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Diacylglycerol Kinase-α Mediates Hepatocyte Growth Factor-induced Epithelial Cell Scatter by Regulating Rac Activation and Membrane Ruffling

Federica Chianale,*† Santina Cutrupi,*†‡§ Elena Rainero,* Gianluca Baldanzi,*‖ Paolo E. Porporato,* Sara Traini,* Nicoletta Filigheddu,¶ Viola F. Gnocchi,* Massimo M. Santoro,# Ornella Parolini,‖ Wim J. van Blitterswijk,@ Fabiola Sinigaglia,* and Andrea Graziani*

Departments of *Medical Sciences, ¶Clinical and Experimental Medicine, and #Scienze dell'Ambiente e della Vita, University of Piemonte Orientale "A. Avogadro," 28100 Novara, Italy; ‡Department of Animal and Human Biology and §Center for Complex System in Molecular Biology and Medicine – SysBioM, University of Torino, 10123 Torino, Italy; ‖Centro Ricerche "E. Menni," Ospedale Poliambulanza, 25124 Brescia, Italy; and @The Netherlands Cancer Institute, Amsterdam, 1066 CX Amsterdam, The Netherlands

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Diacylglycerol kinases (Dgk) phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), thus turning off and on, respectively, DG-mediated and PA-mediated signaling pathways. We previously showed that hepatocyte growth factor (HGF), vascular endothelial growth factor, and anaplastic lymphoma kinase activate Dgkα in endothelial and leukemia cells through a Src-mediated mechanism and that activation of Dgkα is required for chemotactic, proliferative, and angiogenic signaling in vitro. Here, we investigate the downstream events and signaling pathways regulated by Dgkα, leading to cell scatter and migration upon HGF treatment and v-Src expression in epithelial cells. We report that specific inhibition of Dgkα, obtained either pharmacologically by R59949 treatment, or by expression of Dgkα dominant-negative mutant, or by small interfering RNA-mediated down-regulation of endogenous Dgkα, impairs 1) HGF- and v-Src-induced cell scatter and migration, without affecting the loss of intercellular adhesions; 2) HGF-induced cell spreading, lamellipodia formation, membrane ruffling, and focal adhesions remodeling; and 3) HGF-induced Rac activation and membrane targeting. In summary, we provide evidence that Dgkα, activated downstream of tyrosine kinase receptors and Src, regulates crucial steps directing Rac activation and Rac-dependent remodeling of actin cytoskeleton and focal contacts in migrating epithelial cells.

INTRODUCTION

Epithelial tissues are characterized by monolayers of highly polarized cells, whereas in vitro epithelial cells grow to form discrete colonies. During embryonic development and tissue repair, as well as through cancer progression, epithelial cells acquire a highly motile and invasive phenotype in a process commonly known as epithelial-mesenchymal transition (EMT) (Thiery, 2002; Thiery and Sleeman, 2006). In vitro, the scattering of epithelial cells, i.e., the dispersal of colonies due to loss of intercellular adhesion and acquisition of cell motility, is triggered by growth factors stimulation and by oncogenes activation, recapitulating the early phases of EMT (Avizienyte and Frame, 2005).

Hepatocyte growth factor (HGF) and oncogenic Src induce in vitro cell scatter of several epithelial cells, whereas in vivo their inappropriate activation is associated to progression and acquisition of a metastatic phenotype in several epithelial-derived cancer (Irby and Yeatman, 2000; Danilkovitch-Miagkova and Zbar, 2002). Within hours from stimulation of their tyrosine kinase activities, both HGF and v-Src induce scattering of epithelial cell colonies through loss of cadherin-mediated cell–cell adhesions and increase of their motility, due to formation of lamellipodia and remodeling of cortical actin and focal adhesions (Beherens *et al*., 1993; Lamorte *et al*., 2002). The signaling pathways by which HGF and v-Src stimulate EMT, cell scattering, and invasiveness have been extensively investigated in several epithelial cells (Thiery, 2002). Recruitment of Gab-1, along with activation of phosphatidylinositol (PI) 3-kinase, phospholipase C (PLC)γ, Ras, and Rac are required (Lamorte *et al*., 2002, and references therein). Src plays a crucial role in HGF signaling because its activity is required for HGF-mediated cell motility, anchorage-independent growth, and tumorigenesis. Indeed, Src mediates HFG-induced tyrosine phosphorylation of catenins, leading to down-regulation of cadherin-mediated cell–cell adhesions, and of several focal adhesion proteins required for cell motility and invasiveness, such as focal adhesion kinase (FAK), paxillin, and p130Cas (Beherens *et al*., 1993; Rahimi *et al*., 1998; Nakaigawa *et al*., 2000).

Diacylglycerol kinases, which phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), comprise a family of 10

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† These authors contributed equally to this work.

Address correspondence to: Andrea Graziani (andrea.graziani@med.unipmn.it).

distinct enzymes, grouped in five classes, each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich atypical C1 domains (Topham and Prescott, 1999; Imai *et al*., 2005). DG is an established activator of several typical C1 domain-containing proteins, such as protein kinase C (PKCs), Ras guanyl nucleotide releasing proteins (RasGRPs), and chimaerins. Similarly, several signaling proteins have been reported to be regulated by PA, including serine kinases, such as mTor, Raf, and atypical PKCs; small GTPase-regulating proteins, such as SOS, Rho guanine nucleotide dissociation inhibitor protein (RhoGDI), Ras- and Rho-GTPase–activating proteins (GAPs); and signaling lipid-metabolizing enzymes, such as phosphatidylinositol 4-phosphate 5-kinase [PI(4)P 5-kinase] and PLCγ (Topham, 2006; Zhao *et al*., 2007). However, a common specific PA binding domain has not been identified yet. Thus, by regulating in a reciprocal manner the level of both DG and PA lipid second messengers, diacylglycerol kinase (Dgk) enzymes may act as terminators of DG-mediated signals as well as activators of PA-mediated signals.

Recent evidence showed that α, ζ, and θ Dgk isoforms are regulated by extracellular ligands and that they play a role in signal transduction (van Blitterswijk and Houssa, 2000; Luo *et al*., 2003). T cells derived by Dgkα −/− mice feature enhanced DG-mediated RasGRP activity upon T cell receptor (TCR) activation, leading to overactivation of the Ras pathway and a defect in anergy, whereas overexpression of Dgkα in T cells impairs TCR signaling (Olenchock *et al*., 2006a). Evidence in T cells indicates that Dgkα and ζ, by interacting, respectively, with RasGRP and PKC, up-regulate cell sensitivity to TCR activation by negatively modulating the intensity and the kinetic of DG-mediated signaling (Luo *et al*., 2003; Sanjuan *et al*., 2003; Zhong *et al*., 2003). Conversely, mast cells derived from Dgkζ −/− mice feature a diminished high-affinity IgE receptor-mediated degranulation, correlating with impaired PLCγ activation and calcium response, both likely dependent on PA production (Olenchock *et al*., 2006b).

We have previously shown that in endothelial and leukemia cells, activation of Dgkα downstream from tyrosine kinase receptors, such as HGF-receptor, vascular endothelial growth factor (VEGF) receptor-2, and anaplastic lymphoma kinase (ALK), is required for either chemotactic or proliferative signaling induced by their respective ligands and for cell proliferation upon interleukin-2 stimulation of T cells (Cutrupi *et al*., 2000; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005). Growth factors stimulate Dgkα through a mechanism requiring complex formation with Src and phosphorylation of Dgkα on Tyr^{335} by Src itself (Cutrupi *et al*., 2000; Baldanzi *et al*., 2007). The specific signaling pathways regulated by activation of Dgkα still await elucidation.

Herein, we investigate the role of Dgkα in HGF-induced cell migration of epithelial cells. We show that Dgkα activation is required for HGF- and v-Src-induced scattering of Madin Darby canine kidney (MDCK) cells, and particularly in those mechanisms leading to cell spreading and F-actin cytoskeleton and focal adhesions remodeling. By further investigating the role of Dgkα in HGF early signaling, we show that upon 15 min from HGF stimulation, Dgkα activity is necessary for membrane targeting and activation of Rac, and for Rac-regulated formation of membrane ruffles.

These data, by indicating Dgkα as a key signal transducer of motility signals downstream HGF and v-Src, strongly suggest that it may represent a key regulator in the processes of invasion and metastasis.

MATERIALS AND METHODS

Cell Culture

MDCK and MDCK-*ts*-v-Src (Baldanzi *et al*., 2004) are a kind gift of W. Birchmeier (Max-Delbrück-Centrum, Robert-Rössle-Str. 10, 13125 Berlin). Cells were cultured in high glucose DMEM GlutaMAX medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen) and antibiotic-antimicotic solution (Sigma-Aldrich, St. Louis, MO), in humidified atmosphere with 5% CO_2. MDCK cells were cultured at 37°C, whereas MDCK-*ts*-v-Src were normally grown at 40.5°C (inactive v-Src) and shifted to 35°C to achieve v-Src activation.

