Scrib regulates PAK activity during the cell migration process

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Received June 5, 2008; Revised and Accepted August 15, 2008

Genetic studies have highlighted the key role of Scrib in the development of Metazoans. Deficiency in Scrib impairs many aspects of cell polarity and cell movement although the mechanisms involved remain unclear. In mammals, Scrib belongs to a protein complex containing βPIX, an exchange factor for Rac/Cdc42, and GIT1, a GTPase activating protein for ARF6 implicated in receptor recycling and exocytosis. Here we show that the Scrib complex associates with PAK, a serine–threonine kinase family crucial for cell migration. PAK colocalizes with members of the Scrib complex at the leading edge of heregulin-treated T47D breast cancer cells. We demonstrate that the Scrib complex is required for epithelial cells and primary mouse embryonic fibroblasts to efficiently respond to chemoattractant cues. In Scrib-deficient cells, the pool of cortical PAK is decreased, thereby precluding its proper activation by Rac. Loss of Scrib also impairs the polarized distribution of active Rac at the leading edge and compromises the regulated activation of the GTPase in T47D cells and mouse embryonic fibroblasts. These data underscore the role of Scrib in cell migration and show the strong impact of Scrib in the function of PAK and Rac, two key molecules implicated in this process.

INTRODUCTION

Scrib is a cytoplasmic multimodular protein targeted to epithelial adherens junctions and neuronal presynaptic compartments in mammals (1–3). The Drosophila homologue of Scrib (Scribble) is located at the basolateral membrane and the septate junctions (homologues of mammalian tight junctions) of epithelia, and is also found at synapses (4,5). Scrib belongs to the evolutionarily conserved LAP protein family that comprises Scrib, Erbin, Densin-180 and Lano in mammals (6).

LAP proteins contain 16 N-terminal Leucine Rich Repeats (LRR), two LAP specific domains (LAPSD) and one or four PDZ domains in the C-terminal position. Lano has no PDZ domain but is able to interact with PDZ proteins through its C-terminal sequence (7). LRR and PDZ domains are largely distributed in genomes and act as protein interaction domains for a wide range of ligands (8,9). Several interactors...
have been identified for the PDZ domains of LAP proteins. For example, the PDZ domains of Scrib bind to βPIX and ZO-2 while Erbin is associated with ErbB2 and members of the p120-catenin family through a PDZ domain interaction (1,10,11). Studies in invertebrates and vertebrates demonstrated the importance of the LRR domains to retain the LAP proteins at the basolateral membrane (12–14). Recent reports described Sur-8 and Lgl as potential ligands for the LRR domains of Erbin and Scrib, respectively (15,16).

Genetic studies in invertebrates and vertebrates have shown that Scrib and Scribble, as well as LET-413, the worm homologue of Erbin, play a major role in embryonic development and participate in cell polarization of epithelial tissues (4,17,18). Mutant mice lacking Scrib (circletail mutant mice) develop abnormal planar cell polarity. They also show defects in the orientation of the stereociliary bundles in the cochlea (19), and impaired convergent extension, a process required for proper gastrulation and neurulation (17,20). Loss of Scrib also provokes major defects in neural migration and convergent extension movements in zebrafish embryos (17,20). In Drasophila, scribble null mutants present disorganization of simple epidermis such as epidermis, ovarian follicles and imaginal discs (4,21). This results in neoplastic growth and multilayering of epithelial cells. scribble is genetically linked to discs large (dlg) and lethal giant larvae (lgl), two other neoplastic tumor suppressing genes (4). There is some evidence that Scrib participates in oncogenic processes in humans. Indeed, Scrib interacts with the E6 oncoprotein encoded by High Risk Human Papillomaviruses type 16 (HPV16), and promotes its oncogenic activity (22,23). In addition, loss of Scrib is correlated with loss of E-cadherin in a subset of breast cancers, suggesting a tumor suppressing role in humans (2). Interestingly, knockdown of Scrib in Madin-Darby Canine Kidney (MDCK) epithelial cells impairs E-cadherin-mediated cell adhesion (24).

A better understanding of Scrib-associated signaling pathways is needed to reveal how Scrib acts at the molecular level. We previously identified βPIX as a partner for the PDZ domains of Scrib (1). βPIX is a guanine nucleotide exchange factor (GEF) for Rac and Cdc42, and is tightly associated to GIT1, a GTPase activating protein (GAP) for ARF small GTPases (25,26). The Scrib-βPIX-GIT1 protein complex, hereafter called ‘the Scrib complex’, is implicated in vesicle trafficking in neurons and receptor recycling in thyroid cells (1,27).

We show here that PAK (p21-activated kinase) proteins are binding partners for the Scrib complex. This protein complex is located at the leading edge of migratory epithelial cells, where it promotes actin polymerization and contributes to cytoskeleton reorganization. PAKs exhibit oncogenic and pro-migratory properties (28) and are involved, with βPIX and GIT1, in focal adhesion dynamics (29,30) and centrosome maturation (31). While PAK activity is regulated by Rac and Cdc42 GTPases, other mechanisms are required to achieve full enzymatic activity of the kinase. For example, localization of PAK by the βPIX/GIT1 protein complex in discrete subcellular compartments is a dominant process to regulate the kinase (32–34). Furthermore, the enzymatic activity of PAK is regulated by autophosphorylation (28), and by transphosphorylation on tandem serine or threonine residues present in the PAK activation loop (35,36).

Using dominant negative constructs and siRNA or shRNA that specifically knock-down expression of members of the Scrib complex as well as primary murine embryonic fibroblasts (MEF) deficient for Scrib, we demonstrate that Scrib is required for cell migration upon a chemoattractant cue. Scrib anchors βPIX and PAK at the front of motile cells and is required for PAK activity. Decrease of PAK activation is accompanied by a compromised regulation of Rac activity in Scrib-deficient cells. These data provide novel insights into the signaling machinery associated with Scrib, and highlight the central role of Scrib as a regulator of PAK and small GTPase activities at the plasma membrane of migrating cells.

