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Tetraspanin CD81-Regulated Cell Motility Plays a Critical Role in Intrahepatic Metastasis of Hepatocellular Carcinoma

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Background & Aims: **Human hepatocellular carcinoma (HCC) can invade the portal vein and metastasize to other parts of the liver. Currently, the molecular and cellular mechanisms underlying intrahepatic metastasis of HCC are poorly understood. Tumor invasiveness could be considered an aspect of dysregulated motility, and the mechanisms that inhibit cell movement are considered to counteract the spreading of cancer cells through the liver. Accumulating observations suggest that the CD81 tetraspanin may have an inhibitory effect on cell movement.** ***Methods:*** **In the present study using both loss- and gain-of-gene function approaches, we verified that the functional interaction of tetraspanin CD81 with type II phosphoinositide 4-kinase (PI4KII) suppressed HCC cell motility by promoting the formation of CD81-enriched vesicles, non-endosomal intracellular structures, that sequestered actinin-4 with consequent remodeling of actin cytoskeleton.** ***Results:*** **We reported that HCC cells expressing CD81 showed an inability to metastasize compared with HCC cells with undetectable levels of CD81.** ***Conclusions:*** **Taken together, these findings indicate that CD81 functions as a molecular organizer of membrane microdomains, whereby proteins such as PI4KII control actin remodeling and cell motility, establishing a role for these genes as negative modifiers of oncogenicity and HCC progression.**

Hepatocellular carcinoma (HCC) is known to arise in many patients with end-stage liver disease and is one of the most frequent cancers worldwide.[1] The long-term survival of patients with HCC is poor because of the high incidence of recurrence within the liver after treatment. Pathologic studies have identified 2 types of recurrence of HCC, namely multicentric development of new tumors and intrahepatic metastasis.[2]

Intrahepatic metastasis of HCC is frequently observed during advanced stages of the disease, and it is thought to develop through tumor cell dispersal via the portal vein. There is a strong statistical correlation between the presence of intrahepatic metastasis and the frequency of vascular invasion.[3] However, the pathogenesis of the high frequency of intrahepatic metastasis in HCC has not been elucidated.

Tumor invasiveness may be considered a disease of cell motility. In fact, cell motility plays a central role during carcinoma cell dissemination, and the cytoskeleton, a key structure of cell machinery, is continuously remodeled during cell movement. In this context, it is not clear what role is played by the "endosomal compartment" in controlling actin organization and cell movement. Recently, in attempts to shed light on this issue several studies have shown that several members of the tetraspanin protein family, such as CD9, CD63, and CD82, can affect cell motility.[4–7] Furthermore, a few of these proteins have been associated with intracellular vesicles lacking endosomal markers.[8]

CD81 is a ubiquitously expressed tetraspanin protein that has been described to be involved in a broad range of cellular functions, including cellular fusion, exocytosis, and cell motility. CD81 has been proposed to play a role in determining the metastatic behavior of tumors, and in particular the decreased expression of CD81 is associated with the presence of distant extrahepatic metastasis in patients with HCC.[9] Previously, our laboratory has shown that CD81 is involved in controlling the motility of liver cells (ie, hepatic stellate cells); however, the mechanisms by which CD81 regulates this process are not completely known.[10]

An important feature of CD81 is its ability to form oligomers and to associate with a variety of other signaling proteins into membrane microdomains termed tetraspanin-enriched microdomains.[11] We and others have previously shown that type II phosphoinositide 4-kinase (PI4KII) is one of the various signaling proteins with which CD81 can create functional complexes.[12–14] However, the importance of this interaction in influencing cell migration has not been investigated. Whether CD81 reg-

Abbreviations used in this paper: cDNA, complementary DNA; CEV, CD81-enriched vesicle; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; IGF-1R, insulin-like growth factor type 1 receptor; PCR, polymerase chain reaction; PI4KII, type II phosphoinositide 4-kinase; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P2, phosphatidylinositol (4,5)-bisphosphate; RNAi, RNA interference; RT-PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering RNA.

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ulates cell motility directly or by modulating the function of associated molecules is an issue that remains unclear.

In the present study, using both loss- and gain-of-function approaches, we show that CD81 interacts with PI4KII to suppress tumor cell motility. The functional cooperation between CD81 and PI4KII contributes to the formation of intracellular vesicles lacking endosomal markers. These vesicles are able to sequester actinin-4 from the plasma membrane and impair proper assembly of the actin cytoskeleton. Intriguingly, we found that the lipid kinase activity of PI4KII is necessary for the interaction with the cytoskeleton and the suppression of cell motility. This represents an unexpected function of this protein in regulating cell migration.

Materials and Methods

Cell Lines, Antibodies, and Constructs

HepG2 and SW480 cells were purchased from American Type Culture Collection (Rockville, MD). Huh7 cells were obtained from Dr Tetsu Watanabe, Department of Environmental Health, Tokai University. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma Aldrich, St Louis, MO) and supplemented with 10% fetal bovine serum, 5 mmol/L sodium pyruvate, and 5 mmol/L nonessential amino acids at 37°C in a humidified incubator containing 5% CO_2. The following antibodies were used: rabbit polyclonal anti-PI4KIIβ (Abgent, San Diego, CA), rabbit polyclonal IG701 anti–actinin-4 (ImmunoGlobe, Wurzberg, Germany), mouse monoclonal phycoerythrin-conjugated anti–insulin-like growth factor type 1 (IGF-1R; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-hemagglutinin (12CA5), anti-Myc (9E10) (Santa Cruz Biotechnology), rabbit polyclonal (H-121), mouse monoclonal anti-CD81 (5A6) (Santa Cruz Biotechnology), mouse monoclonal anti-green fluorescent protein (GFP; Molecular Probes, Carlsbad, CA), rabbit polyclonal anti–early endocytic antigen 1 (Abcam, Cambridge, MA), and tetramethylrhodamine isothiocyanate–phalloidin (Sigma). Monoclonal anti-CD81 (5A6) was labeled with fluorochrome Alexa Fluor 488 using Zenon mouse immunoglobulin G (IgG) labeling kit (Molecular Probes).

CD81-GFP was obtained by polymerase chain reaction (PCR) amplification of the human CD81 complementary DNA (cDNA) expressing GFP in frame with CD81 in the amino terminal position. The construct was subcloned into the pCDM8 plasmid for transient expression. pcDNA3.1-CD81 was previously described.[13] Myc-PI4KIIβ was generously provided by HL Yin. Ha-Rab5Q79L and Ha-Rab5S34N were generously provided by JL Bos. Myc-RabGAP-5 was generously provided by FA Barr. Eps15Δ95-295 was a generous gift of A Benmerah.

RNA Isolation and Reverse Transcription–PCR

Total RNA was extracted from HepG2 cells using TRIzol (Invitrogen, Carlsbad, CA). Total RNA (5 μg) was used for first-strand cDNA synthesis. The cDNA was then used as a template for PCR amplification of human PI4KII isoforms. All primers used for the amplification were verified in the BLAST database to ensure that each sequence was specific for the target gene; PI4KIIα: forward primer, 5′-ACCCAAGAATGAAGAGCCCTAT-3′, reverse primer, 5′-GGAGCAGTAGTTGCCGGTTAGT-3′; PI4KIIβ: forward primer, 5′-AAACCCAAATCAGAAGAGCC-3′, reverse primer, 5′-GAGGGA GTCCTATCCTATGAAACT-3′. The following thermal cycling variables were used: 94°C for 30 seconds, 55°C for 1 minute, 68°C for 1 minute (35 cycles), and a final extension at 68°C for 10 minutes. Data are representative of reverse transcription–PCR (RT-PCR) from 3 separate preparations.

Chemotaxis Assay

Cell migration assays were performed using a Boyden chamber equipped with 8-μm porosity polyvinylpyrrolidone-free polycarbonate filters (13-mm diameter). Polycarbonate filters were precoated with 20 μg/mL of human type I collagen for 1 hour at 37°C and placed between the upper and the bottom chambers. The lower chamber was filled with serum-free Dulbecco's modified Eagle's medium (control) or IGF-1 (100 ng/mL). Serum-starved cells (1×10^5 cells in 0.2 mL migration media) were added to the upper chamber. After 12 hours of incubation at 37°C, cells on the upper surface membrane were removed with a cotton-tip applicator. Migratory cells on the lower membrane surface were fixed by treatment with methanol and then stained with 0.1% crystal violet, 0.1 mol/L borate pH 9.0, and 2% ethanol. Cells that migrated to the underside of the filters were counted as the mean number of cells in 10 high-power fields. All experiments were run in triplicate.