Reagents

Recombinant human HGF was purchased from Peprotech (Rocky Hill, NJ), and R59949 (diacylglycerol kinase inhibitor II) was from Sigma-Aldrich. Dimethyl sulfoxide, vehicle for R59949, was always used in control samples at the same dilution as R59949. Anti-Myc and anti-Rac1 were from Upstate Biotechnology (Charlottesville, VA); anti-paxillin was from BD Biosciences Transduction Laboratories (Lexington, KY); anti-paxillin $pTyr^{31}$ and $pTyr^{118}$ and anti-Akt $pSer^{473}$ were from BioSource International (Camarillo, CA); anti-Akt was from Cell Signaling Technology (Beverly, MA); anti-α-tubulin was from Sigma-Aldrich; anti-vinculin was from Novus Biologicals (Littleton, CO); anti-FAK was from Calbiochem (San Diego, CA); and Alexa Flour 546/633-phalloidin was from Invitrogen. Anti-Dgkα was kindly provided by W. J. van Blitterswijk (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Secondary horseradish peroxidase-conjugated antibodies were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA); and secondary fluorescein isothiocyanate (FITC)- and tetramethylrhodamine B isothiocyanate (TRITC)-conjugated antibodies were purchased from Dako Denmark (Glostrup, Denmark).

Expression Vectors, Transfections, and Infections with Retroviral Vectors

Myc-Dgkα cDNA cloned into pMT2 expression vector has been described previously (Cutrupi *et al*., 2000). Green fluorescent protein (GFP)-Dgkα-wild type (WT) was obtained by cloning Dgkα in pcDNA-DEST53 (Invitrogen) by using Gateway kit (Invitrogen) according to manufacturer's instructions. Briefly, Dgkα cDNA was inserted in pDONOR 2.11 vector by polymerase chain reaction (PCR) and BP recombination (recombination of an *att*B substrate with an *att*P substrate to create an *att*L-containing entry clone). LR recombination (recombination of an *att*L substrate with an *att*R substrate to create an *att*B-containing expression clone) was performed to transfer Dgkα in pcDNA-DEST53 for N-terminal GFP fusion; detailed information and protocols are available on www.invitrogen.com. $G_{434}D$ point mutation on Dgkα to obtain the kinase-defective dominant-negative mutant (GFP-Dgkα-DN) was performed using QuikChange site-directed mutagenesis kit 22 (Stratagene, La Jolla, CA) as described previously (Cutrupi *et al*., 2000). PINCOS retroviral vector, PINCOS/Dgkα-DN and PINCOS/Dgkα-WT, expressing both GFP and the inserted gene, have been described previously (Cutrupi *et al*., 2000). Transient transfections were performed using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's instructions.

MDCK cells stably expressing PINCOS/empty vector or PINCOS/Dgkα-DN or PINCOS/Dgkα-WT were obtained by infection. Briefly, GP2-293 packaging cell line (Clontech, Mountain View, CA; kindly provided by R. Piva, University of Torino) was transiently cotransfected, by Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions, with the envelope vector pVSV-G (Clontech) together with PINCOS or PINCOS/Dgkα-DN or PINCOS/Dgkα-WT. The next day, the medium was changed to normal growth medium. Forty-eight hours after infection, the retroviral supernatant was collected, the debris was removed by centrifugation at 1500*g*, and the supernatant was filtered by a 0.45-μm pore filter and added with Polybrene (8 μg/ml). Cells were plated in a six-well plate and infected by adding 2 ml of retroviral supernatant and 1 ml of growth medium. The day after the first infection cells were reinfected as described briefly. Sixteen hours later, cells were placed and maintained in growth medium. Efficiency of infection was ~80%, as measured by fluorescence-activated cell sorting (FACS) analysis and/or observation with fluorescence microscope of GFP-expressing cells.

The murine Dgkα, resistant to canine Dgkα small interfering RNAs (siRNAs), was cloned in the lentiviral vector pLenti4V5 (Invitrogen). Lentiviruses were produced following the manufacturer's instructions and used to infect MDCK cells, which were then selected in Zeocin-containing medium to obtain a stably expressing cell line.

RNA Interference

siRNAs against canine Dgkα were chemically synthesized as double-strand RNA (Ambion, Austin, TX). Sequences were as follows: C1, sense GCUCAGAAGUGGACAGGAUtt and antisense AUUCUGUCCACUUCUGAGCtg; C2, sense CCCAGACAUCCUGAAAACCtt and antisense GGUUUUCAGGAUGUCUGGGtc; C3, sense CCUUCCACACCACAAAAACtt and antisense

GUUUUUGUGGUGUGGAAGGtg. A glyceraldehyde-3-phosphate dehydrogenase scramble siRNA (Ambion) was used as negative control.

The BLOCK-iT Fluorescent Oligo (Invitrogen) is a fluorescein-labeled double-stranded RNA oligomer and was used to obtain indication of the transfection efficiency with siRNAs.

Dgk Assay

Dgkα activity was assayed in anti-Myc immunoprecipitates as described previously (Cutrupi *et al*., 2000). Briefly, after immunoprecipitation and extensive washing in lysis buffer (25 mM HEPES, pH 8, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 10% glycerol supplemented with protease inhibitors [Protease Inhibitors Cocktail; Sigma-Aldrich]), lithium chloride buffer (500 mM LiCl and 25 mM Tris-HCl, pH 8) and TNE (25 mM Tris, pH 8, 150 mM NaCl, and 1 mM EDTA), all supplemented with fresh 1 mM Na_3VO_4, the immunocomplexes were assayed at room temperature for 10 min by incubation with 1 mg/ml diolein (Fluka, Buchs, Switzerland; dried in nitrogen atmosphere, resuspended, and sonicated in 1 mM EGTA, 25 mM HEPES, pH 8), 5 mM ATP, 10 μCi/sample [γ-^{32}P]ATP (GE Healthcare, Chalfont St. Giles, United Kingdom), 10 mM $MgCl_2$, and 1 mM $ZnCl_2$. Lipids were then extracted as described previously (Graziani *et al*., 1991), and PA was separated by thin layer chromatography (TLC) in chloroform:methanol:water:32% ammonium hydroxide (60:47:10:3). TLC plates had been coated previously with (1.3% potassium oxalate, 5 mM EDTA):methanol (3:2). [^{32}P]PA was identified by comigration with nonradioactive PA standards (Fluka) stained by incubation in a iodine chamber. Radioactive signals were detected and quantified with GS-250 Molecular Imager and its Phosphor Analyst Software (Bio-Rad, Hercules, CA). One-half of immunoprecipitated lysates was assayed for Dgk activity, whereas the other half was heat-denatured in Laemmli buffer, separated in SDS-polyacrylamide gel electrophoresis (PAGE), blotted, and probed with anti-Myc antibody.

Scatter, Chemotaxis, and Wound Healing

For HGF-induced cell scatter, MDCK cells were plated at low density in 24-well plates, and they were allowed to growth in small colonies. Cells were stimulated in serum-free medium with 2 ng/ml HGF for 24 h, in presence or absence of 1 μM R59949, fixed with 3% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS), and then photographed with phase-contrast optics with a 20× objective (Carl Zeiss, Jena, Germany). For v-Src–induced cell scatter, MDCK-*ts*-v-Src cells were shifted to the permissive temperature of 35°C in 0% fetal bovine serum (FBS) medium for 24 h, in presence or absence of 1 μM R59949.

Chemotaxis assay was performed in a NeuroProbe standard 48-well chemotaxis chamber according to manufacturer's instructions (NeuroProbe, Gaithersburg, MD). Briefly, the bottom chamber was filled with serum-free DMEM containing 50 ng/ml HGF as chemoattractant, in presence or absence of 1 μM R59949. Cells (10^5) were seeded in the upper chamber and let migrate overnight through a polycarbonate filter coated with 0.1% gelatin. Migrated cells were fixed and stained with Diff-Quick (Dade Behring, Deerfield, IL) before counting.

In wound healing assay, cells grown to confluence were scratched using a pipette tip. Cells were then allowed to migrate into the wound for 7 h in serum-free medium containing 2.5 ng/ml HGF, in presence or in absence of 1 μM R59949, and then they were photographed with phase-contrast optics with a 20× objective (Carl Zeiss). Migration was quantified by calculating the area of wound at time points t_0 (time of wound) and 7h (7 h after wound). Normalization was obtained by the formula $[\text{area}(t_0) - \text{area}(7h)]/\text{area}(t_0)$.

Invasion

Invasion assays were performed in serum-free medium in 6.5-mm Transwells with 8-μm pore size membranes. The Transwell membrane was precoated with 10 μg of Matrigel (BD Biosciences, San Jose, CA) in 50 μl of cold serum-free medium and dried overnight at room temperature. Cells (10^5) were seeded in the upper chamber of the Transwell apparatus. The lower chamber was filled with DMEM and 2% FBS in presence or absence of 100 ng/ml HGF, and cells were allowed to migrate for 48 h. After washing with PBS, the cells on the upper surface of the Transwell membrane were removed using a cotton-tipped swab, whereas those cells onto the lower surface were fixed in glutaraldehyde and stained with crystal violet. Fixed cells were then photographed, and invasion was quantified by optical densitometry.