RESULTS

PAK proteins associate to the Scrib complex

We previously demonstrated that Scrib interacts with the βPIX/GIT1 cytoplasmic complex and that this association regulates exocytosis in neuroendocrine cells (1) and G Protein-coupled-receptor trafficking in thyroid cells (27). βPIX (PAK-interacting exchange factor) is a GEF for Rac and Cdc42 which interacts with PAK (25), a serine–threonine kinase family implicated in cell migration (29,30). To test whether PAK proteins associate with the Scrib/βPIX/GIT1 complex, communoprecipitation assays were performed using MCF-10.2A (non-tumorigenic mammary epithelial cells) (Fig. 1A) and T47D (breast carcinoma cells) (Fig. 1B) cell lysates, and two different antibodies that precipitate Scrib (Scrib1 and Scrib2). Proteins present in the immunocomplexes were resolved by SDS–PAGE and subjected to western blotting analysis. As shown in Figure 1A, Scrib coimmunoprecipitates with PAK, GIT1 and βPIX but not with desmoplakin (DSP) used as a control. Scrib binds to two PAK isoforms, PAK1 (65 kD) and PAK2 (60 kD) recognized by the anti-PAK C antibody, that are present in MCF-10.2A and T47D cells. We also immunoprecipitated proteins extracted from T47D cells with antibodies directed against PAK2 or PAK1/PAK2 (PAK-N and PAK-C) (Fig. 1B). The anti-PAK1/PAK2 antibody efficiently immunoprecipitated βPIX and Scrib, while a longer exposure of the blot was needed to visualize Scrib in the anti-PAK2 immunoprecipitate (data not shown). In the same experiment, the anti-Scrib antibody immunoprecipitated again PAK1 and PAK2 (Fig. 1B). Association between the Scrib complex and PAK1/2 was also evidenced in primary mouse embryonic fibroblasts (MEF) where PAK1 is the major PAK isoform (Fig. 1C). The Scrib/βPIX/PAK complex is thus recovered in epithelial cells and in embryonic fibroblasts. PAK1 and PAK2 are part of this complex in a proportion that is relative to their abundance in cells.

βPIX scaffolds PAK and Scrib in a common protein complex

Mapping of the interaction between Scrib and PAK was first done by communoprecipitation using GFP-tagged Scrib constructs transiently expressed in T47D cells. Proteins were immunoprecipitated with anti-GFP antibody and were analyzed by western blot. As shown in Figure 2A, only constructs containing the PDZ domains of Scrib (GFP-Scrib and GFP-Scrib...
PDZ) were able to precipitate endogenous βPIX and PAK. As previously demonstrated, the βPIX C-terminal sequence containing the TNL motif directly interacts with the Scrib PDZ domains (1). On the other hand, the βPIX SH3 domain binds to a PAK proline-rich sequence (25). We evaluated the hypothesis that the interaction between Scrib and PAK is mediated by βPIX (Fig. 2B). To test this possibility, we added increasing amounts of a 15-mer soluble peptide mimicking the βPIX C-terminal motif (PIX peptide) to cell extracts before anti-Scrib immunoprecipitation (Fig. 2C). This peptide efficiently competed for the Scrib–βPIX–PAK interaction, whereas no competition was found with a control peptide (PIX peptide deleted of the C-terminal TNL motif) even at the highest concentration used for the PIX peptide (Fig. 2C). In the same manner, increasing concentrations of a soluble proline-rich PAK peptide (PAK peptide) reduced the amounts of βPIX and Scrib coprecipitated with PAK (Fig. 2D), suggesting that βPIX bridges Scrib to PAK.

To further confirm the key role of βPIX in the formation of the complex, we stably decreased the expression of βPIX in T47D cells using a specific βPIX shRNA construct. In total lysates, expression of βPIX was 70% lower than in cells transfected with a control Luciferase shRNA (shLuc) (Fig. 2E), whereas amounts of Scrib, PAK and GIT1 remained unchanged. Protein extracts were subjected to immunoprecipitation with anti-Scrib or control (anti-FLT3) antibodies and presence of Scrib, GIT1, βPIX and PAK were analyzed by western blot. Amounts of coprecipitated proteins were quantified by scan densitometry. While amounts of immunoprecipitated Scrib were equal in shLuc and shPIX cell lysates, the decrease of βPIX expression paralleled the decrease of amounts of βPIX-GIT1-PAK associated to Scrib, demonstrating that βPIX is a limiting factor for the Scrib–PAK interaction. Altogether, these results show that the Scrib PDZ domains interact with the C-terminal sequence of βPIX, and recruit PAK via βPIX.
Scrib is required for epithelial cells or primary mouse embryonic fibroblasts to efficiently respond to chemoattractant cues

PAK and βPIX are major players in cell migration (32). Furthermore, Scrib was recently shown to be involved in this process (24,37,38). We evaluated the role of the Scrib-PAK complex in cell migration using Boyden chamber assays and T47D cells that respond to heregulin β1 (HRG), a growth factor triggering cell motility (39). Cell migration was quantified by determining the number of cells transmigrating through the collagen-treated filter to the lower chamber containing HRG. As seen in Figure 3A, HRG is a potent cell promigratory growth factor in T47D cells transfected with control siRNA (siGFP). Its migratory potency is dependent on Scrib as we could repress cell migration following transfection of two different synthetic Scrib siRNA (siScrib1 and siScrib2). Analysis of siRNA-treated cell extracts by western blot confirmed the specific extinction of the protein, whereas expression of βPIX was not modified (Fig. 3A, lower panel). Moreover, a recently published human shScrib sequence (37) was used to stably knockdown Scrib in T47D cells. As for synthetic siScrib, expression of Scrib was dramatically reduced in shScrib transfected cells compared with the control shLuc construct (Fig. 3B, lower panel). Again, downregulation of Scrib dramatically impaired cell migration (Fig. 3B, upper panel). To rule out potential shScrib off-target effects, we re-expressed a shScrib-resistant form of Scrib (GFP-rScrib) in shScrib cells. Motility of GFP-rScrib cells was nicely restored compared with the control cells (shScrib cells transfected with GFP alone) (Fig. 3B, upper panel). Expression of proteins was verified by western blot (Fig. 3B, lower panel). Of note, cell migration of T47D cells induced by serum treatment instead of HRG stimulation gave identical results (data not shown) suggesting that the role of Scrib in cell migration is not restricted to HRG signaling.

