Active Mek2 as a regulatory scaffold that promotes Pin1 binding to BPGAP1 to suppress BPGAP1-induced acute Erk activation and cell migration

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Summary

BPGAP1 is a multidomain Rho GTPase-activating protein (RhoGAP) that promotes Erk activation and cell motility. However, the molecular mechanism of how these two processes are linked and regulated remains unclear. Here, we show that the RhoGAP domain of BPGAP1 interacts with the peptidyl-prolyl *cis/trans* isomerase (PPI) Pin1, leading to enhanced GAP activity towards RhoA. BPGAP1 also interacted with wild-type and constitutively active Mek2, but not with its kinase-dead mutant. However, only active Mek2 could bind Pin1, acting as a scaffold to bridge Pin1 and BPGAP1 in a manner that involves the release of an autoinhibited proline-rich motif, 186-PPLP-189, proximal to the RhoGAP domain. This allows the non-canonical 186-PPLP-189 and 256-DDYGD-260 motifs of the proline-rich region and RhoGAP domain of BPGAP1 to become accessible to concerted binding by the WW and PPI domains of Pin1, respectively. Interestingly, Pin1 knockdown led to 'super-induction' of BPGAP1-induced acute, but not chronic, Erk activation upon epidermal growth factor stimulation, in a process independent of GAP modulation. Reintroducing Pin1, but not its catalytic or non-binding mutants, reversed the effect and inhibited cell migration induced by coexpression of BPGAP1 and active Mek2. Thus, Pin1 regulates BPGAP1 function in Rho and Erk signalling, with active Mek2 serving as a novel regulatory scaffold that promotes crosstalk between RhoGAP, Pin1 and Erk in the regulation of cell migration.

Key words: BPGAP1, RhoGAP, Mek, Erk, Pin1, WW, PPI, Proline-rich

Introduction

Rho GTPases are molecular switches that control the dynamics of the cytoskeletal network and cell signalling during morphogenesis, motility, growth and differentiation. Their aberrant expression, mutation or impaired regulation by their immediate regulators, such as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), can often lead to cancer and developmental, neurological and immunological disorders (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005; Heasman and Ridley, 2008; Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). GAPs function by catalysing the conversion of GTPases from their active GTP-bound state to the inactive GDP-bound state (Tcherkezian and Lamarche-Vane, 2007; Bos et al., 2007; Moon and Zheng, 2003). Besides the highly conserved enzymatic GAP domain, many RhoGAPs carry distinctive arrays of protein modules, mostly unexplored, that could potentially connect to various signaling nodes. Furthermore, little is known about how the function of a specific GAP domain is regulated.

We previously showed that BPGAP1 induces cell protrusions and cell migration via the interplay of its BCH domain, a proline-rich region (PRR; 176-PPPTKTPPPRPPLP-189) and a GAP domain (Shang et al., 2003). Through the PRR, BPGAP1 mediates translocation of cortactin from the cytosol to the membrane periphery for cell migration (Lua and Low, 2004) or engages endophilin II (also known as EEN) to increase epidermal growth factor (EGF) receptor internalisation and Erk activation (Lua and Low, 2005a). Intriguingly, the RhoGAP domain could also independently lead to Erk activation, but the precise underlying mechanism(s) remain unknown (Lua and Low, 2005a). Because Erk can regulate cell motility by acting on diverse substrates, including p90 ribosomal S6 kinase (RSK), myosin light-chain kinase (MLCK), focal adhesion kinase (FAK) and paxillin, all of which could lead to membrane protrusions and turnover of focal adhesions (Frodin and Gammeltoft, 1999; Hunger-Glaser et al., 2003; Totsukawa et al., 2004; Ishibe et al., 2004; Huang et al., 2004), it remains to be determined how Erk activation by BPGAP1 could be linked to cell motility and how these two important cellular processes are regulated.