Lipid Kinase Assay

HepG2 cells transiently transfected with empty vector or CD81-GFP using Lipofectamine (Invitrogen) were serum-starved overnight and lysed in buffer containing 1% CHAPS, 20 mmol/L HEPES (pH 7.5), 200 mmol/L NaCl, 5 mmol/L $MgCl_2$, 200 μM Na_3VO_4, 2 mmol/L NaF, 10 mmol/L $Na_4P_3O_7$, 2 mmol/L phenylmethylsulfonyl fluoride. After immunoprecipitation with an anti-GFP monoclonal antibody, immunocomplexes were washed 4 times in lysis buffer. Thereafter, the reaction was performed directly on protein A-sepharose beads. The reaction buffer consisted of 20 mmol/L HEPES (pH 7.5), 10 mmol/L $MgCl_2$, 50 μmol/L adenosine triphosphate, 0.3% Triton X-100, 10 μCi [^{32}P]adenosine triphosphate, and 200 μg/mL sonicated L-α phosphatidylinositol (Avanti polar lipids, Alabaster, AL) as a substrate. Lipid products were resolved by thin-layer chromatography. Thin-layer chromatography plates were exposed to O-Xmat film.

RNA Interference

The small interfering RNA (siRNA) sequence targeting human CD81 was previously described.[14] The siRNA sequences targeting human PI4KIIβ (GenBank accession number AY065990) spanned nucleotides 657–678 (5′-AATGCGATTGACCGTGCAAAA-3′) and 918–939 (5′-AATGATAATTGGTTAGTCAGA-3′). The control luciferase GL2 siRNA target sequence was 5′-AACGTACGCGGAATACTTGGA-3′. The siRNAs were chemically synthesized by Qiagen (Valencia, CA). For transfection, Amaxa nucleofection technology (Amaxa, Koeln, Germany) was used. Cells were resuspended in the nucleofector V solution, available as part of the Amaxa cell optimization kit, according to the Amaxa guidelines for cell line transfection. Seventy-two hours after transfection, protein expression levels were analyzed by flow cytometry, immunoblotting, or immunofluorescence. Transfection efficiency was at least 80% as detected by Alexa Fluor 488–labeled control siRNA.

Lipid Delivery

Long-chain (di-C16) synthetic phosphatidylinositol 4-phosphate [PtdIns(4)P] or BODIPY FL-PtdIns(4)P (Echelon Biosciences, Salt Lake City, UT) was freshly prepared at 300 μmol/L in 150 mmol/L sodium chloride, 4 mmol/L potassium chloride, and 20 mmol/L HEPES pH 7.2 and resuspended by bath sonication. Polyamine carrier–phospholipid complexes were prepared following the Echelon Shuttle PIP kit guidelines.

Live-Cell Microscopy and Immunofluorescence

HepG2 cells transfected with control or PI4KIIβ siRNA were plated in 60-mm dishes containing a sterile 40-mm circular cover glass and were maintained in culture as described above. Seventy-two hours after siRNA transfection, cells were transfected again with the plasmid encoding the CD81-GFP fusion protein. On the following day, the cover glass with the cell monolayer was transferred to a medium-filled chamber fitted to the microscope stage; thereafter, the living cells were stimulated with 100 ng/mL IGF-1. Live imaging was performed at 37°C using a Zeiss LSM 510 confocal microscope. Fluorescent images were captured with a cooled camera operated by Laser Scanning Microscope LSM510 software. The images were arranged using Adobe Photoshop software (Adobe, San Jose, CA).

Animal Experiments

Athymic nude rats were obtained from Charles River Laboratories and maintained in a pathogen-free environment. The human hepatoma cell line Huh7 was selected for the development of xenograft orthotopic HCC in nude rats. Groups of 5 rats were used. Tumor size and body weight of rats were evaluated twice a week. For intrahepatic injection, 50 μL of trypsinized Huh7-mock transfected cells or Huh7-CD81–positive cells (5×10^6) were injected intrahepatically by a 27-gauge needle. Five weeks after inoculation, rats were killed and examined for the presence of orthotopic tumors. Intrahepatic metastatic lesions were defined as lesions that were clearly distinct from the principal tumor.

Immunohistochemistry

Immunohistochemistry was performed using standard procedures as previously described.[13,14]

Statistical Analysis

All data are expressed as means ± SDs. Statistical analysis was performed by one-way analysis of variance and when the *F* value was significant, indicated by Duncan's test.

Results

To elucidate the involvement of CD81 in cell motility, we first used SW480, a colon carcinoma cell line endogenously expressing CD81. In these cells CD81 was silenced using siRNA molecules. Interestingly, CD81-siRNA cells displayed an increase in motility (Figure 1*A*). To gain insights into the role of CD81 regulation of HCC cell motility, we transfected HepG2 cells, an HCC cell line expressing undetectable levels of CD81, with a GFP-tagged CD81 vector. HepG2 CD81-GFP–positive cells were then allowed to migrate in response to IGF-1 in a Boyden chamber chemotaxis assay. We observed that CD81 strongly reduced the IGF-1–induced chemotaxis in these cells (Figure 1*B*). To exclude a hypothetical interference by GFP on CD81 function, we also performed chemotaxis assays with HepG2 transfected with untagged CD81. Again, CD81 was found to inhibit cell migration (data not shown). Taken together, these results suggest that CD81 functions as a suppressor of cell motility. In addition, immunofluorescence analysis showed that CD81 was localized in both plasma membrane and small vesicular structures scattered throughout the cytoplasm after expression of CD81-GFP in HepG2 cells. Notably, the expression levels of CD81-GFP obtained by transfection were comparable to the endogenous levels of CD81 expressed by SW480 cells (Figure 1*C*).

The formation of vesicles enriched in CD81 and the capability of CD81 to inhibit cell motility argued in favor of a possible interaction of CD81 with the endosomal compartment. To address this point, we compared the effect of endocytic pathway components such as Rab5, RabGAP-5, and Eps15 on CD81 in IGF-1–mediated chemotaxis. The guanosine triphosphatase–deficient mutant Rab5Q79L generates abnormally large endosomes by stimulating homotypic fusion of early endosomes.[15] Ectopic expression of Rab5Q79L in HepG2 cells reduced IGF-1–stimulated cell migration. Conversely, over expression of constitutively inactive Rab5 (Rab5S34N) or RabGAP-5, a Rab5 guanosine triphosphatase–activating protein, had no effect on IGF-1–induced cell migration