To confirm these results, we evaluated these parameters in scrib deficient mice (circletail mouse strain). These mice develop abnormal planar cell polarity and neural tube defects (17,19,20). The circletail (Crc) mouse strain has a frameshift mutation in the scrib gene giving rise to an early translation stop codon in between the second and third PDZ domains (Fig. 3C). Wild-type MEF (MEF+/+) and mutant scrib (MEF−/−) derived from the same littermate were first analyzed for Scrib expression by western blot using two anti-Scrib antibodies. In MEF+/+ lysates, the presence of Scrib was revealed by Scrib1, a goat antibody raised against a C-terminal peptide, and by mAb8.1, a mouse monoclonal antibody raised against the LRR domains (2). In MEF−/− lysates, no signal was obtained with the antibodies confirming the total loss of Scrib expression (Fig. 4D). RT–PCR was performed on total mRNA of MEF+/+ and MEF−/− using Scrib or β-Actin specific primers (Fig. 4E). We observed a dramatic drop in the level of scrib messenger in MEF−/− extracts compared with MEF+/+, potentially explained by a nonsense-mediated decay process (40). MEF−/− therefore represent a suitable cell system to study the consequences of loss of Scrib expression. We carried out Boyden chamber assays with MEFs using serum (FCS) as a chemoattractant cue. As shown in Figure 3F, MEF−/− transmigrate two times less compared with MEF+/+ on collagen coated filters following serum stimulation, and five times less when fibronectin was used as a substrate. Taken together, these results obtained from complementary approaches demonstrate that Scrib is a key player in cell migration in epithelial cells and primary fibroblasts.

Formation of the Scrib complex is required for HRG-induced cell migration

We next evaluated the role of members of the Scrib complex in cell migration. As previously shown (29), the migratory potency of HRG is dependent on PAK. Indeed, we could repress T47D cell migration by expressing a dominant negative kinase-dead PAK (data not shown) or by using siRNAs specific for either PAK1 or PAK2 isofrom (Fig. 4A, upper panel). HRG-induced cell migration was also impaired when we expressed a siRNA specific for βPIX. Efficiency of siRNAs was confirmed by western blot (Fig. 4A, lower panel).

To evaluate if the association between components of the Scrib complex is required for cell migration of T47D cells, we stably expressed dominant-negative constructs (GFP-Scrib PDZ and GFP-βPIXpepCter) designed to interfere with endogenous Scrib-βPIX (and thus Scrib-PAK) association. GFP-Scrib PDZ encompasses the four PDZ domains of Scrib able to interact and therefore to titrate endogenous βPIX. Conversely, GFP-PIXpepCter contains the 15 last residues of βPIX that bind to the Scrib PDZ domains and thus compete with endogenous βPIX (see Fig. 2B). T47D cells transfected with various constructs (GFP, GFP-Scrib PDZ, GFP-PIXpepCter or a version deleted of the last three residues GFP-PIXpepCterΔ) were cell-sorted by FACS according to the expression of the GFP proteins. Populations of GFP-positive cells were obtained (~90% of GFP positive cells for each construct) and comparable levels of protein expression were evidenced by probing lysates with anti-GFP antibody (Fig. 4B and C, lower panels). Expression of GFP-Scrib PDZ and GFP-PIXpepCter constructs efficiently impaired the coimmunoprecipitation between Scrib and βPIX, and between Scrib and PAK confirming their dominant negative effect (Fig. 4B and C, lower panels). GFP alone or GFP-PIXpepCterΔ did not provoke a detectable inhibition. Sorted GFP-Scrib PDZ or GFP-PIXpepCter cell populations were subjected to Boyden chamber assays in the presence or absence of HRG. As shown in Figure 4B and C (upper panels), cell migration was drastically impaired by expression of these dominant-negative constructs, whereas GFP alone or GFP-PIXpepCterΔ constructs did not affect the migratory behavior. Taken together, these data demonstrate that the HRG-dependent migration of T47D cells is controlled by the ability of Scrib to interact with the βPIX–PAK complex.

PAK, βPIX and Scrib colocalize at the leading edge of HRG-stimulated epithelial cells

Cell migration requires polarized organization of the cortical actin/myosin cytoskeleton, and localization of proteins at the leading edge of migratory cells. Resting serum-starved T47D
Figure 3. Downregulation of Scrib impairs cell migration. (A) T47D cells were submitted to transmigration assays in Boyden chambers without (white bars) or with (black bars) 1 nM HRG after transfection with the mentioned siRNA. Western blot analysis (lower panel) shows levels of Scrib and βPIX in cells. Data are represented in number of transmigratory cells per mm². (B) shLuc and shScrib T47D stable cell populations, and shScrib T47D cells transfected with GFP alone (GFP) or with a Scrib mutant insensitive to shRNA (GFP-rScrib) were submitted to Boyden chamber assays. Western blot analysis (lower panel) shows levels of Scrib and βPIX, as well as levels of GFP constructs. (C) Scheme of the mutation present in the circletail (Crc) mouse strain and location of epitopes recognized by the Scrib antibodies used in this study. (D) Expression level of Scrib in MEF derived from wild-type (+/+) or Scrib-deficient circletail mice (Crc/Crc) by immunoblot with anti-Scrib antibodies. Anti-alpha-tubulin (α-tub) antibody is used as a loading control. (E) Level of Scrib mRNA in MEF derived from wild-type (+/+) or Crc/Crc mice was evaluated by RT–PCR. β-actin mRNA is used as a control. (F) Transmigration of wild-type (+/+) and Crc/Crc MEF in Boyden chamber assays using serum (FCS) as a chemoattractant. Filters were coated with collagen or fibronectin. Asterisks represent significant (*P < 0.01) or highly significant (**P < 0.001; ***P < 0.0001) differences compared with the control. Western blot analysis illustrates the amount of GFP proteins expressed in cells.
cells exhibit a round shape and have poor spreading on collagen substratum (Fig. 5A–C, upper panels) (39). In these cells, βPIX and PAK have a cytoplasmic localization, whereas Scrib is mostly found at the cell periphery. Stimulation of T47D cells by HRG results in the formation of large lamellipodial structures with membrane protrusions characteristic of the leading edge of migrating cells, as well as the formation of actin meshwork. After 5 min of HRG stimulation, βPIX and PAK partially relocate to the leading edge of cells where Scrib concentrates (Fig. 5A and B, middle panels). Cortical PAK is recognized by anti-phospho-PAK antibody suggesting that a fraction of PAK present at the leading edge is active (Fig. 5C, middle panels). After 15 min of HRG treatment, the nascent protrusions turn into a large polarized lamellipodium characteristic of migrating cells in which Scrib, βPIX and PAK (total and phospho-PAK) are less present (lower panels in Fig. 5A–C).