Immunofluorescence

MDCK cells were seeded in small colonies on glass coverslips (Marienfeld, Lauda-Königshofen, Germany) in 24-well cell culture plates. Cells were overnight starved and then stimulated with 10 ng/ml HGF for the indicated times. R59949 (1 μM) was given as pretreatment in short-time HGF experiments (15 min), whereas in long-time experiments (from 4 h onward), it was given together with stimulus. After stimulation, cells were washed twice in PBS and fixed by incubation with PBS 3% paraformaldheyde-4% sucrose. Cells were then permeabilized in cold HEPES-Triton buffer (20 mM HEPES, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100), washed with PBS containing 0.2% bovine serum albumin (BSA), and incubated for 15 min with PBS containing 2% BSA. Then, 15 μl of primary antibody (1:100 in PBS containing 2% BSA) was added directly onto each glass coverslip in a humidified chamber for 30 min, and excess antibody was washed away with PBS containing 0.2% BSA. Cells were then incubated for an additional 15 min with PBS containing 2% BSA and FITC-/TRITC-conjugated secondary antibodies and/or Alexa Fluor 546/633-phalloidin (1:30 and 1:200 in PBS containing 2% BSA, respectively) was added for 30 min in the humidified chamber. After washes, each glass coverslip was washed briefly in water and blocked onto a glass microscope slide by Mowiol (20% Mowiol 4-88, 2.5% 1,4-diazabicyclo[2.2.2]octane in PBS, pH 7.4) and let polymerize. Confocal images were acquired with the Leica confocal microscopy TSP2 and LCS Leica confocal software (Leica, Wetzlar, Germany). Basal planes are shown.

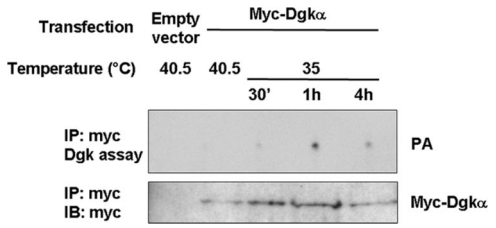

Figure 1. v-Src activates Dgkα. MDCK-*ts*-v-Src maintained at the nonpermissive temperature of 40.5°C were transiently transfected with Myc-Dgkα, starved overnight in 0% FBS medium, and shifted to the permissive temperature of 35°C for the times indicated. Cell lysates were immunoprecipitated with an anti-Myc antibody. Half of each immunoprecipitate was separated by SDS-PAGE and after blotting it was probed with anti-Myc; the other half was assayed for Dgk activity as described in *Materials and Methods*.

Western Blotting and Cell Fractionation

Cell lysates were prepared after cold PBS washing by scraping on ice in lysis buffer (25 mM HEPES, pH 8, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 10% glycerol supplemented with fresh 1 mM Na_3VO_4, and protease inhibitors [Protease Inhibitors Cocktail; Sigma-Aldrich]). Clarified lysates were denatured by boiling in Laemmli buffer for direct Western blotting.

Detergent-soluble and insoluble fractions were obtained according to Potempa and Ridley (1998). Briefly, cells were lysed in NP-40 buffer (25 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 1% NP-40, 4 mM EDTA, 25 mM NaF, 10% glycerol supplemented with fresh 1 mM Na_3VO_4, and protease inhibitors) for 30 min on a rotating wheel at 4°C. The lysates were centrifuged at 10,000*g* for 30 min, and the supernatant was collected as the NP-40–soluble fraction (S). The pellet was resuspended in 100 μl of 25 mM HEPES, pH 7.5, 4 mM EDTA, 25 mM NaF, 1% SDS, and 1 mM Na_3VO_4. After addition of 900 μl of the NP-40 buffer, the homogenate was passed 10 times through a 27-gauge needle and left for 30 min on a rotating wheel at 4°C. The lysates were then centrifuged at 10,000*g* for 30 min, and the supernatant was collected as the NP-40–insoluble fraction (I). Equal sample volumes were loaded for SDS-PAGE.

RacGTP Pull-Down Assay

RacGTP pull-down assays were performed according to Zondag *et al*. (2000). Briefly, MDCK cells were seeded in 15-cm-diameter cell culture plates and overnight starved in 0% FBS medium before stimulation with 100 ng/ml HGF for 15 min. R59949 (1 μM), when used, was added with a 30 min pretreatment and maintained during the subsequent HGF stimulation. Cells were then washed in ice-cold PBS and lysed with glutathione transferase (GST)-fish buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% glycerol, 0.1% Triton X-100 supplemented with fresh 1 mM Na_3VO_4, protease inhibitors, and 1 mM dithiothreitol) and harvested by scraping. The clarified lysates were incubated for 45 min with purified GST-PAK-BD at 4°C, precoupled to glutathione-Sepharose beads (GE Healthcare). After three washes with GST-fish buffer, samples were resuspended in Laemmli buffer, heat-denatured, and separated by SDS-PAGE in a 12% polyacrylamide gel. A small amount of each sample was directly denatured in Laemmli buffer for whole cell lysate proteins analysis.

Statistical Analysis

At least triplicates were analyzed when quantification was performed. Couples of conditions were compared using Student's *t* test. Histograms represent means ± SEs.

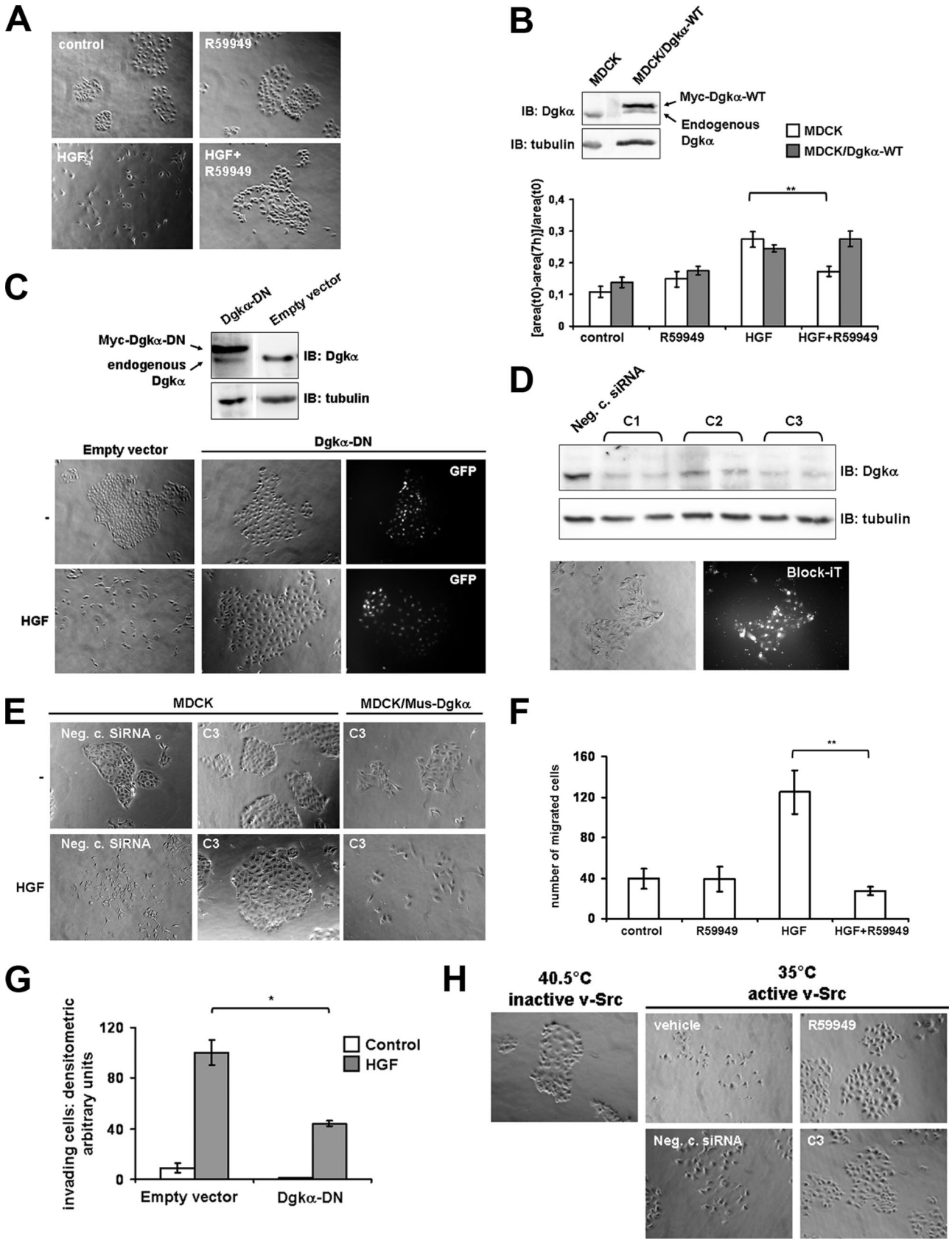

Figure 2. Dgkα is required for HGF-induced cell scatter and migration of MDCK cells. (A) MDCK cell colonies were treated, in 0% FBS medium, with 2 ng/ml HGF in presence or absence of 1 μM R59949 for 24 h. Representative fields are shown. (B) Control or MDCK/Dgkα-WT cells were allowed to migrate into the wounded area in 0% FBS medium with 2.5 ng/ml HGF in presence or absence of 1 μM

RESULTS

Dgkα Activation Mediates HGF-induced Scatter and Migration of MDCK Cells

We showed previously that activation of Dgkα in endothelial cells is required for VEGF and HGF-induced chemotaxis (Cutrupi *et al*., 2000; Baldanzi *et al*., 2004). However, the role of Dgkα in epithelial cell scattering has never been investigated, as well as the signaling pathways involved.