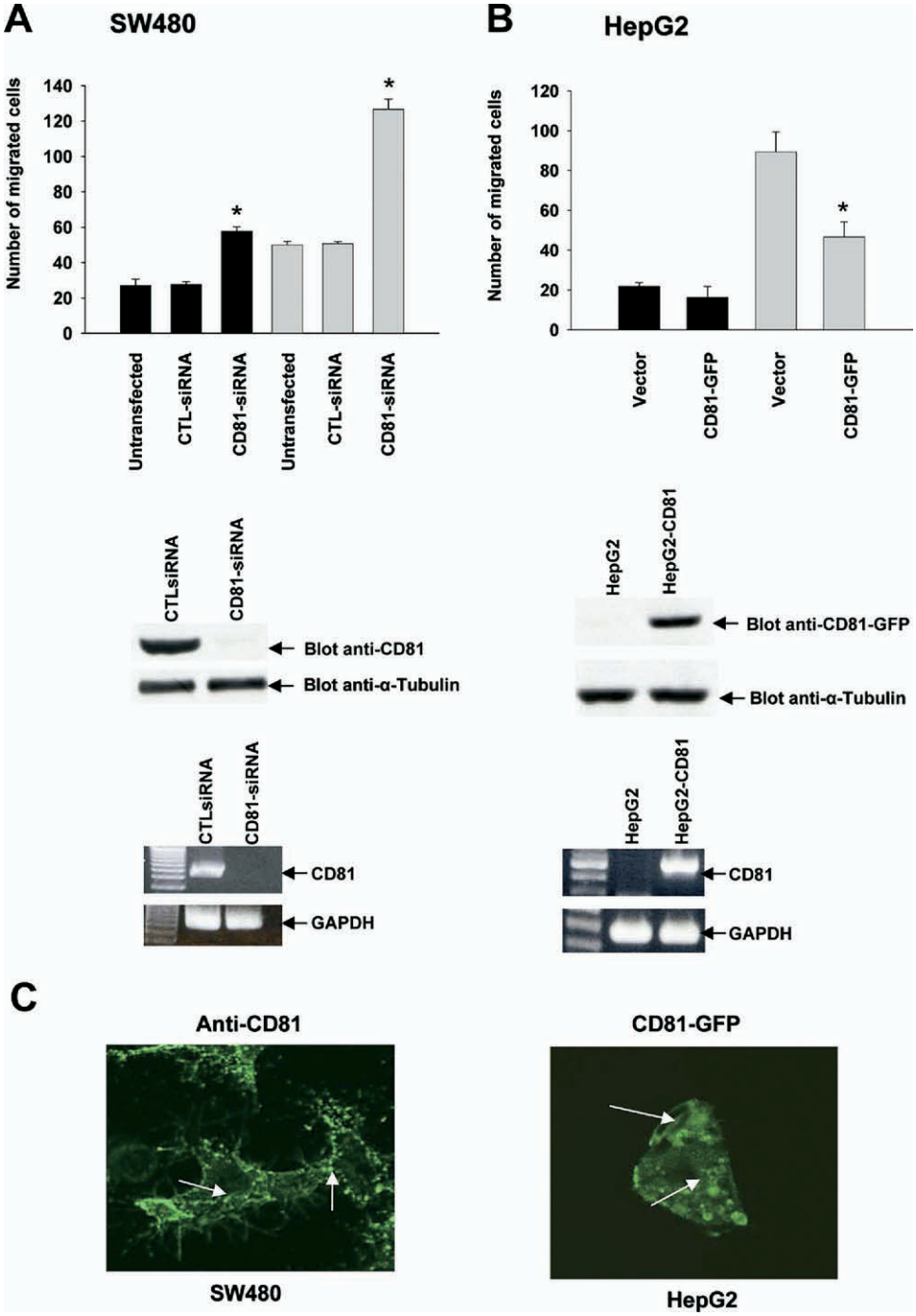


Figure 1. CD81 inhibits IGF-1–induced cell migration. (*A*) CD81 expression in SW480 was knocked down by RNA interference (RNAi) as shown by immunoblotting (*middle panel*) and RT-PCR (*bottom panel*) then cell migration was evaluated by Boyden chamber technique without (*black bars*) or with IGF-1 (*gray bars*). Endogenous GAPDH mRNA level was measured as the internal control. All results are representative of 3 independent experiments. The values are expressed as means ± SDs. **P* < .001 vs CTL-siRNA. (*B*) HepG2 cells expressing undetectable levels of CD81 as indicated by immunoblotting (*middle panel*) and RT-PCR (*bottom panel*) were transfected with CD81-GFP and IGF-1–independent (*black bars*) and IGF-1–induced (*gray bars*) cell migrations were evaluated. The endogenous GAPDH mRNA level was measured as the internal control. All results are representative of 3 independent experiments. The values are expressed as means ± SDs. **P* < .001 vs vector. (*C*) CD81-GFP–transfected HepG2 cells analyzed by immunofluorescence showed that protein was localized at the plasma membrane and in small vesicular structures scattered throughout the cytoplasm (*arrows*). The expression levels of CD81-GFP obtained by transfection were comparable to CD81 endogenous levels expressed by SW480 (*left panel*).

(Supplementary Figure 1*A*; see supplemental material online at www.gastrojournal.org). In addition, HepG2 cells transfected with Eps15 Δ95-295, a dominant-negative mutant of the endocytic protein Eps15, had no effect on migration. Eps15 acts by sequestering the endocytic adaptor protein AP2 and inhibits both ligand-induced and constitutive endocytosis.[16] Because only Rab5Q79L and CD81 were able to suppress cell motility, we examined a possible intracellular co-distribution of CD81-GFP with Rab5 and its effector early endocytic antigen 1 (EEA1) that is a well-established marker for early endosomes. Interestingly, most of the intracellular CD81-GFP was localized in vesicles that did not overlap with early endosomes (Supplementary Figure 1*B*; see supplemental material online at www.gastrojournal.org). Because several tetraspanins have been shown to localize to late endosomes, we performed colocalization studies of CD81-GFP with tetraspanin CD63, a marker of late endosomes. We found no detectable overlap of CD63 punctate staining with CD81-GFP (Supplementary Figure 1*B*). These findings are in agreement with the recent report by Deneka et al[8] in which they show a lack of colocalization