Scrib is required for the correct subcellular localization of βPIX and PAK at the leading edge of motile cells

Using a dominant-negative approach, we have previously shown that Scrib retains βPIX at the plasma membrane of neuroendocrine cells upon membrane depolarization induced by high KCl concentration (1). We evaluated the role of Scrib in βPIX and PAK localization at the plasma membrane upon HRG treatment using Scrib depleted T47D cells. Cells transiently transfected with control (siGFP) or Scrib (siScrib1) siRNAs were stimulated with HRG. Localization of βPIX and Scrib was evaluated by immunofluorescence and confocal acquisition. In Figure 5D and E, we focused on a representative field showing two adjacent cells, the left one being unaffected by Scrib siRNA treatment while the right one (marked by an asterisk) has been successfully depleted of Scrib. In the Scrib-positive cell, βPIX is located at the leading edge of the lamellipodium and is consistently found in polarized membrane protrusions with Scrib (Fig. 5D). In contrast, in the Scrib-negative cell, neither the cortical reinforcement of βPIX (see higher magnification in inserts), nor the formation of large polarized lamellipodia and protrusions was observed. Similarly, membrane recruitment of PAK was impaired in Scrib-negative cells (Fig. 5E).

Figure 4. Integrity of the Scrib complex is required for cell migration (A–C) T47D cells were submitted to transmigration assays in Boyden chambers without (white bars) or with (black bars) 1 nM HRG (upper panels). (A) Cells were transfected with siRNA and proteins were revealed by western blot with the mentioned antibodies (lower panels). (B and C) Dominant negative GFP-Scrib PDZ or GFP-βPIXpepCter and controls (GFP and GFP-βPIXpepCterΔ) were stably expressed in T47D cells as described in more details in the text. Comparable levels of GFP proteins were present in lysates (lower panels). The Scrib complex was immunoprecipitated with the Scrib1 antibody, and proteins were revealed with the mentioned antibodies. Error bars represent standard deviation from at least three independent experiments. Asterisks mark significant (*P < 0.01) or highly significant (**P < 0.001; ***P < 0.0001) differences compared with the control.
lation and palmitoylation sequences of Fyn kinase (Fyn-GFP) that localize the GFP at the plasma membrane (41). After fixation of cells and staining with Scrib and PAK antibodies, membrane localization of Scrib and PAK was defined using the Fyn-GFP signal as a reference. The Metamorph software (LineScan function) was used to quantify the overlap between Fyn-GFP, Scrib and PAK signals at the plasma membrane. By counting more than 100 events per condition, we...

Figure 5. Scrib is required for the subcellular localization of βPIX and PAK at the leading edge. Unstimulated (upper panels), 5 or 15 min HRG-treated (middle and lower panels) T47D cells were fixed and labeled with anti-Scrib and anti-βPIX (A) or anti-PAK (B) or anti phospho-T423PAK (C) antibodies. Actin was stained with TRITC-labeled phalloidin. Confocal acquisition shows the localization of Scrib, βPIX and PAK at the leading edge of 5 min HRG-treated T47D cells. (D and E) T47D cells transiently transfected with siScrib1 were stimulated 5 min by HRG and subjected to immunofluorescence with the mentioned antibodies. In this representative field, the level of Scrib expression was unaffected in one cell (left) and decreased in another one (right, asterisk). Inserts are magnified views of the boxed areas. (F) Cell fractionation performed on resting or HRG-stimulated T47D cells (5 min of HRG treatment) depleted of Scrib (shScrib) or Luciferase (shLuc) as a control. Levels of expression and distribution of the mentioned proteins were evaluated by western blotting in total lysates (TL), cytosolic (Cy) and membrane-associated (Mb) fractions. Purity of cytosolic and membrane fractions was evaluated by anti-GDI and anti-E-cadherin (E-Cadh) immunoblots, respectively. (G) shLuc or shScrib T47D cells stimulated 5 min with HRG were analyzed by confocal microscopy following costaining with anti-βPIX (green), anti-Paxillin (blue), anti-Scrib (purple) antibodies and Texas Red-phalloidin-labeled F-actin (red). Higher magnification of leading edges (white squares) are shown in inserts. (G') Two optical slices of 1 μm thick are shown: focal adhesion plan (P1) and 1 μm above (P2). Green and red lines in xy images indicate the position of xz projections. Scale bars, 5 μm.
found that, in the presence of heresulin, more than 68% of Scrib positive cells (shLuc) had cortical recruitment of PAK compared with 26% in Scrib depleted cells (Fig. S1). To biochemically confirm these results, we performed cell fractionation of shScrib or shLuc T47D cells stimulated or not by HRG (Fig. 5F). In shLuc cells, Scrib is mainly associated with the membrane fraction (Mb) in the presence or absence of HRG stimulation and is absent from the cytosolic fraction (Cy). In contrast, βPIX and PAK are present in the membrane and cytosolic fractions in unstimulated cells and are enriched in the membrane fraction upon HRG stimulation. This recruitment was reduced in Scrib depleted cells (Fig. 5F). Membrane and cytosolic pools of two control proteins, E-cadherin, an intrinsic membrane protein, and GDI, a cytosolic protein, were weakly affected by HRG treatment or Scrib depletion.