MDCK cells express endogenous Dgkα and feature an R59949-sensitive Dgk activity associated to anti-phosphotyrosine immunoprecipitates upon HGF stimulation (data not shown). On v-Src activation, obtained by shifting MDCK-*ts*-v-Src cells to the permissive temperature, Dgkα is activated in a time-dependent manner, reaching a maximum activity after 1 h (Figure 1). Activation of Dgkα by v-Src was evaluated by assaying Dgk activity in anti-Myc immunoprecipitates of MDCK-*ts*-v-Src cells transiently transfected with Myc-Dgkα. Similarly, Myc-Dgkα was also activated by HGF in MDCK cells (data not shown), as reported previously in endothelial cells (Cutrupi *et al*., 2000).

MDCK cells form discrete compact colonies that, upon either HGF stimulation or v-Src activation, undergo scatter, which involves cell spreading, dissolution of intercellular adhesions and migration of cells away from one another (Beherens *et al*., 1993; Weidner *et al*., 1993; Palacios and D'Souza-Schorey, 2003).

To investigate the role of Dgkα in cell scattering and migration, Dgkα activity was inhibited in MDCK cells by R59949, a pharmacological isoform-specific Dgk inhibitor. Cell treatment with 1 μM R59949 (Figure 2A), severely impair HGF-induced cell scatter. The specificity of Dgkα inhibition by R59949 cell treatment was verified in a wound healing assay. Indeed, overexpression of Dgkα in MDCK cells fully reestablishes HGF-induced cell migration even in presence of 1 μM R59949 (Figure 2B).

Figure 2 (cont). R59949 for 7 h. Quantification was performed as described in *Materials and Methods*. Means of at least four experiments with SEs are shown. **p < 0.005. The Western blot shows the level of Myc-Dgkα-WT expression. (C) MDCK/empty vector or MDCK/Dgkα-DN were treated, in 0% FBS medium, with 2 ng/ml HGF for 24 h. Representative fields are shown. The Western blot shows the level of Myc-Dgkα-DN expression. (D) Lysates of MDCK cells transiently transfected with negative control siRNA or canine Dgkα siRNAs C1, C2, and C3 were separated by SDS-PAGE and after blotting they were probed for Dgkα and tubulin. MDCK cell colonies were transfected with BLOCK-iT Fluorescent siRNA to evaluate the efficiency of transfection. (E) MDCK and MDCK/Mus-Dkgα cell colonies were transiently transfected with negative control siRNA or canine Dgkα siRNAs and treated, in 0% FBS medium, with 2 ng/ml HGF for 24 h. Representative fields are shown. (F) MDCK cells were seeded in the top part of a chemotaxis chamber and induced to migrate in presence of 50 ng/ml HGF in the bottom part, in presence or absence of 1 μM R59949. The histograms represent the number of migrated cells, means of eight different wells with SEs. **p < 0.005. A representative experiment is shown. (G) MDCK/empty vector or MDCK/Dgkα-DN cells were seeded in the top chamber of a Transwell apparatus. Invasion through a Matrigel-covered porous membrane was induced in 48 h in 2% FBS medium by the presence of 100 ng/ml HGF in the bottom chamber. Fixed cells on the Transwells lower surface were stained with crystal violet, photographed, and quantified by optical densitometry. Means of three experiments are shown, with SEs; *p ≤ 0.05. (H) MDCK-*ts*-v-Src cell colonies, maintained at the nonpermissive temperature of 40.5°C, were transiently transfected with negative control siRNA or canine Dgkα siRNAs, as indicated, placed in 0% FBS medium, and shifted to the permissive temperature of 35°C for 24 h. The same experiment was performed with untransfected cells, in presence or absence of 1 μM R59949, as indicated. Representative pictures are shown.

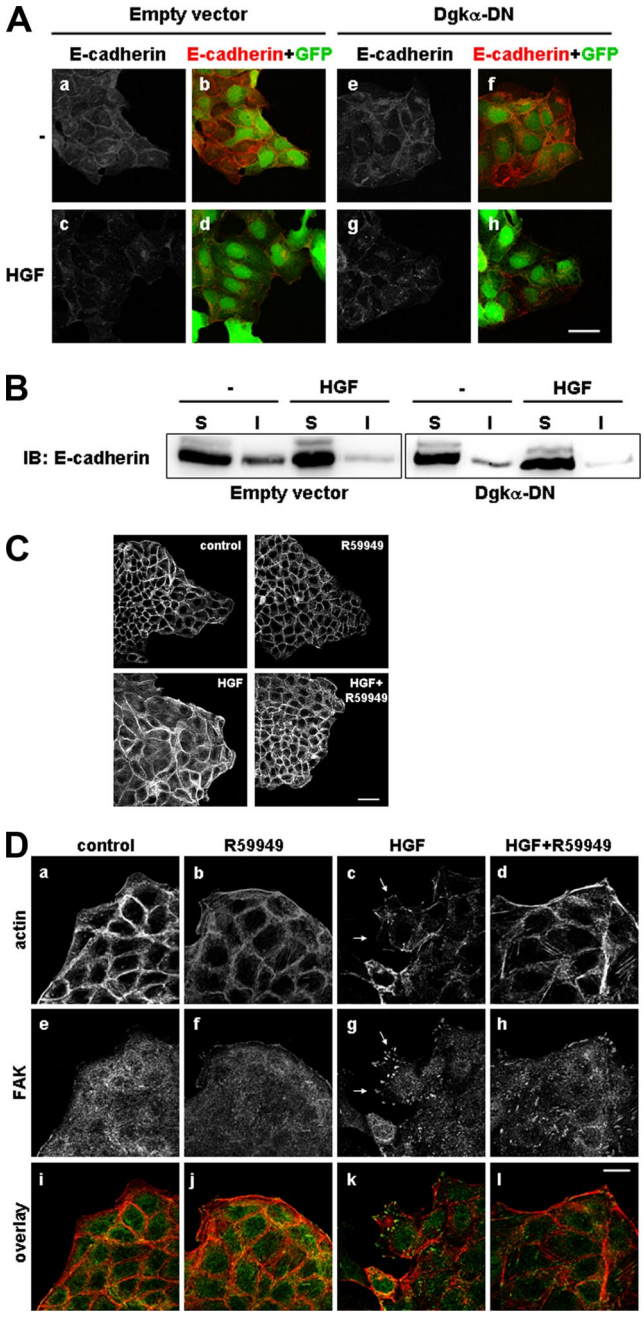

Figure 3. Dgkα is required for HGF-induced cell spreading and lamellipodia formation, but not for down-regulation of E-cadherin–mediated intercellular adhesions. (A) MDCK/empty vector or MDCK/Dgkα-DN cells were treated with 10 ng/ml HGF for 6 h, fixed, and stained for E-cadherin. Representative pictures are shown. Bar, 20 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were treated with 10 ng/ml HGF for 6 h. Cell lysates were fractionated into an NP-40–soluble (S) and a NP-40–insoluble (I) fraction. Equal sample volumes were loaded, separated by SDS-PAGE, and probed for E-cadherin. (C) MDCK cell colonies were starved overnight in a 2% FBS medium and treated with 10 ng/ml HGF for 4 h, in presence or absence of 1 μM R59949. Fixed cells were stained for actin filaments with phalloidin. Bar, 40 μm. (D) MDCK cells treated as described in C were fixed and stained for actin (red, a–d) and FAK (green, e–h). Bar, 16 μm. Representative pictures are shown.