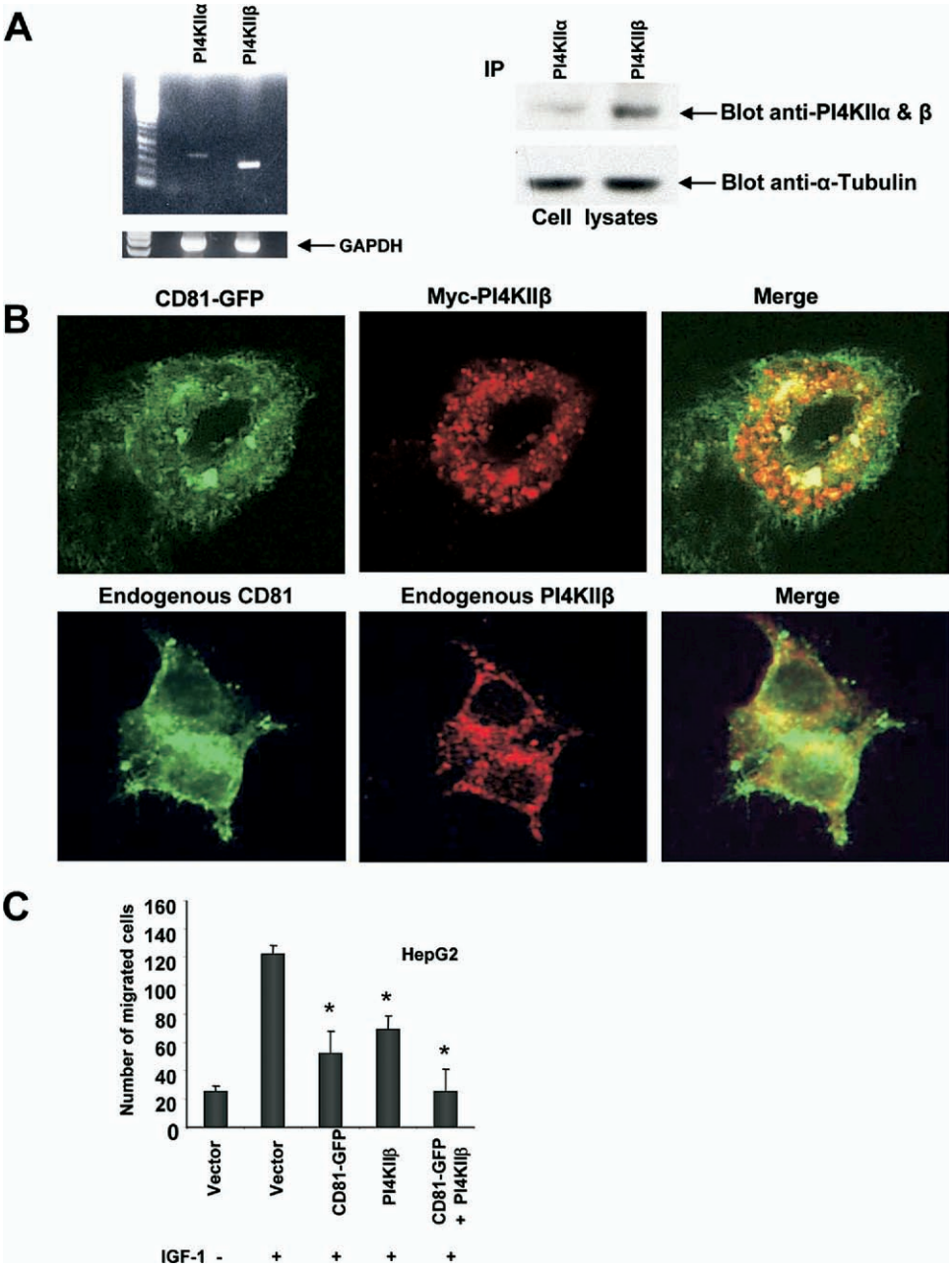


Figure 2. PI4KIIβ colocalizes in intracellular vesicles with CD81 and cooperates to inhibit cell migration. (*A*) HepG2 cells predominantly express PI4KIIβ as detected by RT-PCR and immunoprecipitation. The endogenous GAPDH mRNA level was measured as the internal control. Cell lysates were immunoblotted with anti–α-tubulin antibody as loading control. (*B*) HepG2 cells (*top panels*) transfected with CD81-GFP (*green*) and myc-tagged PI4KIIβ (*red*) were fixed, and indirect immunofluorescence was performed. Transfectants displayed colocalization of CD81 and PI4KIIβ (*yellow*). SW480 cells (*bottom panels*) stained with antibodies anti-CD81 and with anti-PI4KIIβ showed colocalization of endogenous CD81 and PI4KIIβ (*yellow*). (*C*) HepG2 cells were transfected with CD81-GFP, myc-PI4KIIβ, or in combination. After transfection, serum-starved cells were allowed to migrate toward IGF-1 in a chemotaxis assay. Values are means $\pm$ SDs of at least 3 separate experiments. $P < .05$ or higher degree of significance vs empty vector.

of CD63 and CD81 in human monocytes-macrophages. Taken together, these results indicate that vesicles containing CD81, hereafter termed CD81-enriched vesicles (CEVs), differ from both early and late endosomes and suggest the presence of a distinct vesicular trafficking pathway responsible for the inhibition of cell motility.

Because IGF-1 was used as a motogenic factor, we also tested the hypothesis that the inhibitory effect of CD81 on cell migration was due to changes in the expression of the IGF-1R on the plasma membrane. CD81 could sequester the IGF-1R within CEVs and thereby affect the internalization process. This is substantiated by the capability of CD81 to form oligomers and associate with various transmembrane receptors.[11] However, fluorescence-activated cell sorting (FACS) analysis showed that CD81-GFP did not cause any significant difference in the expression levels of IGF-1R (Supplementary Figure 2; see supplemental material online at www.gastrojournal.org). We therefore excluded the possibility that CD81 alters internalization and endocytic recycling of IGF-1R.

Taking advantage of previous studies that showed an association between CD81 and PI4KII,[12,13] we sought to characterize which PI4KII isoform associates with CD81 in HepG2 cells. RT-PCR and immunoblotting analysis showed that HepG2 cells predominantly express PI4KIIβ (Figure 2*A*). When PI4KIIβ was over expressed in these cells, confocal analysis showed prominent vesicular localization that overlaps with CEVs. Importantly, similar

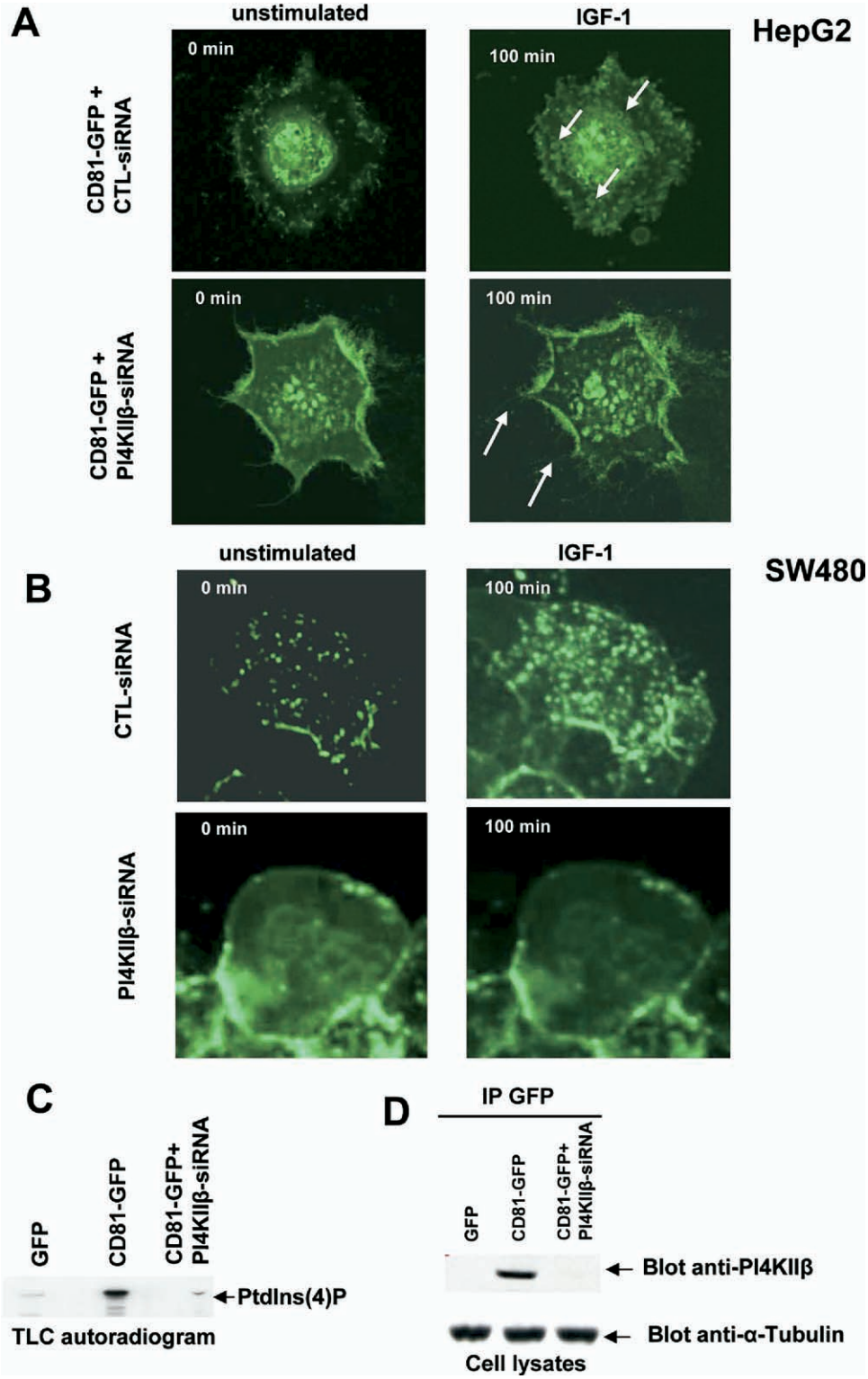


Figure 3. PI4KIIβ is required to produce CD81-enriched vesicles. (*A*) Live-cell imaging was performed on HepG2 cells transfected with control siRNA (CTL-siRNA) or PI4KIIβ-siRNA. Seventy-two hours after transfection, cells were again transiently transfected with CD81-GFP, serum-starved, and then stimulated with IGF-1. As shown in the *top panel*, HepG2 cells formed intracellular vesicles (*arrows*). In PI4KIIβ-knockdown cells, the formation of CEVs was impaired. Conversely, these cells displayed an increased capability to form filopodia (*arrows*). Individual frames from a confocal time series are shown. (*B*) SW480 cells were transfected with CTL-siRNA or PI4KIIβ-siRNA. Seventy-two hours after transfection, SW480 cells were serum-starved, incubated with antibodies anti-CD81 Alexa Fluor 488 conjugated, and stimulated with IGF-1. As shown in the *top panel* SW480 cells increased the intracellular vesicles after IGF-1 stimulation. In PI4KIIβ-knockdown cells, the formation of CEVs was impaired. (*C*) HepG2 cells were treated with CTL-siRNA and PI4KIIβ-siRNA. Seventy-two hours after siRNA treatment, cells were transfected with GFP-tagged CD81 or GFP alone. Equal amounts of proteins were immunoprecipitated using anti-GFP antibody and subjected to lipid-kinase assay using phosphatidylinositol as substrate. One representative experiment is shown (n = 3). (*D*) HepG2 cells were treated with CTL-siRNA and PI4KIIβ-siRNA. Seventy-two hours after siRNA treatment, cells were transfected with GFP-tagged CD81 or GFP alone. Anti-GFP immunoprecipitates (*top panel*) and cell lysates (*bottom panel*) were immunoblotted with anti-PI4KIIβ and anti–α-tubulin, respectively.