Because the PRR of BPGAP1 contains multiple potential binding sites for Src homology 3 (SH3) or WW domains (Zarrinpar et al., 2003; Sudol and Hunter, 2000), it could become a target for further regulation by certain proteins containing these domains. Using candidate screens with full-length BPGAP1, Pin1 was identified as one such candidate. Pin1 is a ubiquitous regulator of protein conformation that uses its WW domain to target specific phosphorylated Ser/Thr-Pro motifs and help isomerise the phosphorylated Ser/Thr-Pro peptide bond through its peptidyl-prolyl *cis/trans* isomerase (PPI) domain (Zhou et al., 1999; Lu et al., 2002). It regulates diverse cell fates, including cell proliferation, cell stress, neuronal function and survival, whereas its deregulation is linked to cancer and neurological disorders (Lu and Zhou, 2007; Ryo et al., 2001; Liou et al., 2002; Wulf et al., 2001). Recent studies show

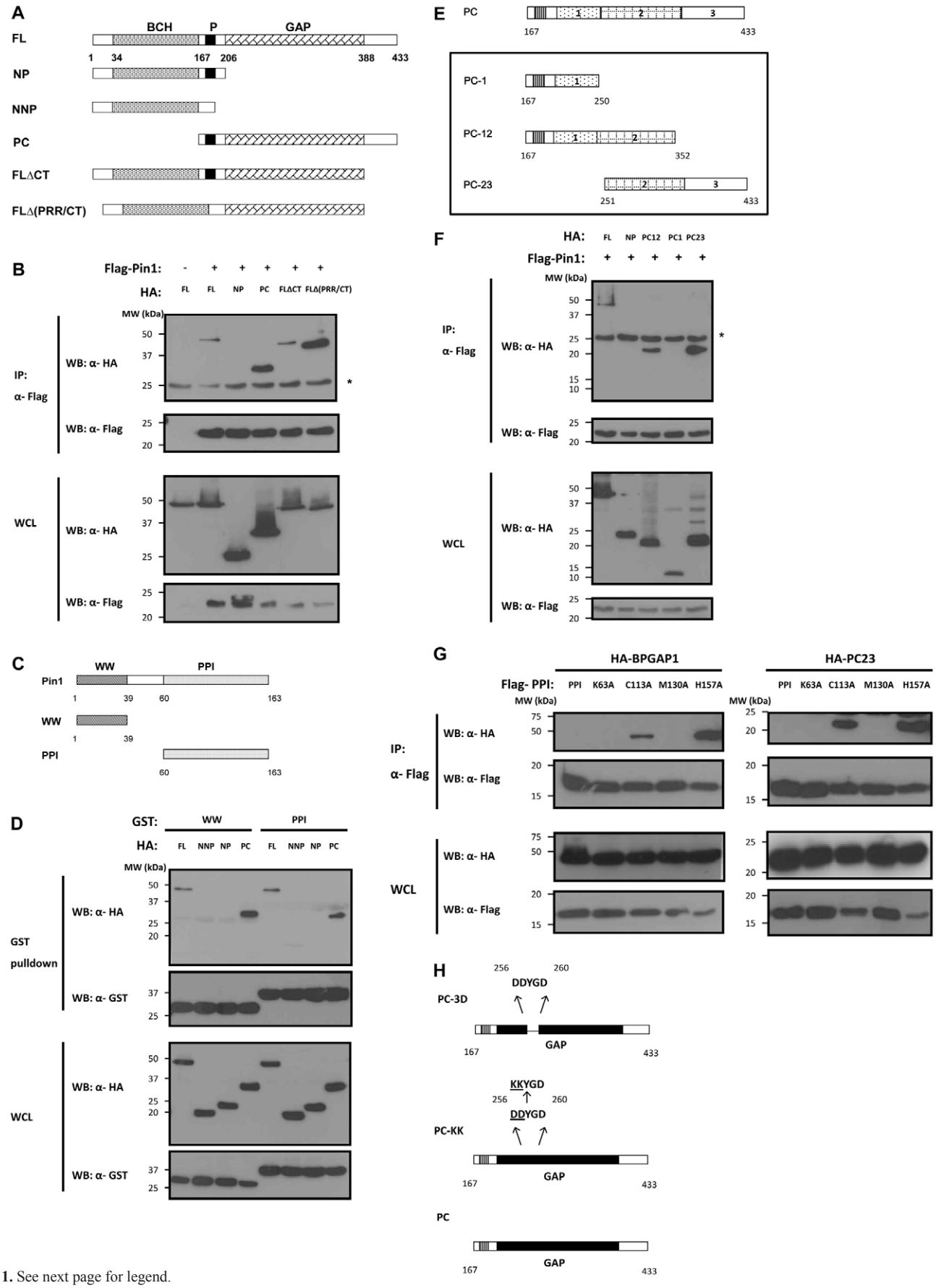

Fig. 1. See next page for legend.

that Pin1 also regulates various signalling events that are linked to cell motility and cell invasion. These include regulating the feedback loop of the Raf-Mek-Erk pathway (Dougherty et al., 2005), the stability of $p27^{Kip1}$, a Rho regulator (Zhou et al., 2009; Brenkman et al., 2008; Besson et al., 2004), the activation of Notch1 (Rustighi et al., 2009) and the turnover of FAK (Zheng et al., 2009). Despite such important associations, no direct link has been established between Pin1 and Rho-RhoGAP signalling in cell motility.

Here, we show that Pin1 uses its WW and PPI domains to interact with specific motifs within the PRR and RhoGAP domain of BPGAP1, downregulating the impact of BPGAP1 on acute Erk activation and consequently cell motility. Intriguingly, binding was enhanced by active Mek2 acting as a scaffold and could involve the release of an autoinhibitory PRR motif for binding to WW domains. Loss-of-function Pin1 mutants failed to suppress BPGAP1-induced acute Erk activation and cell motility. Such concerted action provides a novel feedback mechanism for crosstalk between RhoGAP, Pin1 and Erk signalling, at least for the control of cell migration.