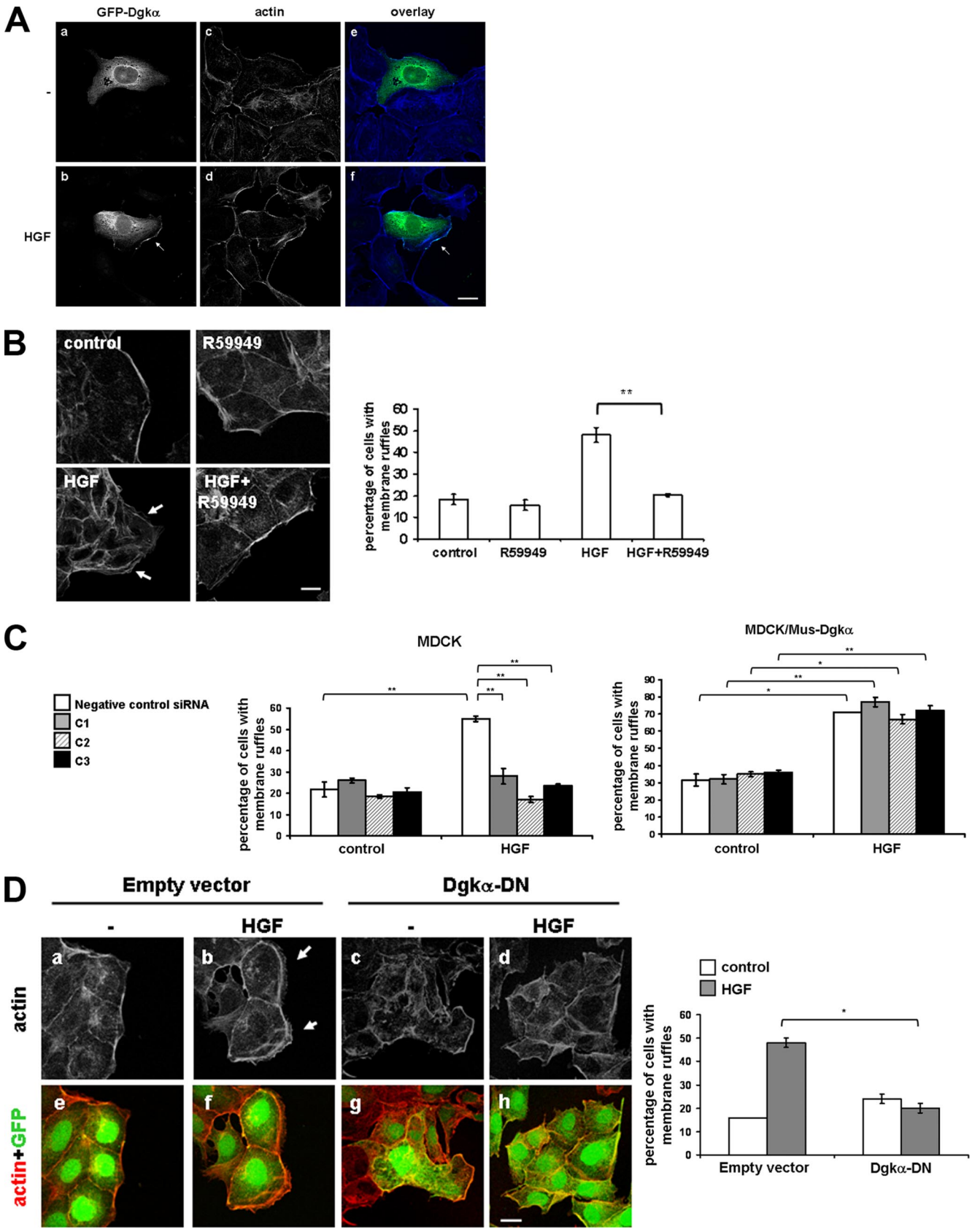

Figure 4. Dgkα is required for HGF-induced membrane ruffling of MDCK cells. (A) MDCK cells were transiently transfected with GFP-Dgkα, starved overnight in 0% FBS medium, stimulated with 10 ng/ml HGF for 15 min, fixed, and stained for actin. Bar, 8 μm. (B) MDCK cell colonies were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, fixed, and stained for actin. Bar, 16 μm. Confocal acquired images were observed and cells at the edge of colonies were scored for

Moreover, HGF-induced cell scatter was also impaired by stable expression of Dgkα kinase-defective mutant, acting as dominant negative (Dgkα-DN) (Figure 2C). About 80% of cells were infected with PINCOS/Dgkα-DN, as measured by FACS analysis (data not shown) and as shown in GFP panels; global overexpression of Dgkα-DN is shown by Western blot (Figure 2C).

To further verify the specificity of Dgkα requirement in HGF-induced cell scatter, the endogenous protein was down-regulated by transient transfection of specific siRNAs. Three siRNAs were designed (C1, C2, and C3), transiently transfected in MDCK cells, and they proved to be effective in knocking down canine Dgkα, as verified by Western blot; negative control siRNA does not affect Dgkα expression (Figure 2D). Transfection of MDCK cells, with the same conditions, with BLOCK-iT Fluorescent Oligo demonstrates that the efficiency of siRNA internalization into MDCK cells is near to 100% (Figure 2D). Similarly to R59949 treatment and expression of Dgkα-DN, C3 siRNA-mediated down-regulation of endogenous Dgkα inhibits HGF-induced MDCK cell scatter (Figure 2E). Similar results were obtained with C1 and C2 (data not shown). To provide further evidence of the specificity of Dgkα requirement for cell scatter, we generated MDCK cells stably expressing murine Dgkα, whose expression is not affected by any of three siRNAs directed against the canine hortologue (MDCK/Mus-Dgkα). Indeed, transient transfection of C3 (Figure 2E), C1 or C2 (data not shown) in these cells does not affect HGF-induced cell scatter.

We further verified that Dgkα is required for HGF-induced cell migration in a quantitative chemotaxis assay. Indeed, 1 μM R59949 abolishes HGF-induced chemotaxis of MDCK cells toward the HGF-filled lower chamber (Figure 2F), whereas it does not affect cell basal migration.

A motile phenotype is essential also for the acquired ability of scattering MDCK cells to invade the extracellular matrix, a typical feature of metastatic carcinoma. Thus, we verified the role of Dgkα in HGF-induced invasion of MDCK cells through a Matrigel barrier, a common assay to investigate the signaling pathways leading to metastatic progression (Birchmeier *et al*., 2003). Indeed, inhibition of Dgkα by expression of Dgkα-DN, strongly impairs HGF-induced in vitro invasiveness of MDCK cells (Figure 2G).

Similarly to HGF-induced cell scatter, inhibition of Dgkα, either by R59949 treatment or down-regulation of the endogenous protein by C3 siRNA, strongly impairs MDCK cell scattering induced upon *ts*-v-Src activation (Figure 2H). Similar results were obtained with C1 and C2 siRNAs (data not shown).

Dgkα Inhibition Uncouples Spreading, Cytoskeletal Remodeling, and Lamellipodia Formation from Down-Regulation of E-Cadherin–mediated Intercellular Adhesions

In HGF-induced cell scattering, loss of cell–cell contacts is preceded by internalization of E-cadherins at 4–6 h from HGF stimulation (Beherens *et al*., 1993; Potempa and Ridley, 1998; Kimura *et al*., 2006), which occurs concomitantly to colony spreading, so that the area covered by each colony increases two- to threefold. At the same time, cells at the colony outer edge undergo dramatic morphological changes, featuring extended lamellipodia, where focal adhesion proteins, such as paxillin and FAK, are recruited at new sites of adhesion and at the tips of stress fibers (Weidner *et al*., 1993; Ridley *et al*., 1995; Palacios and D'Souza-Schorey, 2003).

We observed that inhibition of Dgkα, either by 1 μM R59949 treatment (data not shown) or by expression of Dgkα-DN, does not affect the HGF-induced internalization and removal of E-cadherins from cell–cell contacts (Figure 3Ag), occurring upon 6 h of cell stimulation. In addition, we performed fractionation of MDCK cell lysates in NP-40–soluble and NP-40–insoluble fractions. On 6 h of treatment, HGF induces a decrease in the amount of E-cadherin in the insoluble fraction, independently from Dgkα-DN expression (Figure 3B).

Conversely, inhibition of Dgkα by 1 μM R59949 results in a remarkable reduction of HGF-induced colony spreading upon 4 h of cell stimulation (Figure 3C). Moreover, staining for F-actin clearly shows that Dgkα inhibition strongly affects HGF-dependent morphological changes such as lamellipodia formation (Figure 3Da–d). Consistently with inhibition of lamellipodia formation, R59949 treatment severely affects HGF-induced remodeling of focal adhesions spatial organization, as visualized by staining for FAK (Figure 3De–h). Inhibition of Dgkα in unstimulated MDCK cells does not affect their morphology concerning all of the analyzed aspects.

These data strongly suggest that Dgkα is not involved in the mechanisms by which HGF down-regulates E-cadherin–mediated intercellular adhesions and that its inhibition uncouples HGF-induced events, leading to loss of intercellular adhesions from the signaling pathways mediating cell spreading, F-actin remodeling, lamellipodia formation, and eventually cell migration.

Dgkα Is Required for HGF-induced Membrane Ruffle Formation and Focal Adhesions Remodeling

On few minutes of HGF stimulation, MDCK cells at the outer edge of colonies undergo intense ruffling. They eject small membrane protrusions, whose formation relies on regulated recruitment of molecular scaffolds to growing focal complexes at new adhesion sites, coupled to the coordinated organization of actin filaments into lamella network and bundled arrays. Eventually, membrane ruffles evolve in wider lamellipodia driving and providing direction to cell migration (Small *et al*., 2002). Thus, we verified whether the effects of Dgkα inhibition observed after hours of HGF stimulation derived from impairment of events occurring at earlier time points, such as formation of membrane ruffles and new focal complexes.

We ascertained Dgkα localization in resting or HGF-treated MDCK cell by transiently transfecting a GFP-Dgkα fusion protein. In untreated cells Dgkα displays cytoplasmic localization, but upon 15 min of HGF treatment it accumu-

Figure 4 (cont). presence of membrane ruffles (arrows). The percentage of cells with membrane ruffles was calculated. Means of three experiments with SEs are shown. $^{**}p < 0.005$. (C) MDCK and MDCK/Mus-Dgkα were transiently transfected with negative control siRNA or canine Dgkα siRNAs C1, C2, or C3, starved overnight in 0% FBS medium, and treated with 10 ng/ml HGF for 15 min. Confocal acquired images were observed, and cells at the edge of colonies were scored for presence of membrane ruffles. The percentage of cells with membrane ruffles was calculated. Means of three experiments with SEs are shown. $^{*}p < 0.05$; $^{**}p < 0.005$. (D) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min, fixed, and immunostained for actin (red, a–d). The arrows indicate membrane ruffles in empty vector-infected cells. Bar, 16 μm. Representative pictures are shown. Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles. Means of three experiments with SEs are presented. $^{*}p < 0.05$.

lates at the cell periphery, in correspondence of the protruding plasma membrane (Figure 4A). This observation suggests that Dgkα may play a role in HGF-induced earlier events leading to membrane ruffle formation.