results were also obtained in SW480 cells (Figure 2*B*). Moreover, over expression of PI4KIIβ reduced the migratory capability of HepG2 toward IGF-1, and an additive effect was observed when PI4KIIβ was coexpressed with CD81 (Figure 2*C*). These results indicate that PI4KIIβ is required by CD81 to inhibit cell motility. The relevance of the CD81-PI4KIIβ interaction was further investigated using gene-specific siRNA constructs to knockdown PI4KIIβ in HepG2 cells, followed by CD81-GFP expression and stimulation with IGF-1. Time-lapse confocal imaging of CD81-GFP cells transfected with nonsilencing control (siRNA) showed the formation of intracel-

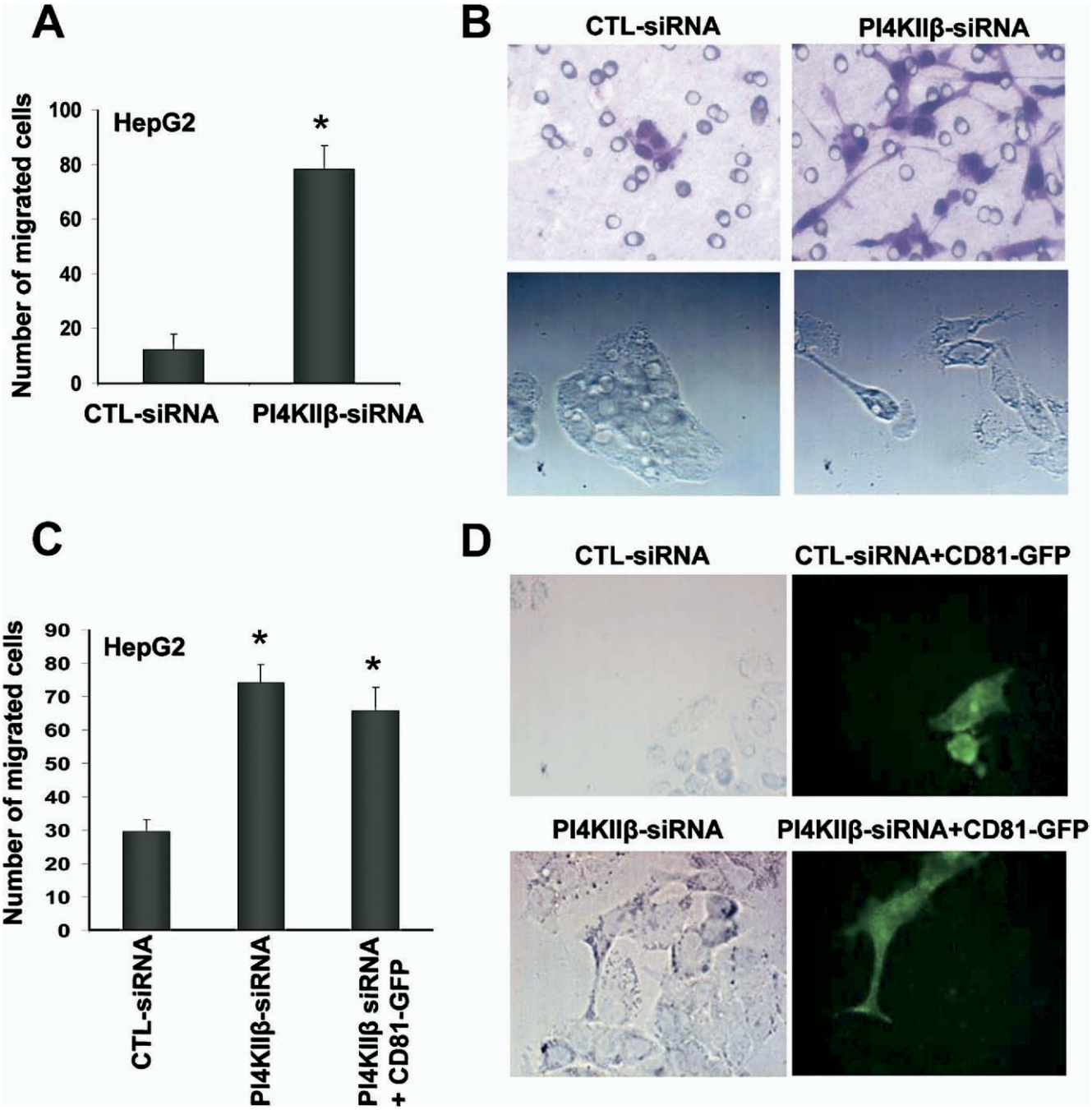
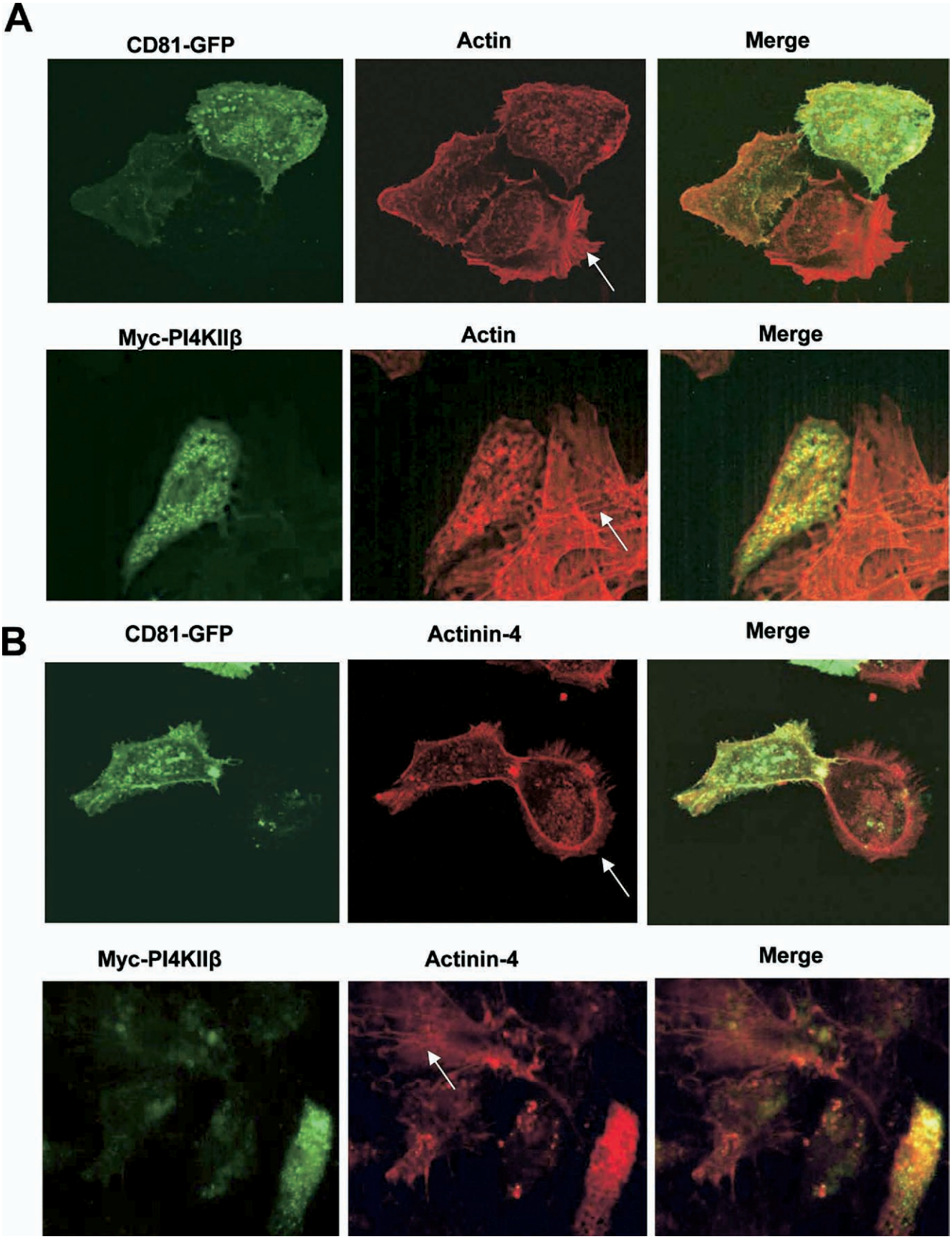


Figure 4. PI4KIIβ plays an essential role in controlling cell motility. (*A*) PI4KIIβ-knockdown HepG2 cell migration was evaluated in an IGF-1–induced chemotaxis assay. PI4KIIβ-knockdown HepG2 cells showed a dramatic ability to migrate. (*B*) The membrane filter used in the chemotaxis assay was stained with crystal violet (*top panel*), and the PI4KIIβ-knockdown migratory cell shape is shown. A scatter appearance was also evidenced by phase-contrast microscopy (*bottom panel*). HepG2 cells were treated with control siRNA (CTL-siRNA) or PI4KIIβ-siRNA. Seventy-two hours after siRNA treatment, cells were transfected with CD81-GFP. As shown, CD81 was unable to revert the functional (*C*) and morphologic (*D*) aspect of PI4KIIβ-knockdown cells. The *values* are expressed as means ± SDs. *$P < .001$ compared with CTL-siRNA.