Results

RhoGAP domain of BPGAP1 harbours a cryptic Pin1-binding site

The PRR of BPGAP1, 176-PPPTKTPPPRPPLP-189, contains multiple potential binding sites for SH3 and WW domains. Our candidate screens with full-length BPGAP1 identified the WW-containing Pin1 as a novel partner. To establish the nature of their interaction, different hemagglutinin (HA)-tagged fragments of BPGAP1 were coexpressed with Flag-tagged Pin1 in 293T cells and immunoprecipitated with M2 beads (Fig. 1A). Fig. 1B shows that the NP fragment (N terminus plus the PRR) of BPGAP1 did not co-immunoprecipitate with Pin1, whereas full-length (FL) BPGAP1 and the PC fragment (C terminus plus PRR) of BPGAP1 did. This indicates that the PRR did not contribute to Pin1 binding under the basal conditions tested. In addition, the interactions between Pin1 and FLΔCT (full-length BPGAP1 minus the short C terminus) or between Pin1 and FLΔ(PRR/CT) (the PRR and short C terminus were both removed) remained intact. These results show that the Pin1-binding sites of BPGAP1 reside within its RhoGAP domain.

Pin1 contains a WW domain and a PPI domain that binds and isomerizes phosphorylated Ser/Thr-Pro motifs (Zhou et al., 1999; Lu et al., 2002). To investigate their relative contributions to BPGAP1 binding, GST fusions of the WW or PPI domains of Pin1 (Fig. 1C) were coexpressed in cells with various HA-tagged BPGAP1 fragments and then precipitated with glutathione beads. Fig. 1D shows that both Pin1 domains interacted with full-length BPGAP1 and the PC fragment, but not with NP or NNP (containing the N terminus without PRR) fragments, suggesting that both the WW and PPI domains of Pin1 target the C terminus of BPGAP1.