Thus, we set out to investigate earlier changes in F-actin cytoskeleton organization in response to HGF. On 15 min of HGF treatment, small membrane ruffles develop on the outer membranes of cells at colony edge (Figure 4B, arrows). The percentage of cells featuring membrane ruffles raises from <20% in control cells (vehicle- and R59949-treated cells) to ~50% in HGF-treated cells. In presence of 1 μM R59949, the percentage of membrane ruffle-displaying cells upon HGF stimulation is reduced to almost the control value (Figure 4B). To further verify the specificity of Dgkα requirement in HGF-induced ruffle formation, we showed that transient transfection of either C1, C2, or C3 siRNA impairs HGF-induced membrane ruffling and that this inhibition is completely overridden by the expression of the Dgkα murine hortologue, which is not affected by any of three siRNA (Figure 4C). Consistently, HGF fails to induce membrane ruffles in cells expressing Dgkα-DN compared with cells expressing the vector alone (Figure 4D). In conclusion, these data demonstrate that the formation of membrane ruffles occurring upon 15 min of HGF treatment depends on stimulation of Dgkα activity.

Membrane ruffle formation implies the recruitment of focal adhesion proteins at new adhesion sites within the ruffle itself. In epithelial cells, Paxillin recruitment to newly formed focal complexes, where it acts as a scaffold for signaling molecules, is required for HGF-induced signaling leading to cell migration (Lamorte *et al*., 2003; Ishibe *et al*., 2004; Chen *et al*., 2005).

In resting MDCK cells, paxillin is partially diffuse in the cytoplasm, whereas in cells at colony edge it is also localized in focal adhesions along the outer plasma membrane (Figure 5, Aa and Ba). On 15 min of HGF stimulation, paxillin condensates to the newly formed focal complexes in correspondence of membrane ruffles (Figure 5Ac and Bb). On inhibition of Dgkα by either 1 μM R59949 (Figure 5Ad) or by expression of Dgkα-DN (Figure 5Bf), Paxillin accumulates along the outer plasma membrane instead of being recruited in the area of ruffling, whereas ruffle formation is impaired. Inhibition of Dgkα in unstimulated cells does not significantly affect paxillin localization either in the cytoplasm or at focal adhesions along the outer plasma membrane.

To verify that paxillin indeed accumulates in structures identifiable as focal complexes, we analyzed its colocalization with vinculin, a resident protein whose function is to stabilize them (Ziegler *et al*., 2006). In unstimulated cells vinculin and paxillin colocalize at focal complexes along the outer plasma membrane of colony-edge cells, and upon HGF stimulation they are both recruited to newly formed focal complexes in the area of ruffling, in a manner fully dependent on Dgkα activity. In fact, inhibition of Dgkα, although impairing HGF-induced neoformation of ruffles and focal complexes at membrane ruffles, does not affect vinculin and paxillin colocalization (Figure 5C).

On growth factor stimulation Src- and FAK-mediated phosphorylation of paxillin is required to recruit and coordinate multiple signaling complexes, regulating events at the leading edge of migrating cells (for review, see Brown and Turner, 2004). Phosphorylation of paxillin on tyrosine 31 and 118 mediates its association with Crk, and it is required for growth factor-induced paxillin-mediated migratory signals (Nakamura *et al*., 2000; Petit *et al*., 2000). Thus, we verified whether inhibition of Dgkα affects HGF-induced phosphorylation of paxillin Tyr^{31} and Tyr^{118}, identified by anti-phosphotyrosine–specific antibodies. Western blot analysis of paxillin tyrosine phosphorylation reveals that HGF induces paxillin phosphorylation of both Tyr^{31} and Tyr^{118} in control MDCK cells (Figure 5D). Surprisingly, basal phosphorylation of paxillin in both residues is enhanced in cells expressing Dgkα-DN, and it is not further affected by HGF stimulation (Figure 5D).

In summary, these data demonstrate that upon minutes of HGF stimulation, activation of Dgkα is required for the formation of membrane ruffles and for the succeeding remodeling of paxillin- and vinculin-containing focal complexes.

Dgkα Is Required for HGF-induced Rac Activation and Membrane Targeting

The data presented above strongly suggest that activation of Dgkα is involved in the signaling mechanisms leading from HGF-receptor activation to ruffle formation.

Membrane ruffle formation is dependent on the activation of Rac small GTPase, which acts upstream of the recruitment of WAVE and Arp2/3 complexes at new adhesion sites promoting F-actin polymerization (Takenawa and Suetsugu, 2007). In migrating cells, active Rac localization at leading edge is enhanced and allows the coupling with its downstream effectors (Kurokawa and Matsuda, 2005). In MDCK cells, HGF activates Rac, whose function is required for HGF-induced cell scatter, spreading, and for ruffles and lamellipodia formation (Ridley *et al*., 1995; Royale *et al*., 2000).

Activation of endogenous Rac was assayed by GST-PAK pull-down to purify active GTP-bound Rac from lysates of either control or HGF-stimulated MDCK cells. HGF treatment results in activation of endogenous Rac. Inhibition of Dgkα, by either 1 μM R59949 or by Dgkα-DN expression, severely impairs HGF-induced Rac activation, without affecting Rac basal state of activation (Figure 6A). Rac activation requires the coordinated activity of its direct upstream regulators, which are recruited in multimolecular complexes at the cell leading edge. Because several Rac guanine nucleotide exchange factors (GEFs) are regulated through their pleckstrin homology domain by D-3 phosphoinositides (Welch *et al*., 2003), we verified whether inhibition of Dgkα affects the PI 3-kinase pathway, as measured by Akt phosphorylation. Indeed, HGF induces Akt phosphorylation in both control and Dgkα-DN–expressing MDCK cells (Figure 6B), demonstrating that Dgkα does not mediate Rac activation by regulating PI 3-kinase.

Rac activation is tightly coupled to its targeting to specific cholesterol-enriched membrane microdomains, defined by ligand-activated integrin signaling (Grande-Garcìa *et al*., 2005). Thus, we verified whether inhibition of Dgkα may interfere with HGF-induced targeting of Rac to the plasma membrane. By confocal microscopy, we observed the localization of endogenous Rac in MDCK cells (Figure 7A). In most unstimulated cells, Rac is both cytoplasmic and at intercellular contacts, whereas only ~20% of colony-edge cells feature Rac at the outer plasma membrane (Figure 7Aa). After 15 min of HGF stimulation, the percentage of colony-edge cells featuring Rac at the outer plasma membrane raises to >40% (Figure 7Ac), whereas localization of Rac at cell–cell contacts is not affected. Inhibition of Dgkα by 1 μM R59949 treatment abolishes HGF-induced Rac membrane targeting (Figure 7Ad), whereas it does not significantly affect Rac localization in unstimulated cells (Figure 7Ab) nor Rac localization at cell-cell contacts. Similar results were obtained when Dgkα was inhibited upon expression of Dgkα-DN (Figure 7B). On HGF stimulation Rac is properly membrane localized in cell infected with the empty vector (Figure 7Bb), whereas it