Figure 5. CD81 and PI4KIIβ affect the remodeling of both actin and actinin-4. (*A*) HepG2 cells transfected with CD81-GFP (*green*) and myc-PI4KIIβ (*green*) were fixed and stained with tetramethylrhodamine isothiocyanate–phalloidin (*red*). Both CD81 and PI4KIIβ colocalized with actin. HepG2 cells expressing CD81-GFP or myc-PI4KIIβ exhibited a reduced ability to form stress fibers (*arrow*). (*B*) HepG2 cells transfected with CD81-GFP (*green*) and myc-PI4KIIβ (*green*) were fixed and stained with an antibody directed against actinin-4 (*red*). Both CD81 and PI4KIIβ colocalized with actinin-4. Note that actinin-4 was localized in stress fibers and plasma membrane in HepG2 cells nonexpressing CD81-GFP or myc-PI4KIIβ (*arrow*).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lular CEVs and a marked reduction in the number of filopodia at the plasma membrane (Figure 3*A*). Conversely, imaging of live PI4KIIβ-siRNA cells showed that these cells were incapable of forming CEVs, suggesting that the presence of PI4KIIβ is required for the formation of these intracellular membrane structures. In addition, we observed an increase in the number of filopodia, a typical feature of cell motility. Similar data were obtained with SW480 cells which express endogenous levels of CD81 (Figure 3*B*). Additional experiments carried out with these cells confirmed our observations made on HCC cells, thus excluding the possibility that the effect seen in HepG2 cells could be due to an artifact merely caused by the ectopic expression of CD81.

PI4KIIβ-siRNA–treated HepG2 cells also showed a reduction of both CD81-associated PI4KIIβ activity and protein expression (Figure 3*C* and *D*), clearly showing that PI4KIIβ is necessary for the formation of CEVs.

The formation of CEVs is implicated in cell motility as shown by experiments in which siRNA knockdown of PI4KIIβ potently increased the chemotactic-induced migration of HepG2 cells (Figure 4*A*) and dramatically altered the shape of these cells that appeared scattered and elongated, characteristics consistent with the motile phenotype (Figure 4*B*). Surprisingly, this phenotype was not reversed by restoring CD81 expression in these cells (Figure 4*C* and *D*), thus emphasizing the relevance of the CD81-PI4KIIβ interaction in controlling cell migration.

Cell migration is a highly complex process that is mediated by dynamic changes in the actin cytoskeleton.[17] To determine whether both CD81 and PI4KIIβ affected the actin cytoskeleton rearrangement, we over expressed either CD81-GFP or Myc-PI4KIIβ vector in HepG2 cells. Confocal analysis showed that CD81-GFP as well as Myc-PI4KIIβ produced a disassembly of actin filaments and showed colocalization of actin with both CD81 and PI4KIIβ (Figure 5*A*). In addition to analyzing the colocalization of actin, we also examined the localization of actinin-4 in relation to CD81 and PI4KIIβ. Actinin-4 is an antiparallel homodimer with an actin-binding domain and a binding site for phosphoinositides and has been shown to be involved in the regulation of actin bundling in cell motility.[18] Accordingly, we investigated the interaction of both CD81 and PI4KIIβ with actinin-4. Confocal analysis showed that CD81 and PI4KIIβ colocalize with actinin-4 after the expression of either CD81-GFP or Myc-PI4KIIβ in HepG2 cells. Moreover, this resulted in altering their location along the plasma membrane (Figure 5*B*). Consistent with this finding, coimmunoprecipitation experiments of endogenous CD81 in SW480 cells showed that CD81 is associated with both actinin-4 and PI4KIIβ (Figure 6*A*). Furthermore, SW480 cells transfected with PI4KIIβ-siRNA showed a marked reduction in the number of CEVs. This was associated with a decreased colocalization of CD81 with actinin-4 in these

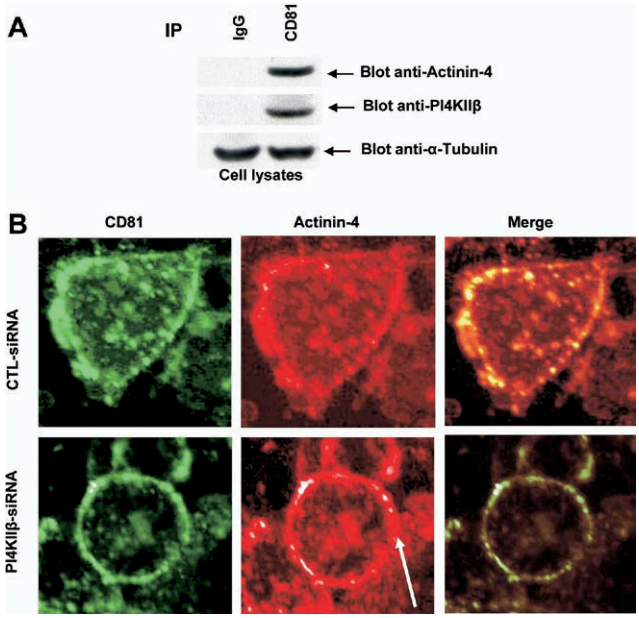

Figure 6. CD81 and actinin-4 colocalize at the plasma membrane after PI4KII knockdown. (*A*) SW480 cells were lysed and proteins were immunoprecipitated (IP) with antibodies immunoglobulin G1 (IgG1) isotype (Control) and anti-CD81 (5A6) antibodies. Immunoprecipitates were immunoblotted with rabbit polyclonal anti–actinin-4 and anti-PI4KIIβ antibodies. Cell lysates were immunoblotted with anti–α-tubulin antibody as loading control. (*B*) SW480 cells were transfected with CTL-siRNA and PI4KIIβ-siRNA. Seventy-two hours after transfection cells were fixed and stained with antibodies directed against CD81 (*green*) and actinin-4 (*red*). Note the reduction of CD81-enriched vesicles and the prominent localization of actinin-4 to the plasma membrane (*arrow*) in PI4KII-siRNA–transfected cells.

vesicles, as well as a partial colocalization of these molecules at the plasma membrane (Figure 6*B*). Thus, CD81 and PI4KIIβ affect the cytoskeleton through the rearrangement of actin.