To further delineate the specific Pin1-binding motif of BPGAP1, the HA-tagged PC was further subdivided into three regions (Fig. 1E), namely PC1 (amino acids 167-250), PC12 (amino acids 167-352) and PC23 (amino acids 251-433), which were tested for

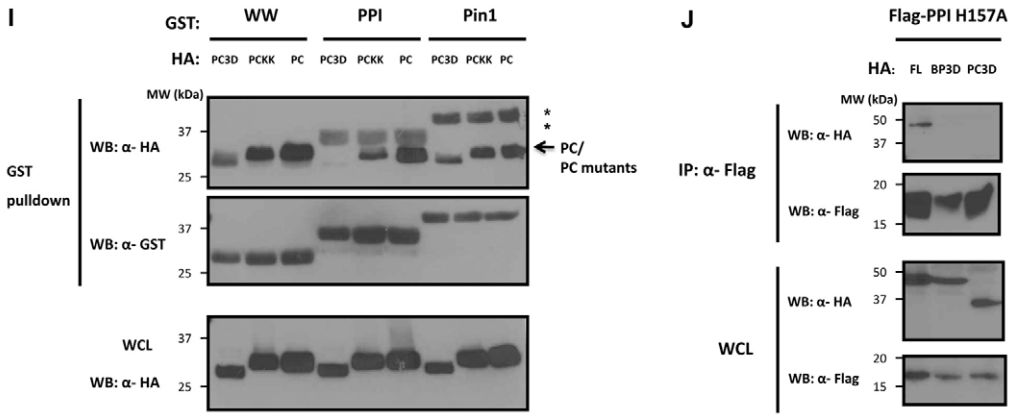

Fig. 1. RhoGAP domain of BPGAP1 harbours a cryptic Pin1-binding site. (**A**) Schematic diagram of BPGAP1 fragments: FL (full length), NP (N terminus with PRR; a.a. 1-206), NNP (N terminus without PRR; a.a. 1-167), PC (proline-containing C-terminal half; a.a. 168-433), FLΔCT (FL without C-terminal tail; a.a. 1-388) and FLΔ(PRR/CT) (FL without the PRR and C-terminal tail). (**B**) 293T cells were transfected with HA-tagged constructs and Flag-tagged Pin1. Lysates were immunoprecipitated (IP) with anti-Flag M2 beads and analyzed by western blot (WB) for bound HA-tagged BPGAP1 fragments (first panel) or re-probed with anti-Flag for the loading control (second panel). Protein expression was verified with anti-HA (third panel) and anti-Flag (fourth panel). The asterisk indicates the light chain of the Flag antibody. (**C**) Schematic diagram of Pin1 and its WW (a.a. 1-39) and PPI (a.a. 60-163) domains. (**D**) 293T cells were transfected with HA-tagged BPGAP1 constructs and GST-tagged WW or PPI domains. Lysates were precipitated with glutathione beads, and analyzed for bound HA-tagged BPGAP1 fragments using anti-HA (first panel) and anti-GST (second panel). Protein expression was verified with anti-HA (third panel) and anti-GST (fourth panel). (**E**) Schematic diagram of PC1 (a.a. 167-250), PC12 (a.a. 167-352) and PC23 (a.a. 251-352). (**F**) 293T lysate expressing HA-tagged constructs with Flag-tagged Pin1 was immunoprecipitated by anti-Flag M2 beads and analyzed with HA antibody (first panel) and re-probed with anti-Flag (second panel). Expression of HA-tagged (third panel) and Flag-tagged (fourth panel) proteins was verified. The asterisk indicates the light chain of the Flag antibody. (**G**) 293T cells were transfected with HA-tagged full-length BPGAP1 or HA-tagged PC23 and Flag-tagged PPI mutants, immunoprecipitated with M2 beads, analyzed by western blot with anti-HA (first panel) and re-probed with anti-Flag (second panel). Expression of HA-tagged (third panel) and Flag-tagged (fourth panel) proteins was verified. (**H**) Schematic diagram of deletion mutant PC3D and point mutant PCKK. (**I**) 293T cells were co-transfected with GST-tagged full-length Pin1, WW domain or PPI domain with HA-tagged PC3D, PCKK or PC, precipitated with glutathione beads and analyzed for bound proteins with HA antibody (first panel) and re-probed with anti-GST (second panel). Expression of HA-tagged proteins was verified with anti-HA (third panel). The asterisk indicates the shadow staining to GST fusion proteins. (**J**) Cell lysates coexpressing HA-tagged BPGAP1, BP3D (BPGAP1 devoid of the motif 256-DDYGD-260) or PC3D and Flag-PPI-H157A were immunoprecipitated with M2 beads and analyzed for bound proteins with anti-HA (first panel) and re-probed with anti-Flag (second panel). Expression of proteins is verified with anti-HA (third panel) and anti-Flag (fourth panel).