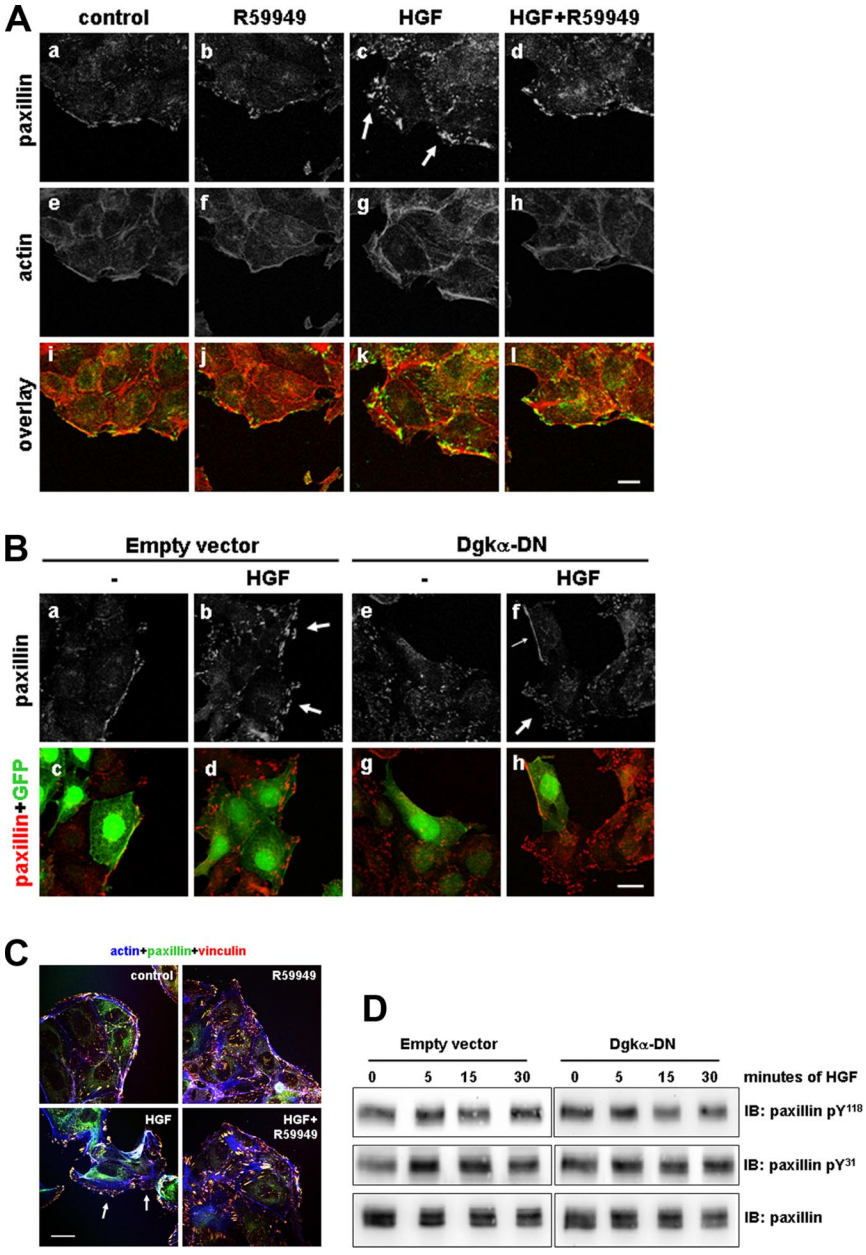

Figure 5. Dgkα is required for HGF-induced paxillin localization to newly formed focal complexes. (A) MDCK cell colonies were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, fixed, and stained for paxillin (green, a–d) and actin (red, e–h). Representative pictures are shown. Bar, 16 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min, fixed, and immunostained for paxillin (red, a, b, e, and f). Thick arrows indicate paxillin localization at focal adhesions in the areas of membrane ruffling, whereas the thin arrow indicates paxillin localization at cell periphery in a Dgkα-DN–infected cell, without membrane ruffles. Bar, 16 μm. Representative pictures are shown. (C) MDCK cell colonies were treated as described in A, fixed, and stained for paxillin (green), vinculin (red), and actin (blue). Representative pictures are shown. Bar, 16 μm. (D) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium and treated with 50 ng/ml HGF for 15 min. Whole cell lysates were separated by SDS-PAGE and probed with anti-paxillin pTyr[31] and pTyr[118] and anti-paxillin.

remains predominantly cytoplasmic in Dgkα-DN–expressing cells (Figure 7Bf).

In summary, these data demonstrate that Dgkα is required for HGF-induced activation and targeting of Rac to the plasma

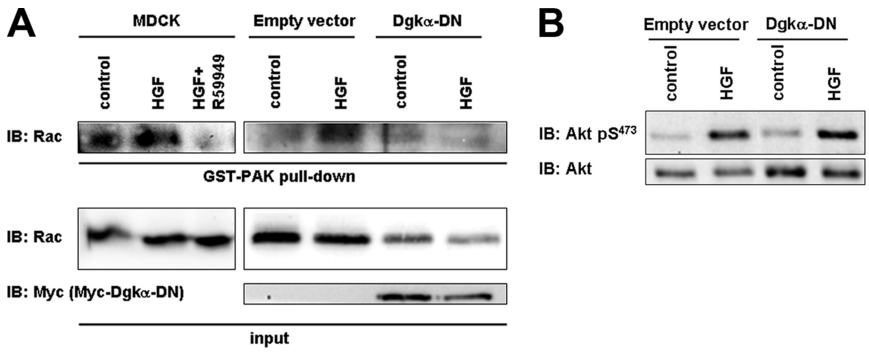

Figure 6. Dgkα is required for HGF-induced Rac activation. (A) MDCK cells were starved overnight in 0% FBS medium, treated with 100 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, and lysed. GTP-bound active Rac was purified in each sample by pull-down with GST-fused PAK CD domain. MDCK/empty vector and MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with 100 ng/ml HGF for 15 min, and pull-down assays were performed as described in text. (B) MDCK/empty vector and MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with 50 ng/ml HGF for 15 min, and lysed. Whole cell lysates were separated by SDS-PAGE and probed with anti-Akt pSer[473] and Akt.

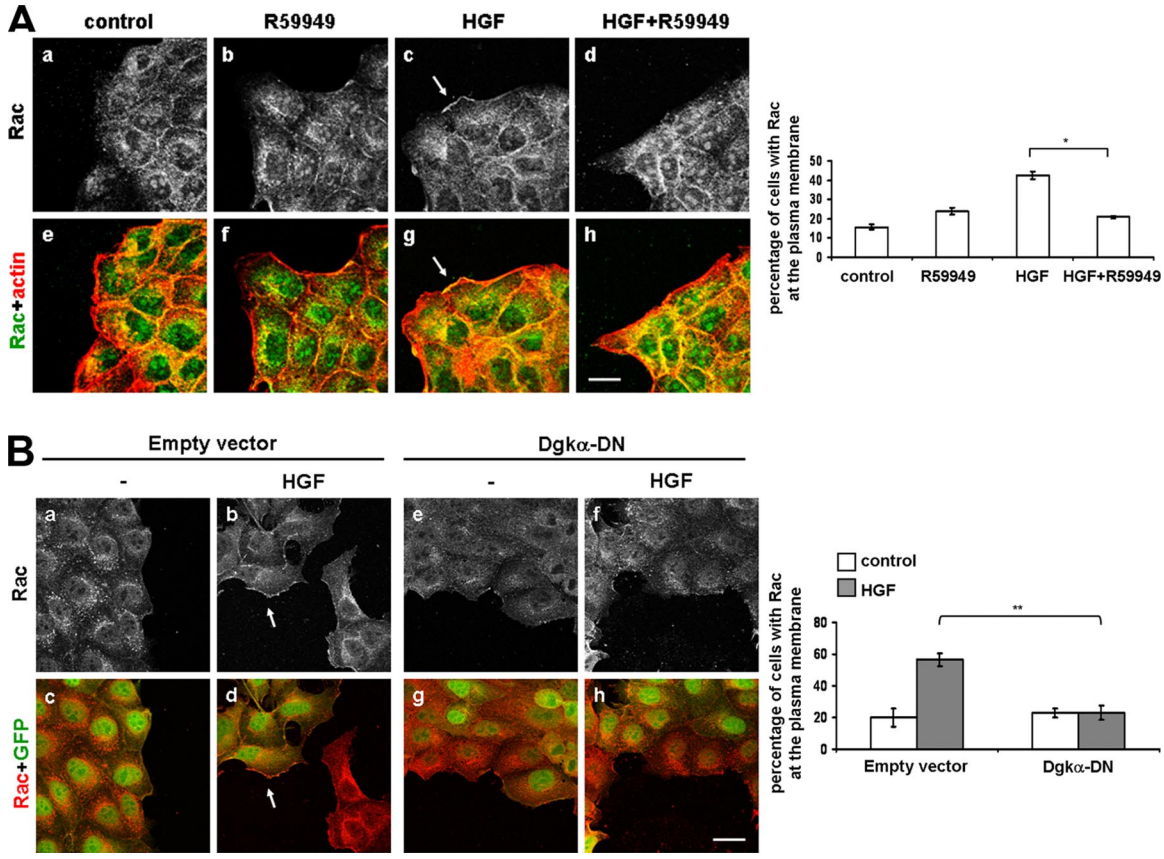

Figure 7. Dgkα is required for HGF-induced Rac localization to the plasma membrane. (A) MDCK cell colonies were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, fixed, and stained for Rac (green, a–d) and actin (red). Representative pictures are shown. Bar, 16 μm. Confocal acquired images were observed and the percentage of cells at the edge of colonies featuring Rac at the outer plasma membrane was calculated. Means of three experiments with SEs are shown. *$p < 0.05$. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min, fixed, and immunostained for Rac (red, a, b, e, and f). Representative pictures are shown. Bar, 20 μm. Confocal acquired images were observed, and the percentage of edge-of-colony cells featuring Rac at the outer plasma membrane was calculated. Means of three experiments with SEs are presented. **$p < 0.005$.

membrane and for the following formation of membrane ruffles, thus strongly suggesting that Dgkα is involved in the signaling pathways regulating Rac function and targeting upon activation of HGF receptor. These data demonstrate that Dgkα plays a pivotal role in the migratory signaling downstream HGF, being involved in early molecular events such as Rac activation, membrane ruffle protrusion, and formation and organization of new focal adhesions, and that it consequently regulates the acquisition of a migratory phenotype in epithelial cells.

DISCUSSION

In this study, we investigated the role of Dgkα in HGF- and v-Src-induced cell migration. We show that Dgkα-specific inhibition, obtained either pharmacologically, or by expression of a kinase-defective dominant-negative mutant, or by siRNA-mediated down-regulation of the endogenous protein, impairs both HGF- and v-Src-induced cell scatter and migration. This finding is consistent with previous demonstrations from our laboratory that Dgkα is activated by growth factors through a mechanism requiring its tyrosine phosphorylation mediated by Src family tyrosine kinases and that its function is required for migration of endothelial cells (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004, 2007; Bacchiocchi *et al.*, 2005). Moreover, these data suggest that Dgkα represents a crucial node in the signaling network downstream Src regulating epithelial cell scattering and switching to a motile mesenchymal phenotype.