To understand the connection between the CD81-PI4KIIβ complex and the cytoskeleton, we hypothesized that PtdIns(4)P, the catalytic product of PI4KIIβ, could regulate actinin-4. Our hypothesis was supported by studies that had established that actinin isoforms interact with PtdIns(4)P and phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2] and that phosphoinositide binding inhibited the bundling activity of actinin by blocking the interaction of the actin-binding domain with actin filaments.[19] To test this hypothesis, we evaluated the interaction of PtdIns(4)P with actinin-4 by confocal microscopy using a well-established procedure to deliver PtdIns(4)P into the cells.[20,21] PI4KIIβ-siRNA cells treated with BODIPY FL-PtdIns(4)P showed that synthetic PtdIns(4)P associated with actinin-4 in vesicular structures (Figure 7*A*). More importantly, intracellular delivery of PtdIns(4)P was able to revert elongated cell shape and scatter appearance induced by PI4KIIβ silencing (Figure 7*B*). We then determined the effect of PtdIns(4) on IGF-1–stimulated cell migration and found that

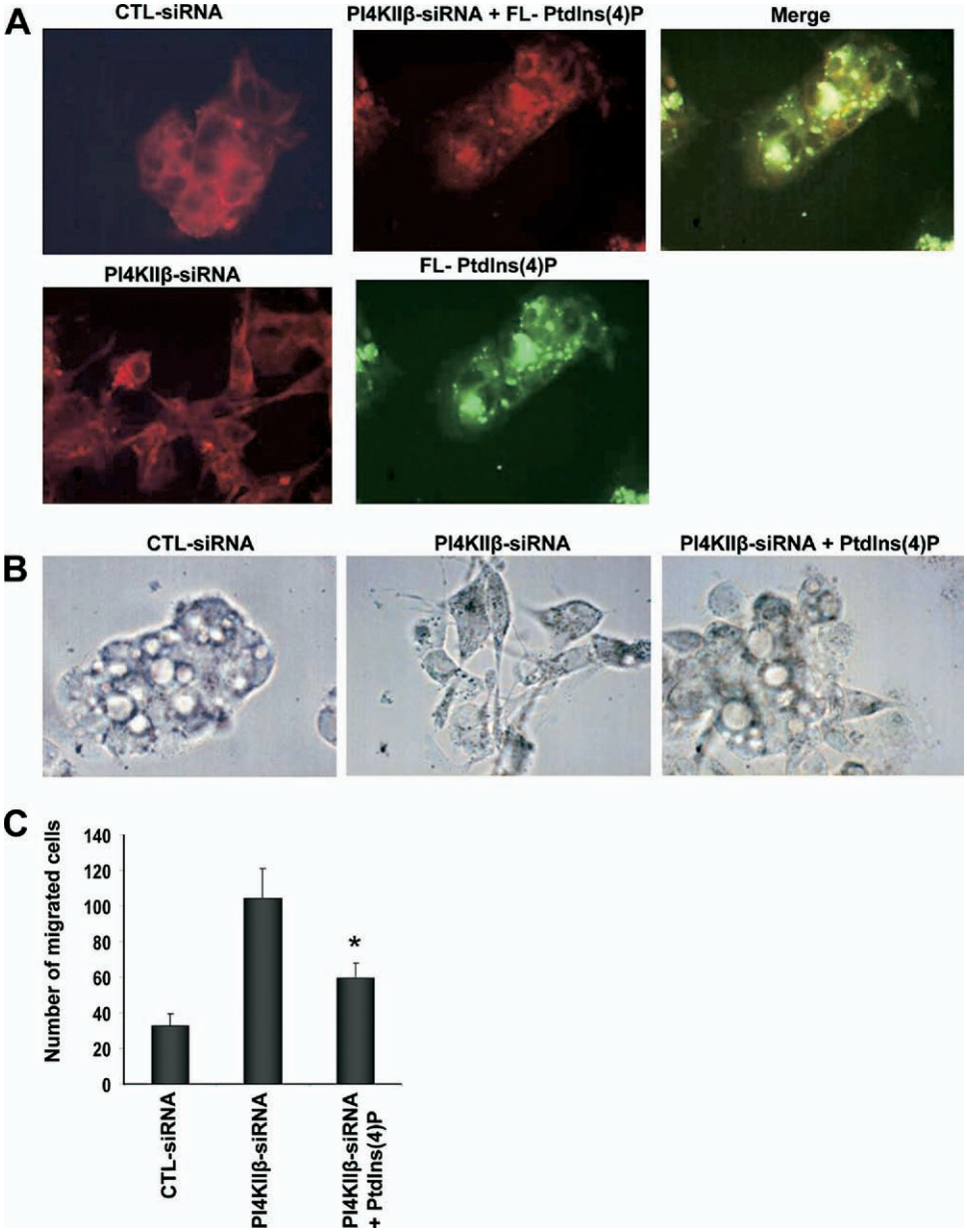

Figure 7. Lipid kinase activity of PI4KIIβ is required for the interaction with actinin-4. (*A*) HepG2 cells were treated with control siRNA (CTL-siRNA) or PI4KIIβ-siRNA and, after 72 hours, stained for actinin-4 (*red*). In some experiments, PI4KIIβ-knockdown HepG2 cells were incubated with polyamine shuttle carrier in the presence of 30 μmol/L BODIPY FL-PtdIns(4)P (*green*) for 12 hours at 37°C. Cells were fixed and stained with an antibody against anti–actinin-4 (*red*). Fluorescent PI(4)P colocalized with actinin-4 (*yellow*). (*B*) HepG2 cells were treated with CTL-siRNA or PI4KIIβ-siRNA. Seventy-two hours after the start of siRNA transfection, cells were incubated with polyamine carrier alone or in the presence of 30 μmol/L diC16-PI(4)P for 12 hours at 37°C. PI4KIIβ-knockdown HepG2 cells in the presence of shuttled PI(4)P reverted the scatter phenotype. (*C*) HepG2 cells treated with CTL-siRNA or PI4KIIβ-siRNA were incubated with polyamine carrier alone or in the presence of 30 μmol/L diC16-PI(4)P for 12 hours at 37°C. Thereafter, IGF-1–stimulated chemotaxis assay was performed. All results are representative of 3 independent experiments. The *values* are expressed as means ± SDs. *$P < .05$ vs PI4KIIβ-siRNA.

PtdIns(4)P inhibited the increased capability of PI4KIIβ-knockdown cells to migrate (Figure 7*C*). Interestingly, PtdIns(4)P had no effect on the migration of cells expressing endogenous levels of PI4KIIβ (data not shown). Collectively, these data indicate that the catalytic activity of PI4KIIβ is required for the interaction with actinin-4 and control of cell migration.[22]

To further address our previous findings, we made use of an animal model in which HepG2 cells were injected orthotopically into the liver of athymic nude rats. However, in this model HepG2 cells were unable to grow.[23] Therefore, we selected and used a tumorigenic cell population present in the HCC cell line Huh7 that expresses CD81 at undetectable levels (Supplementary Table 1; see supplemental material online at www.gastrojournal.org). When injected into the liver, these cells formed both primary tumors and multiple small tumors (Supplementary Figure 3*A*; see supplemental material online at www.gastrojournal.org). Huh7 were transfected with pcDNA3.1-CD81, and a stable clone expressing high levels of CD81 was sorted by FACScan (Figure 8*A*). In the CD81-expressing cells we performed cell proliferation, apoptosis, and cell-cycle assays. Data shown in Figure 8*A* excluded an inhibition of cell proliferation or induction of apoptosis. Huh7-CD81–positive cells, when injected orthotopically, developed primary tumors (Figure 8*B*) but were unable to form secondary tumors (Supplementary Figure 3*B*; see supplemental material online at www.gastrojournal.org).

These results are in agreement and extend previous clinicopathologic observations in which the expression of CD81 was examined on specimens of human HCC. In