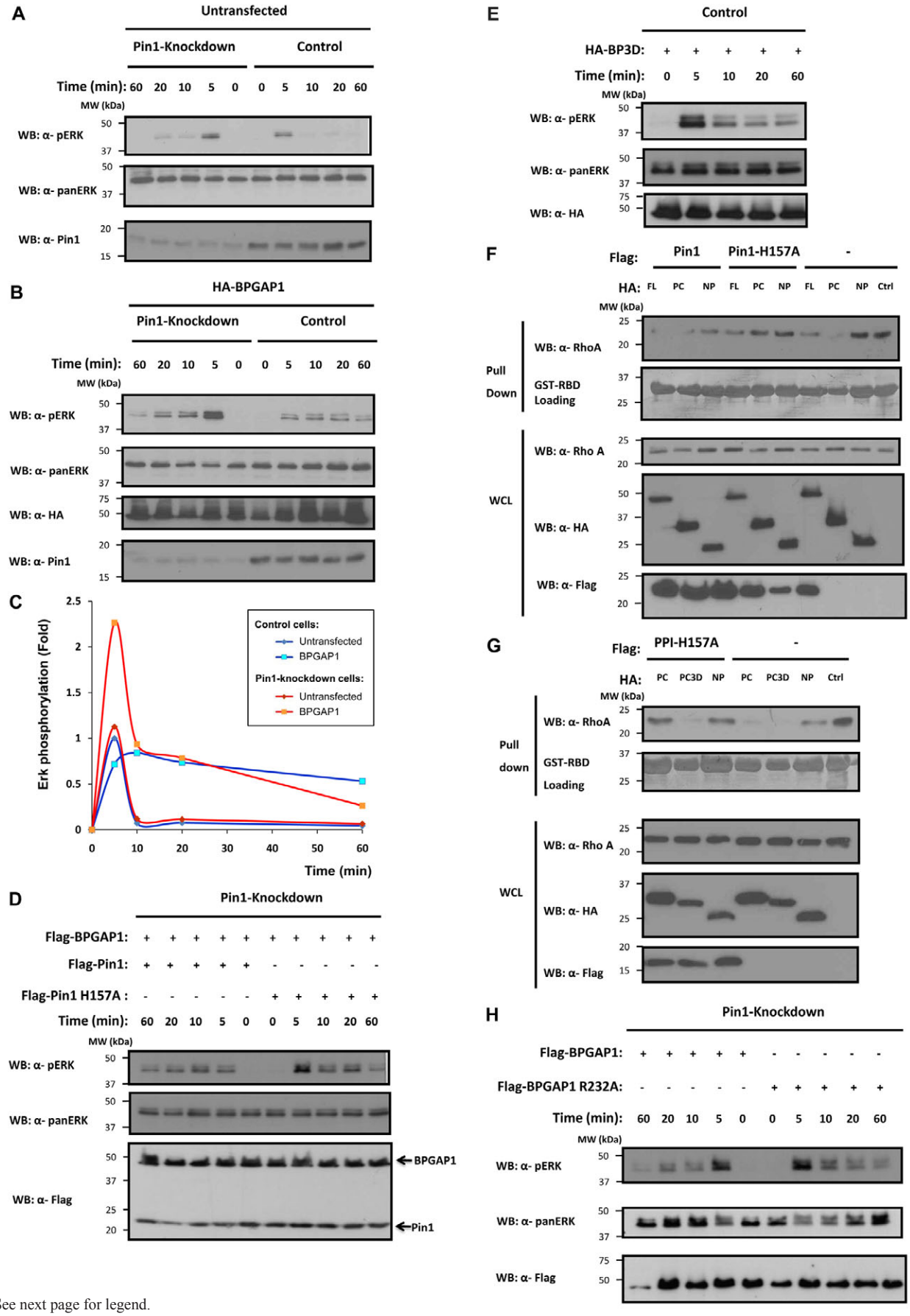

Fig. 2. See next page for legend.