βPIX is present in focal adhesions through its interaction with GIT1 (42,43). We decided to determine if Scrib is also present in these structures. To do this, we immunostained βPIX, Scrib and Paxillin, a focal adhesion component, in shLuc or shScrib T47D cells following 5 min of HRG treatment (Fig. 5G) and performed z-stack confocal microscopy acquisitions. Figure 5G’ shows high magnifications of nascent leading edges (white squares) at two different planes and the x–z projection. At the lower plane (P1) of control cells (shLuc), focal adhesions were visualized by Paxillin staining. βPIX substantially colocalized with this focal adhesion component while Scrib was barely detectable in these structures. In contrast, a 1 μm upper plane (P2), showed bright Scrib staining, especially at the leading edge. In this plane, Scrib colocalizes with βPIX while Paxillin is barely detectable. In Scrib depleted cells (shScrib), colocalization between βPIX and Paxillin still occurred at focal adhesions (plane P1), however, in slice 1 μm above (plane P2), almost no βPIX staining was detected in the absence of Scrib (Fig. 5G’). Altogether these data demonstrate that βPIX and Scrib localize at the nascent leading edge of migrating T47D cells upon HRG stimulation and that Scrib appears required for βPIX localization. Moreover, we show that at least two pools of βPIX coexist in HRG-stimulated cells, one that colocalizes with Paxillin in focal adhesions and one that is associated with Scrib at the nascent leading edge.

Impaired chemotaxis of Scrib deficient cells is rescued by active PAK

When we observed T47D cells with normal amounts of Scrib (Scrib+) following 5 min of HRG treatment, a majority (64%) of cells develop polarized protrusions. Thirty-one of HRG-treated cells have a resting phenotype (no protrusion) or present unpolarized protrusions (5%) (Fig. 6A). In Scrib depleted cells (Scrib-) treated by HRG, only 18% display polarized protrusions while the percentage of cells with unpolarized protrusions (‘pancake’ shape) reached up to 36%. Of note, 46% of Scrib negative cells present a resting phenotype. Mislocalization of the βPIX/PAK complex following depletion of Scrib (Fig. 5C and D) is thus correlated to a decreased formation and polarization of cell protrusions following HRG stimulation. Interestingly, it was previously shown that inhibition of PAK activity by expression of a dominant-negative PAK suppresses the capacity of HRG to reorganize actin cytoskeleton structures in non-invasive breast cancer MCF-7 cells (29). Similarly, transient expression of kinase-dead PAK (PAK KR) in HRG-treated T47D cells strongly impairs HRG responses: 71% of transfected cells have a resting phenotype, and 29% form polarized protrusions (Fig. 6A). Like in MCF-7 cells, PAK thus plays a key role in T47D cell actin cytoskeleton organization mediated by HRG.

We next wanted to evaluate whether PAK activity is a limiting factor in the cell migration defect observed in Scrib-depleted cells. PAK is a serine–threonine kinase locked by an autoinhibitory mechanism which is partially released by active Cdc42 or Rac resulting in a conformational change, autophosphorylation and activation of the kinase (6). Threonine 423 in PAK1 (T423) and threonine 402 in PAK2 (T402) located in the C-terminal domain of the kinases are phosphorylation sites important for kinase activation. Substitution of these residues by a glutamic acid (PAK TE mutants) mimicking the phosphorylation state can relieve the autoinhibitory mechanism. Overexpression of a myc-tagged PAK TE construct in HRG-treated T47D cells gave rise to a slight increase in transmigration (lanes siGFP with or without PAK TE in Fig. 6B). This active form of PAK almost totally rescued the effect of Scrib depletion (lanes siScrib with or without PAK TE in Fig. 6B). A comparable rescue of cell migration was observed in MEFcKO/ −/+ transfected with PAK TE (Fig. 6C). Expression levels of Scrib and mycPAK TE are shown in Figure 6B and C. The fact that the migratory defect of Scrib-deficient cells can be partially rescued by expression of an active form of PAK suggests that PAK activity is a limiting factor for Scrib depleted cells and that Scrib probably lies upstream of PAK.

Absence of Scrib impairs HRG-induced PAK activation and Rac regulation

We next addressed the role of Scrib in PAK activation. To this end, level of PAK phosphorylation at threonine 423 and threonine 402 in PAK1 and PAK2, respectively, that signals complete PAK activation was analyzed (28,44) by western blot using a specific anti-phosphothreonine 423 PAK antibody (T423pPAK). Protein extracts of shLuc or shScrib T47D cells stimulated by HRG were probed with the T423pPAK antibody. In shLuc cells, PAK2 is phosphorylated at T423 5 min after HRG stimulation. In contrast, in shScrib cells, PAK2 is weakly phosphorylated at this site even after 30 min of HRG stimulation (Fig. 7A). Amounts of phosphorylated Akt (S473pAkt), a well-known downstream target of HRG signaling (45,46), and ErbB2 are identical in HRG-stimulated shLuc and shScrib T47D cells ruling out that the absence of Scrib has a general effect on HRG signaling (Fig. 7A). To confirm the specificity of the T423pPAK antibody and point out the involvement of PAK2, we used synthetic siRNAs confirming the specificity of the T423pPAK antibody and point out the involvement of PAK2, we used synthetic siRNAs that specifically decrease PAK1 or PAK2 expression in HRG-stimulated T47D cells. A siGFP was used as a control siRNA. As shown in Figure 7B, downregulation of PAK2 leads to a stronger decrease of phospho-T423pPAK signal than downregulation of PAK1, confirming that PAK2 is the major isoform phosphorylated on T423 in these cells. Next, to directly measure PAK2 kinase activity, we immunoprecipitated PAK2 with a specific anti-PAK2 antibody and we performed an in vitro kinase assay using myelin basic protein (MBP) as a
substrate in the presence of ATP[yP12]. As shown in Figure 7C, PAK2 exhibits a reduced ability to phosphorylate MBP in Scrib depleted cells compared with control as quantified in Figure 7D. Moreover, western blot analysis of resting or serum-stimulated MEF+/- or MEFCrc/Crc with the T423pPAK antibody reveals that the induction of PAK activity in MEFCrc/Crc is reduced in comparison to MEF+/- (Fig. 7E). We conclude that Scrib positively regulates PAK activity.