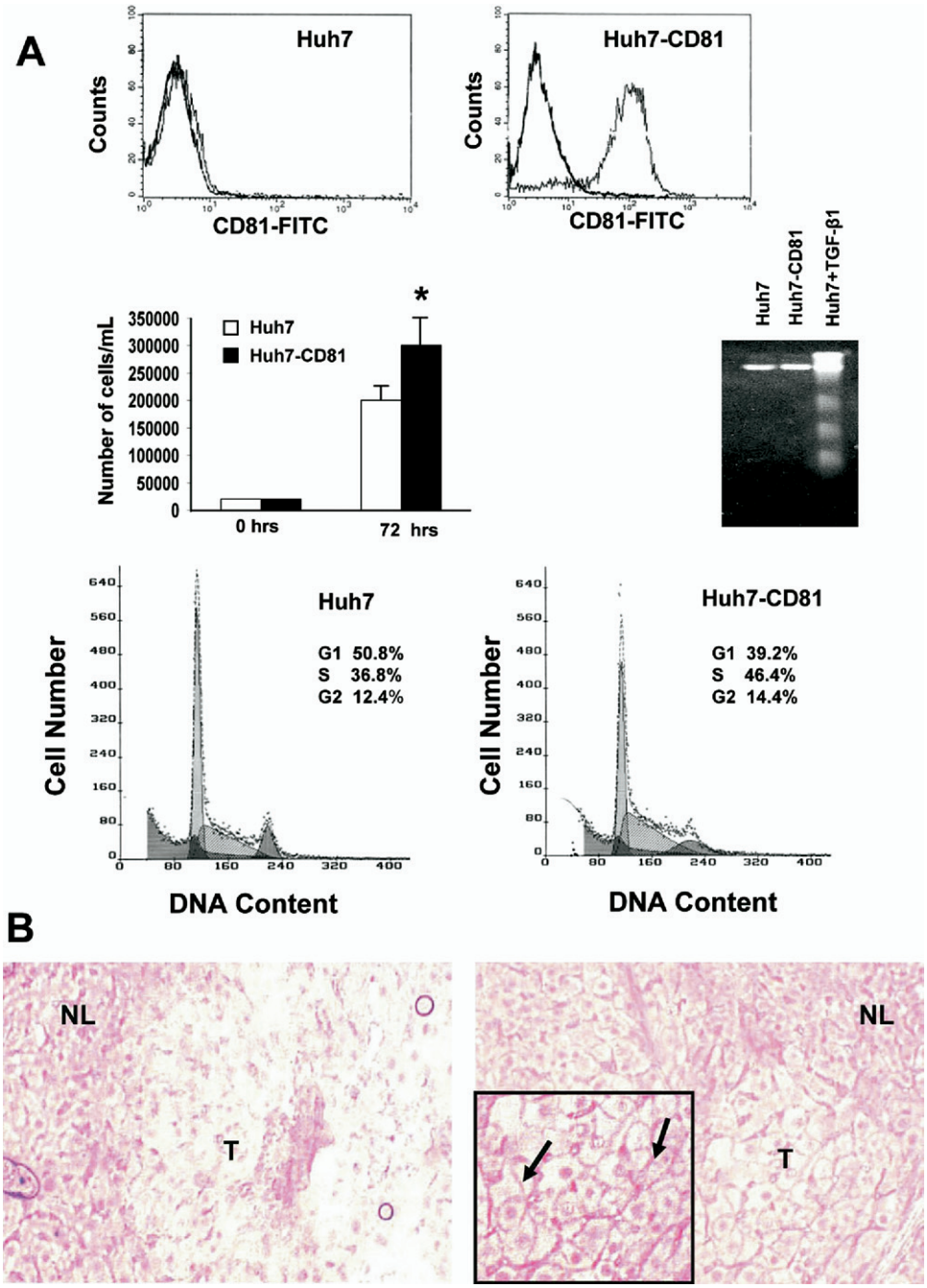

Figure 8. Growth in vitro and in vivo of human HCC line Huh7 expressing CD81. (*A*) Huh7 cells were transfected with the pcDNA3.1-CD81 vector, and single colonies were isolated by antibiotic selection in geneticin. The antibiotic resistant clones were subsequently amplified and sorted by FACScan. Cells were then grown and newly sorted to create a clonal cell population expressing equal levels of CD81 (*top panels*). Cell proliferation, apoptosis (*middle panels*), and cell-cycle progression (*bottom panels*, the data are representative of 1 clone) were evaluated in the above-mentioned cell population as well as in the control (mock-transfected). The values are expressed as means ± SDs. $^*P < .05$ vs Huh7. (*B*) Immunohistochemistry of primary tumors formed after mock-transfected Huh7 (*left panel*) or Huh7-CD81–expressing cells (*right panel*) were injected into the liver of athymic nude rats. Immunohistochemical staining with anti-CD81 showed that CD81 was expressed specifically in hepatocytes (*left panel*) of normal liver (NL) in comparison with tumors generated by mock-transfected Huh7 cells (T). Immunohistochemical staining with anti-CD81 of a tumor generated from Huh7-CD81 cells (*right panel*) (T) and hepatocytes of normal liver (NL) both stain positive for CD81 (anti-alkaline phosphatase). The *inset* shows a tumoral area enlarged with CD81 staining indicated by *arrows*.

those studies the researchers identified that CD81 was expressed on the surface of hepatocytes in nontumor portions in all patients, whereas the loss of CD81 was evident in poorly differentiated and metastatic HCC. They concluded that the loss of CD81 was directly associated with the differentiation and metastatic ability of HCC.[9,24]

Discussion

In this study, a novel mechanism of cell motility suppression was found involving the combined actions of both CD81 and PI4KIIβ. This is the first demonstration that CD81 and PI4KIIβ synergistically cooperate to inhibit cell migration. Our observations indicate that, in the presence of CD81, PI4KIIβ is prevalently compartmentalized intracellularly and this is concomitant with the inhibition of cell migration. More importantly, the present study highlights the capability of PI4KIIβ to inhibit cell migration, because the silencing of this gene resulted in increased cell migration. This finding discloses a novel implication for PI4KIIβ function. Although PI4KII proteins are tightly membrane bound, a significant portion of the protein is cytosolic especially in the case of the β isoform. Because of this fact, we hy-

pothesize that the mechanisms responsible for the recruitment of PI4KII to intracellular membranes represent important aspects of its function. We propose that CD81 could retain PI4KIIβ within intracellular compartments such as CEVs where the lipid kinase activity is needed for recruitment of actinin-4. We have also shown a direct connection of PI4KIIβ with the cytoskeleton through the interaction of PtdIns(4)P with actinin-4, pointing out the role of PtdIns(4)P as emerging regulatory molecules of the cytoskeleton. Nevertheless, we cannot exclude the presence of PtdIns(4,5)P2 and its possible function as a downstream messenger. Recently, Nasuhoglu et al[25] reported that the over expression of PI4KII resulted in an increase of both PtdIns(4)P and PtdIns(4,5)P2. Studies carried out on cellular phosphoinositides show that these directly affect the actin cytoskeleton.[26] Indeed, according to Fraley et al[19] actin bundling is reduced by phosphoinositides, and in situ evidence using GFP-tagged actinin defective in binding to phosphoinositides suggest that phosphoinositides negatively affect the actin-bundling activity of actinin.

Moreover, the delivery of PtdIns(4)P per se or as an immediate precursor of PtdIns(4,5)P2 or other phosphoinositides is capable of reverting both increased migration and the scattered phenotype induced by PI4KII knockdown. Unlike CEV-mediated actinin-4 compartmentalization, this mechanism avoids proper plasma membrane localization of actinin-4, in which actin fibers are cross-linked into actin networks. The down-regulation of actinin-4 induced by the compartmentalization process is an attractive mechanism for the control of cell motility. It is noteworthy that actinin-4 is found to be coordinately up-regulated with motility genes associated with invasion and metastasis. However, the mechanisms by which this occurs and the location where lipids bind the cytoskeletal machinery is not completely understood. Our study supports these observations and establishes a temporal and spatial mechanism operating in intracellular membranes by which PtdIns(4)P binds to the cytoskeletal machinery.

To spread within tissues, cancer cells must modify their shape and stiffness to interact with the surrounding tissue structures. Cell migration through tissues is a process regulated by a continuous cycle of interdependent steps, dependent on several factors, including cortical actin, a branched actin network below the inner leaflet of the plasma membrane, and actin stress fibers. Stress-fiber assembly is predominantly controlled by the small G-protein Rho and its downstream effectors, including the Rho-associated serine/threonine kinase. In contrast, the cortical actin network is not regulated by Rho,[27,28] and in this scenario CD81 and PI4KII could function as negative regulators of cortical actin assembly. Further studies will be necessary to confirm this important question, in light of the fact that migration of most metastatic cancer cells is sustained by cortical actin dynamics.[27]

Elucidations into the molecular mechanisms that initiate and promote cell motility are important and have been studied a great deal. Just as important, however, is comprehension of mechanisms that prevent or halt cell motility because of their potential in the treatment of metastatic cancers.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.03.024.

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Table 1. Characteristics of the Selected Subpopulation of Huh7 Tumorigenic in Athymic Rat

Morphology:	Epithelial-like		
Markers:	Alpha-1-fetoprotein (AFP)	+++	ELISA
	Cytokeratins (CK)18 and CK19	+++	IF
	Alpha-smooth muscle actin (a-SMA)	−−−	IF
	CD81	−−−	IF, IB
Invasiveness:	Highly invasive on matrigel		
Tumorigenicity:	Yes, in athymic rat (liver primary and secondary tumors) colony-forming ability on soft agar		
Histology:	Moderately differentiated hepatocellular carcinoma		

+++, strongly positive; −, negative; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; FC, flow cytometry