binding to Flag-Pin1. The results show that Pin1 interacted most strongly with PC12 and PC23, but not with PC1 (Fig. 1F), suggesting that the region spanning amino acids 251-352 is important for Pin1 binding. Because the PPI domain is an enzyme, any transient interaction with its partner and/or substrate might not have been captured. We therefore compared the binding profiles of various PPI domain mutants for which the wild-type residues are thought to be crucial to PPI function, that is, K63A, C113A, M130A and H157A (Ranganathan et al., 1997). Unlike previous studies of the interaction of the GST-tagged PPI domain with BPGAP1 (Fig. 1D), we did not detect any strong interaction of the Flag-tagged wild-type PPI domain with BPGAP1 by immunoprecipitation (Fig. 1G), possibly because of the intrinsic nature of these different fusion constructs and their expression levels. Strikingly, such transient interaction between BPGAP1 or PC23 and Flag-tagged PPI could be effectively trapped using PPI catalytic mutants C113A and H157A (Fig. 1G), confirming the involvement of an unorthodox PPI-binding site within the region of the RhoGAP domain comprising amino acids 251-352, as seen in Fig. 1E,F. Next, we modelled the RhoGAP domain and rationalized that certain acidic surfaces within the PC23 region (supplementary material Fig. S1) might be important for binding to the basic catalytic loop and surfaces of the PPI domain, for example, the region surrounding Lys63, Arg68 and Arg69 (Ranganathan et al., 1997). Indeed, when one such polyacidic region, 256-DDYGD-260, was deleted from the PC fragment (PC3D) (Fig. 1H), binding to PPI was completely abolished, whereas binding to the WW domain was partially reduced (Fig. 1H,I). Consequently, PC3D binding by full-length Pin1 was also significantly reduced because of the loss of binding to the PPI domain. In comparison, replacing the first two aspartate residues of the polyacidic region with lysines (PCKK) also significantly reduced binding to the PPI domain, but not to the WW domain or full-length Pin1 (Fig. 1I). Furthermore, when the 256-DDYGD-260 region was deleted from full-length BPGAP1, it could no longer be 'trapped' by the PPI-H157A mutant (Fig. 1J). The 256-DDYGD-260 motif of the RhoGAP domain of BPGAP1 therefore represents a cryptic target for the PPI domain of Pin1.

Fig. 2. Pin1 independently suppresses BPGAP1-induced acute Erk activation and increases its RhoGAP activity. (**A**,**B**) Pin1-knockdown 293T cells or scrambled siRNA control lines were left untransfected (A) or transfected with HA-BPGAP1 (B). Cells were made quiescent by serum depletion for 18 hours and treated with 100 ng/ml EGF for the times indicated. Lysates were analyzed by western blot for activated Erk1/2 using phospho-Erk1/2 antibody and equal loading was verified with anti-Erk1/2, whereas protein expression was verified with anti-HA. (**C**) Densitometry analyses of the ERK phosphorylation signals were expressed as number of fold over the maximal level seen by the untransfected control cells after 5 minutes. The signals were normalized with control-stimulated cell lysates prepared concurrently and analyzed alongside the test constructs. This serves as an internal control for gel variations and also for normalizing the film exposures. (**D**) Pin1-knockdown cells were transfected with Flag-BPGAP1 and Flag-Pin1 or Flag-Pin1-H157A, and starved before stimulation with 100 ng/ml EGF for the times indicated. Lysates were analyzed for Erk1/2 activation as before. (**E**) Control cells were transfected with HA-BP3D and starved before stimulation with 100 ng/ml EGF for the times indicated. Lysates were analyzed for Erk1/2 activation as before. (**F**,**G**) 293T cells were transfected with the indicated HA-tagged constructs in the absence (–) or presence of Flag-tagged Pin1 or Pin1-H157A (F) or with Flag-PPI-H157A (G). Lysates were incubated with GST-Rhotekin-RBD (Rho-binding domain), which recognized only active endogenous RhoA. Bound proteins and lysates were analyzed using anti-RhoA (first and third panels, respectively) or anti-HA for various BPGAP1 fragments (fourth panel) or anti-Flag for Pin1 (fifth panel). Blots were stained with Amido Black to control for GST pull-down (second panel). (**H**) Pin1-knockdown cells were transfected with Flag-tagged BPGAP1 or GAP-inactive BPGAP1-R232A, and serum deprived for 18 hours before stimulation with 100 ng/ml EGF for the times indicated. Activated Erk1/2 was analyzed by anti-phospho-Erk1/2 antibody and normalized upon re-probing with anti-Erk1/2 for their expression levels.