Since PAK activity is commonly considered to correlate with Rac or Cdc42 activity, we wondered if Scrib depletion could impair activation of these small GTPases. We measured Rac and Cdc42 activity by a GST–PAK–CRIB pulldown assay using shLuc and shScrib T47D cell extracts. In starved control cells (shLuc), Cdc42 and Rac have a low activity and are active 5 min after HRG stimulation (Fig. 7F). In Scrib depleted cells (shScrib), basal Rac and Cdc42 activities are significantly higher and are weakly sensitive to HRG stimulation (Fig. 7F). Similar results were obtained in MEFCrc/Crc compared with MEF+/- when cells were stimulated with serum (Fig. 7G). Rac is a small GTPase implicated in localized actin cytoskeleton organization necessary for the formation of lamellipodia. Membrane bound Rac is considered to be in an active state (47). To confirm our biochemical data, we evaluated Rac localization by immunofluorescence and microscopy confocal acquisition in shLuc and shScrib T47D cells. In resting shLuc cells, Rac staining is slightly cortical while the membrane Rac signal appears substantially brighter in shScrib (Fig. 7H). Upon HRG stimulation, Rac was concentrated at the leading edge of shLuc T47D cells and colocalized with Scrib in lamellipodia (Fig. 7H). As shown already in Figure 6A, more than one-third of HRG-stimulated shScrib T47D cells display a ‘pancake’ shape linked to cell motility defects (48).

Like Scrib, βPIX is required for HRG-induced PAK activity and Rac regulation

As shown in Figure 5, Scrib is required for correct βPIX and PAK localization at the leading edge of motile cells. Since βPIX is considered as a GEF for Rac and Cdc42, we asked if mislocalization of βPIX in shScrib T47D cells could be responsible for higher Rac and Cdc42 activity. To answer this question, we used shPIX T47D cells that express low
Figure 7. Absence of Scrib impairs HRG-induced PAK activation (A) shLuc and shScrib T47D cells or (E) MEF^+/+ and MEF^Crc/Crc were submitted to HRG or FCS treatment, respectively, and lysates were analyzed by western blot with the mentioned antibodies. Upon a comparable stimulation controlled by a downstream target phosphorylation (S473pAkt), shScrib T47D cells and MEF^Crc/Crc failed to activate PAK2 (T423pPAK) in contrast to shLuc control cells. This defect can also be monitored by PAK kinase assays (see C and D). (B) T47D cells transfected with the mentioned siRNAs were unstimulated or stimulated with HRG and processed as in (A). (C) In vitro kinase assays. PAK2 was immunoprecipitated by anti-PAK2 antibodies and subjected to kinase assay in the presence of MBP and γP32-ATP. Similar amounts of immunoprecipitated PAK2 were demonstrated by western blot while equal amounts of MBP were verified by Ponceau Red staining. (D) Quantification of kinase assays in fold induction from the level present in resting conditions. Results are means of two independent experiments. (F) and (G) Lysates were incubated with agarose beads coupled to GST-PAK-CRIB (GST-CRIB). Affinity-purified GTP-bound Rac/Cdc42 were analyzed by western blotting (upper panel). Equal amounts of GST-CRIB were loaded as shown by Red Ponceau staining. Quantifications of Rac and Cdc42 activations, based on three independent experiments, are expressed as GTP-Rac/Cdc42 over total Rac/Cdc42 (lower panel). Basal activity was defined as the GTP-Rac or Cdc42 level present in resting shLuc cells. Error bars represent standard deviations. (H) shLuc and shScrib T47D cells were submitted to HRG treatment and stained with anti-Rac (green), anti-Scrib (red) antibodies and phallolidin labelled actin (blue) before confocal microscopy acquisitions. Green lines in XY images indicate the position of XZ projections. Scale bar, 10 μm.
DISCUSSION

Scrib is a scaffold protein conserved during evolution. Based on genetic studies in Drosophila, Scrib is described as a cell polarity protein controlling apico-basal polarity (4). The N-terminal LRR domains are required to target Scrib to the septate junctions (14) where it contributes to segregate apical proteins, such as Crumbs, away from the basolateral membrane. In contrast, scrib mutant mice have no obvious defect of apico-basal polarity and show impaired planar cell polarity (19) and a defect of convergent extension during vertebrate gastrulation (17). The latter phenotype could be due in part to a problem of cell migration. Interestingly, loss of Scrib provokes a defect in neural migration and convergent extension movements in zebrafish embryos (20). Recent reports attribute a role in cell migration to mammalian Scrib. Loss of Scrib promotes random cell migration of MDCK epithelial cells (24), and impairs directed cell migration of primary rat astrocytes (38) and MCF.10A epithelial cells (3). Depletion of Scrib in MDCK cells destabilizes E-cadherin-mediated cell adhesion and leads to increased cell migration independent of βPIX. In MCF10A and rat astrocytes, the default of migration seems due, in part, to a problem of cell orientation and implicates βPIX.

In this report, we show that Scrib interacts with the βPIX/PAK protein complex and is required for the retention of the protein complex at the leading edge of migratory cells. PAK1 and PAK2 isoforms are part of this complex in proportion that is relative to their abundance in the cells. Interestingly, the βPIX/PAK interaction is important for confining protrusions to the migration front (30,49). Accordingly, we found that loss of Scrib impairs the formation of protrusions. The implication of the Scrib complex in cell migration was demonstrated in Boyden chamber assays using two different cell systems, T47D breast carcinoma cells and mouse embryonic fibroblasts (Fig. 3). Using siRNA and dominant-negative construct approaches, we show that the chemotactic activity of HRG was dependent on the presence of Scrib, βPIX and PAK, and on the interaction between Scrib and βPIX-PAK (Figs. 3 and 4). GIT1, a multimodular protein that binds to PIX proteins (50), is associated with the Scrib complex (Figs. 1 and 2) and participates in the cell migration process as highlighted by the strong inhibitory effect of GIT1 siRNA on T47D cell migration (data not shown).