Although both HGF stimulation and v-Src activation promote epithelial cell dispersion by coordinating loss of intercellular adhesions and migration of cells away from one another, the two events are regulated through distinct signaling pathways (Palacios *et al.*, 2001). Intriguingly, Dgkα inhibition uncouples the down-regulation of E-cadherin–mediated intercellular adhesions from cell migration, strongly suggesting that Dgkα may regulate specifically those signaling events required for HGF- and v-Src–stimulated epithelial cell motility. Thus, we investigated the role of Dgkα in well characterized HGF-induced morphological and molecular events leading to cell migration.

Spreading and lamellipodia protrusion with formation of new focal adhesions at the leading edge are mandatory steps in cell migration (Ridley *et al.*, 1995; Small *et al.*, 2002). We show herein that upon Dgkα inhibition, no cell spreading, lamellipodia extension, and remodeling of focal adhesions are observed upon HGF treatment, suggesting that activation of Dgkα is likely to be required for an earlier event. Rapid formation of membrane ruffles, upon minutes from growth factors stimulation, preludes to establishment of extended lamellipodia at the leading edge of migrating cells (Royale *et al.*, 2000). Indeed, upon inhibition of Dgkα, MDCK

cells fail to extend membrane ruffles after HGF stimulation. Intriguingly, although recent findings indicate that Dgkα is enriched in the pseudopodia of spontaneously invasive epithelial MSV-MDCK-INV cells (Jia *et al*., 2005), we show that Dgkα is recruited to membrane ruffles upon HGF treatment. Together, these data provide the first circumstantial evidence that Dgkα may act in growth factors signaling at the leading edge of migrating cells.

Ruffle formation, cell spreading, and lamellipodia protrusion are dependent on Rac small GTPase activation, occurring through its targeting to newly formed focal complexes (Ridley *et al*., 1995; Burridge and Wennerberg, 2004; Rossman *et al*., 2005). Rac targeting and GTP loading are regulated by a complex signaling network involving the recruitment of distinct Rac-regulating proteins to multiple molecular complexes at the leading edge of migrating cells.

An increasing body of evidence suggests that Dgks regulate small GTPases, including Rac, through multiple mechanisms, whose complexity still awaits elucidation. In T cells, Dgkα and -ζ negatively regulate Ras pathway, by finely tuning the access of RasGRP1, a C1 domain-containing Ras GEF, to its activator DG (Jones *et al*., 2002; Olenchock *et al*., 2006a; Topham and Prescott, 2001; Zha *et al*., 2006). However, in epithelial cells, neither the overexpression nor the down-regulation of Dgkα affects the Ras pathway, as detected by extracellular signal-regulated kinase-1/2 phosphorylation (our unpublished data). In addition Dgkγ, but not Dgkα, upon its recruitment to the plasma membrane, negatively regulates platelet-derived growth factor (PDGF)- and epidermal growth factor (EGF)-induced Rac activation and membrane ruffling, by enhancing the activity of β2-chimaerin, a Rac GAP containing a C1 and a Src homology 2 domain (Tsushima *et al*., 2004, Yasuda *et al*., 2007). These observations provide further support to the previous finding that DG-dependent membrane recruitment of β2-chiamerin determines the extent and the kinetic of EGF-induced Rac activation. (Wang *et al*., 2006). Conversely, in neurons and skeletal myoblasts Dgkζ acts in a complex with Rac at specific sites of the plasma membrane and controls the remodeling of F-actin cytoskeleton leading to neurite extension and membrane ruffle protrusion, possibly by facilitating Rac1 activation and/or localization to the cell surface (Abramovici *et al*., 2003; Yakubchyk *et al*., 2005). Furthermore Dgkζ and PI(4)P 5-kinase colocalize with F-actin at lamellipodia protrusions in epithelial cells (Luo *et al*., 2004), where Dgk-generated PA is required for full activation of PI(4)P 5-kinase activity, consistently with a role of both lipid kinases in positive regulation of Rac function. Interestingly a Dgk and a PI(4)P 5-kinase activities were found to associate in a complex with Rac and RhoGDI (Tolias *et al*., 1998). RhoGDI forms a complex with Rac, keeping it in a cytosolic inactive GDP-bound form, and upon Rac activation it contributes to Rac targeting to specific sites at the plasma membrane (Moissoglu *et al*., 2006). Because Rac targeting implies the displacement of the interaction between Rac and RhoGDI, the finding that in vitro PA and $PI(4,5)P_2$ impair RhoGDI affinity for Rac (Chuang *et al*., 1993; Ugolev *et al*., 2006) raises the hypothesis that activation of the RhoGDI-associated Dgk may allow the release of Rac from RhoGDI, and leads to speculation that Dgkα also may regulate Rac activation through this mechanism. Together, these data strongly indicate that distinct Dgk isoforms act as regulators of Rac membrane targeting and activation through multiple mechanisms, whose complexity awaits to be elucidated.

Several Rac GEFs, such as Vav2, DOCK180/Elmo, βPIX, and Tiam1, are regulated either directly or indirectly through Src-dependent tyrosine phosphorylation (Lamorte *et al*., 2002; Servitja *et al*., 2003; Santy *et al*., 2005), and/or interaction with phosphatidylinositol 3,4,5-trisphosphate (Welch *et al*., 2003). Although there is no direct evidence for a role of any Dgk isoforms in the regulation of any Rac GEFs, based on the observations reported herein, we may discuss several hypotheses, providing a framework for further investigation.

Several data indicate that, upon growth factors and v-Src stimulation, rapid Rac-mediated membrane ruffling occurs through the recruitment of βPIX to paxillin-containing focal complexes (Cotton *et al*., 2007). Indeed, βPIX mediates rapid ruffle formation upon PDGF, EGF, and fibroblast growth factor treatment in different cell types (Lee *et al*., 2001; Park *et al*., 2004; Shin *et al*., 2006), and the interaction between βPIX and Rac is necessary and sufficient for Rac recruitment to membrane ruffles and focal adhesions (ten Klooster *et al*., 2006). Crk recruitment to tyrosine-phosphorylated paxillin contributes to βPIX localization to focal complexes (Lamorte *et al*., 2003). Indeed, we show that HGF stimulates phosphorylation of paxillin on both Tyr^{31} and Tyr^{118}, the two major determinants for Crk association. Surprisingly, the inhibition of Dgkα enhances basal phosphorylation of Paxillin on both residues, but it does not affect their phosphorylation upon HGF stimulation, suggesting that Dgkα may affect βPIX function in a complex manner. Moreover, βPIX and Dgkα do not associate in a complex, not even upon HGF stimulation (data not shown).

On minutes of growth factors stimulation, βPIX recruitment and Rac activation are promoted by rapid GTP/GDP cycling of Arf6, suggesting that Arf6 plays a pivotal role in Rac-mediated membrane ruffling (ten Klooster *et al*., 2006; Cotton *et al*., 2007). Furthermore, upon hours of HGF stimulation, ARF6 has been recently shown to regulate Rac targeting at tubule tips of MDCK cells grown in 3D collagen (Tushir and D'Souza-Schorey, 2007). Interestingly, several Arf GAPs are regulated by phospholipids, including PA (Randazzo *et al*., 2000). Moreover, PLD-induced production of PA downstream of Arf6 is required for Arf6-dependent epithelial cell ruffling and migration (Santy and Casanova, 2001). Thus, we may speculate that also Dgkα may contribute to regulate Arf6 function in coordinating Rac activation, focal adhesions remodeling and membrane ruffle formation.

Several Rac and Arf GEFs are regulated by phosphatidylinositol 3,4,5-trisphosphate, the product of PI 3-kinase. However, we can rule out that Dgkα mediates Rac activation by regulating phosphatidylinositol trisphosphate (PIP_3) synthesis, because inhibition of Dgkα does not affect HGF-induced activation of Akt, a major PIP_3 target. Conversely, the finding that PIP_3 might contribute to recruit and activate Dgkα (Ciprés *et al*., 2003) allows speculation that Dgkα might contribute to couple PIP_3 generation to the activation of one of the PIP_3-dependent Rac GEFs, such as Vav2 and Tiam1. However, the expression of a either wild-type or kinase-defective Dgkα in fibroblasts does not affect PDGF-induced Rac activation and ruffle formation (Tsushima *et al*., 2004), both mediated by Vav2 (Liu and Burridge, 2000). Moreover, the Rac GEF Tiam1 is mainly involved in maintaining E-cadherin–mediated epithelial cell–cell adhesions (Mertens *et al*., 2003), events that we showed to not be regulated by Dgkα, making Tiam1 an unlike target of Dgkα activity.

In conclusion, herein we clearly demonstrate that activation of Dgkα is required for HGF- and v-Src-induced cell migration. By exploring some significant molecular events affected by Dgkα inhibition, we raise the hypothesis that Dgkα may act in growth factors migratory signaling by mediating Rac targeting and activation, thus revealing a

novel signaling pathway linking tyrosine-kinase receptors and Src to small GTPases in the context of cell migration.

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