie Curie Excellence grant, contract MEXT-CT-2005-050302 to L. Dupré), by the Ligue Contre le Cancer (grant from the Comité Régional de Haut-Garonne to L. Dupré) and by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC), by a grant from the Fondation ARC pour la Recherche sur le Cancer (SU2010061347), and by the Italian Ministries of Education-University-Research (MIUR-PRIN) grant to G. Scita. J. Viaud is a fellow of “Fondation ARC pour la Recherche sur le Cancer.”

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 13, 2012; revised March 7, 2013; accepted March 14, 2013; published OnlineFirst May 3, 2013.

www.aacjournals.org
Cancer Res; 73(11) June 1, 2013 3423

Published OnlineFirst May 3, 2013; DOI: 10.1158/0008-5472.CAN-12-3564

Downloaded from cancerres.aacrjournals.org on October 22, 2017. © 2013 American Association for Cancer Research.
Aspenstrom P. A Cdc42 target protein with homology to the non-

Etienne-Manneville S. Polarity proteins in migration and invasion.

Torres E, Rosen MK. Contingent phosphorylation/dephosphorylation


Ho HY, Rohatgi R, Lebensohn AM, Le Ma, Li J, Gygi SP, et al. Toca-1


Ringshausen I, Dechow T, Schneller F, Weick K, Oelsner M, Peschel C,


Linder S, Hufner K, Wintergerst U, Aepfelbacher M. Microtubule-


CIP4 Controls CCL19-Driven Cell Steering and Chemotaxis in Chronic Lymphocytic Leukemia

Gema Malet-Engra, Julien Viaud, Loïc Ysebaert, et al.