Depletion of PAK by siRNA or expression of a kinase-dead PAK impaired cell migration in a similar manner to that of Scrib depletion. Like in other cell models (29,51), phosphorylated PAK is present at the leading edge of migratory T47D cells. This recruitment is strongly reduced in Scrib deficient cells and is accompanied by a large reduction of the pool of activated PAK upon HRG treatment (Fig. 7). Interestingly, this defect is specific to PAK since Akt was efficiently phosphorylated in Scrib deficient cells stimulated by HRG. The cell migration defects of Scrib deficient cells are mostly due to a loss of PAK activation since expression of a constitutive activated PAK restored the promigratory effect of HRG (Fig. 6). A recent paper showed that loss of FAK (Focal Adhesion Kinase) impairs polarized keratinocyte migration, PAK activity and localization of the PIX–GIT–PAK complex at focal adhesions (52). Even if Scrib is not present in focal adhesions (Fig. 5G) (53), the similar phenotypes of FAK and Scrib deficient cells suggest that functional relationships may exist between Scrib and FAK.

Deficiency in lamellipodia formation and PAK activation of Scrib-depleted cells is apparently not due to inefficient membrane recruitment or activation of Rac and Cdc42 GTPases. In fact, we observed higher Rac and Cdc42 basal activities in Scrib deficient cells compared with control cells, and a small increase of activity upon HRG stimulation (Fig. 7F and G). Moreover, recruitment of Rac at the plasma membrane was

Figure 8. βPIX is required for HRG-induced PAK activity (A) and (B) shLuc or shβPIX T47D cells were stimulated with HRG and lysates were treated as described in Figure 7. Quantifications of Rac and Cdc42 activations are calculated from the results of three independent experiments.
increased in Scrib deficient cells and correlated to its level of activation (Fig. 7H). We believe that lack of PAK activation due to the absence of Scrib is not due to limiting Rac or Cdc42 activation but more likely to other mechanisms. Interestingly, a recent report demonstrates that increased levels of active Rac at the plasma membrane impair cell motility and trigger profound cell morphological changes (‘pancake’ shaped cells) similar to those observed in Scrib deficient cells (48). PAK activation is described as a multistep process in which active Rac and Cdc42 trigger a conformational change that exposes the N-terminal PIX-binding region of PAK. In this conformation, partially active PAK associates with GIT–PIX oligomers (33) and reaches high local concentration favoring phosphorylation on T423 by nearby kinases such as PDK1 (35), and therefore complete PAK activation (32,34). We observed that cells lacking Scrib or βPIX poorly activate PAK (low T423 phosphorylation). Interestingly, phosphorylation of Akt, a downstream target of PDK1, is not affected in these cells (Fig. 7A and E) suggesting that PDK1 activity is conserved in the absence of Scrib. We believe that impaired localization of PAK at the cell membrane following mislocalization or absence of βPIX is the major event that precludes its phosphorylation by PDK1 (or related kinases) on T423.

The strong effect of Scrib deficiency on PAK activity and results of rescue experiments with constitutively active PAK suggest that Scrib perturbs cell migration by acting upstream of the PAK–βPIX pathway. It was recently reported that the depletion of Scrib has a weak or no effect on Rac or Cdc42 activity, and that wound-induced membrane localization and activation of small GTPases is reduced (3,24,38). In these works, extinction of Scrib not only impairs chemotaxis in Boyden chamber assays, but also provokes a default of directional cell migration and/or a compromised closure of wounds in wound healing assays. Differences in our results are probably due to the diversity of the cell models used in these studies.

Altogether, these data demonstrate that Scrib participates in lamellipodia formation through the activation of PAK at the leading edge of migratory cells. Recently, Horwitz and co-workers (43) identified Paxillin as a substrate for PAK. Paxillin plays a major role in lamellipodia formation through a mechanism that requires βPIX and GIT1. While Scrib is not present in Paxillin-positive structures (Fig. 5G), it is possible that Scrib may control Paxillin phosphorylation through PAK activation. Further studies will address this issue.

**MATERIALS AND METHODS**

**Cell culture and cell transfection**

T47D and MCF-10A2 cells were grown in accordance with ATCC recommendations. Wild type (+/+) and mutant Scrib (Crc/Crc) MEF (17) were derived from the same littermate. 13.5-day-old embryos were dissected, cut into pieces and trypsinized. Individual cells were grown in Dulbecco’s modified Eagle’s medium with 15% fetal calf serum (FCS) supplemented with 1 mM sodium pyruvate, non-essential amino acids and 50 µM β-mercaptoethanol (54). T47D cells were transfected with plasmids or siRNA using Lipofectamine 2000 and Oligofectamine reagents, respectively, according to the manufacturer’s instructions (Invitrogen). MEF nucleofection was done according to the manufacturer’s protocol (Amaxa).

**Plasmids, siRNA and shRNA**

The Scrib constructs have been previously described (1). shScrib (37) and shβPIX (targeting the CTGTGTCCTCCAAATTCAG sequence) cloned into the pSUPER vector were transfected to stably reduce Scrib or βPIX protein expression in T47D cells. Cells were selected in 2 µg/ml puramycin. A negative control targeting the Luciferase (shLuc) was added to these experiments (24). The synthetic siRNA used in this study were the following: siScrib1 (GGCUCUGGAGAUCGGCACTT), siScrib2 (CGAUCUGAAGUCGUCGCCCATT), siβPIX (GAGCUCCGAGAGACACAUUGTTT), siPAK1 (UCUGUAACACACGCGUCUG), siPAK2 from Santa Cruz and siGFP (GFP-22 from Qiagen). Total RNAs from MEF were extracted using Trizol (Invitrogen). RT using SuperScript II with oligo dT were done following recommendations of the manufacturer. Mouse Scrib (fwd CTGGTGATGGGGGCTCCTATTTATAC, rev GCCGGAGCAATTCCTCCACAAGGTGA) or β-actin (fwd GATGACCGCCATCATGTTGAGA, rev AGGATTCGACCTACCAAGAAGGA) were PCR amplified and run on agarose gels.

**Antibodies**

Rabbit anti-PAK1 (N20 and C19), goat anti-Scrib (Scrib1:C20 and Scrib2:K21), goat anti-PAK2 (γ-PAK N19), goat anti-GIT1 (A19) antibodies are from Santa Cruz Biotechnology. Anti-βPIX antibody and anti-DSP are from Chemicon International. Monoclonal antibodies against E-cadherin, Rac and GDI are from Becton Dickinson Bioscience, anti-GFP (clone 4.1) antibody is from Roche, anti-ottoxin is from Sigma. Rabbit polyclonal T423pPAK used in western blot is from Biosource. Monoclonal antibodies against Cdc42 and S473pAkt, and polyclonal antibodies against PAK1(Ser199/204)/PAK2(Ser192/197) are from Cell Signaling. Rabbit polyclonal anti-ErbB2 antibody was kindly provided by Ali Badache. Secondary antibodies coupled to horse radish peroxidase are from Dako. Alexa Fluor®-conjugated antibodies and labeled phalloidin are from Molecular Probes, Invitrogen.

**Protein procedures**

For cell stimulation, cells were seeded on rat-tail collagen I (Roche) coated-vessels, serum-starved overnight and stimulated or not with 1 nM HRG (R&D Systems) followed by protein extraction as previously described (1,55). For immunoprecipitation, after preclearing with agarose beads, and incubation with antibodies, protein G-agarose beads were added to the lysates and bound immune complexes were recovered, washed three times with lysis buffer and separated on SDS–PAGE for western blot analysis. For competitive immunoprecipitation assays, soluble peptides (Neosystem Inc.) were incubated for 1 h at 4°C with the precleared cellular lysates at a final concentration of 100 or 200 µM. The PAK peptide (DATPPVIAVRPEHTKSVYTRS) corresponds to...
the 183–204 PAK1 proline-rich peptide, the PIX peptide (VLKNMNDPAWDETNL) to the 15 last C-terminal residues of BPIIX, and the control peptide to the PIX peptide deleted of the C-terminal TNL motif. For subcellular fractionation, cells were washed with cold PBS, scraped and homogenized with hypotonic buffer containing 10 mM Tris–HCl pH 7.5, 0.2 mM magnesium chloride, 0.2 mM calcium chloride, 5 mM potassium chloride, protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). After 50 strokes, homogenates were spun at 800 g at 4°C for 15 min. Supernatants (cytosolic and particulate fractions) were clarified at 25 000 g for 15 min, whereas pellets (membrane fraction) were washed twice with a hypotonic buffer and then solubilized in RIPA buffer (41).

**PAK kinase assays**

Procedure was done as described in (56). Briefly, 0.5 mg of cell lysates were immunoprecipitated with 3 μg of anti-γPAK antibody and subjected to in vitro kinase reaction for 30 min at 30°C in 15 μl of kinase buffer containing 10 μCi ATP[yP32] and 1 μg of MBP (Sigma) as a substrate. The reaction products were separated on 4–12% gradient gels (Invitrogen), transferred on nitrocellulose and revealed by autoradiography and western blot.

**GTPase assays**

Levels of GTP-bound Rac and Cdc42 in cell lysates were assessed using GST–PAK–CRIB pulldown assays as described elsewhere (24).

**Immunofluorescence procedures**

Cells were grown on rat-tail collagen I (Roche) coated coverslips, stimulated with or without 1 nM HRG and then fixed in 4% paraformaldehyde in PBS for 20 min, permeabilized in 0.1% Triton X-100 for 5 min and blocked with 10%PBS, scraped and homogenized with hypotonic buffer containing 10 mM Tris–HCl pH 7.5, 0.2 mM magnesium chloride, 0.2 mM calcium chloride, 5 mM potassium chloride, protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). After 50 strokes, homogenates were spun at 800 g at 4°C for 15 min. Supernatants (cytosolic and particulate fractions) were clarified at 25 000 g for 15 min, whereas pellets (membrane fraction) were washed twice with a hypotonic buffer and then solubilized in RIPA buffer (41).

**Boyden chamber assays**

Cell migration was evaluated using 8-μm pore polycarbonate membrane Transwell chambers (Corning Costar). The bottom side of the membrane was coated with 25 μg/ml rat-tail collagen I or with 5 μg/ml human fibronectin. Cells were serum-starved for 16 h and then plated in the top chamber. Medium with or without 1 nM HRG was added to the bottom chamber and cells were allowed to migrate for 24 h. Non-migrated cells were scraped from the top of the membrane. Migrated cells were fixed in 4% formaldehyde and stained with 0.1% crystal violet. Cells were counted and migration was expressed as number of cells per mm².

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**FUNDING**

This work was supported by Inserm, Institut Paoli-Calmettes, La Ligue Nationale Contre Le Cancer (Label Ligue), Association pour la Recherche contre le Cancer, Institut National du Cancer and Région PACA. S.N. and M.S. are recipients of a fellowship from la Fondation pour la Recherche Médicale and La Ligue Nationale Contre Le Cancer, respectively.

**ACKNOWLEDGEMENTS**

We thank Marcel Deckert and Martin Schwartz for the gift of myc-tagged PAK vectors and Fyn-GFP construct, respectively. We would like to thank Ali Badache, Michel Aurrand-Lions and Brynn Taylor for their comments, Daniel Isnardon and Rémi Galindo for confocal imaging and FACS cell-sorting, respectively.

**Conflict of Interest statement** None declared.

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