Pin1 independently suppresses BPGAP1-induced acute Erk activation and increases its RhoGAP activity

We previously showed that BPGAP1 induces robust and sustained Erk activation via an endophilin-II-mediated pathway and a RhoGAP-dependent pathway (Lua and Low, 2005a). To examine the biological significance of Pin1 binding to BPGAP1, we compared Erk activation profiles induced by BPGAP1 with normal or reduced levels of endogenous Pin1. Control or Pin1-knockdown 293T cells that did not express HA-tagged BPGAP1 (Fig. 2A) or expressed HA-tagged BPGAP1 (Fig. 2B) were each challenged with EGF for the times indicated. In untransfected control cells, EGF induced a short burst of Erk activation (p-Erk1/2), peaking at 5 minutes and then rapidly decaying. The Erk induction profile remained relatively unchanged for the untransfected Pin1-knockdown cells, indicating that Pin1 alone did not significantly affect the basal Erk activation profile (Fig. 2A,C). In comparison, BPGAP1 expression in the control cells resulted in sustained Erk activation after 5 minutes, before reaching half the maximum at the 60 minute time point. Strikingly, when HA-BPGAP1 was overexpressed in Pin1-knockdown cells, the maximal activation at 5 minutes was greatly enhanced, before the signal decayed by the same amount seen for the Pin1-positive cells (Fig. 2B,C), suggesting that Pin1 could suppress BPGAP1-induced acute, but not chronic, Erk signalling (Fig. 2C). As a control for specificity, although the NNP fragment (containing the N terminus and the BCH domain) is also known to activate Erk (Lua and Low, 2005a), cells expressing the NNP fragment failed to further activate Erk in Pin1-knockdown cells (supplementary material Fig. S2). To substantiate that Pin1 indeed negatively regulates BPGAP1-induced acute Erk signalling, Pin1-knockdown cells were 'rescued' by reintroducing wild-type Pin1 in the presence of overexpressed BPGAP1. Now, the acute 'super-induction' at 5 minutes was fully suppressed (Fig. 2D). By strong contrast, Pin1-H157A, which is defective in PPI function, failed to exert such suppression (Fig. 2D). Furthermore, when full-length BPGAP1 devoid of the PPI-binding motif 256-DDYGD-260 was present in cells that expressed endogenous Pin1, this Pin1-insensitive mutant bypassed Pin1 suppression, leading to the super-induction of Erk in 5 minutes (Fig. 2E). Thus, all these results point to distinct phases of Erk regulation by BPGAP1, whereby Pin1 acts to suppress only the acute, but not the chronic, phase.

To test whether binding of Pin1 to the RhoGAP domain could modulate the intrinsic GAP activity of BPGAP1, which in turn influences Erk activation, cells were transfected with HA-tagged full-length, NP or PC fragments of BPGAP1, and levels of endogenous active RhoA were measured in the absence or presence of Pin1 or Pin1-H157A. The level of active RhoA was reduced by full-length BPGAP1 and, most potently, by PC (Fig. 2F). The level of active RhoA effected by full-length BPGAP1 was further reduced when Pin1 was present. Strikingly, expression of Pin1-H157A almost completely suppressed the RhoGAP activity of PC (Fig. 2F), probably by trapping PC in an inactive form. To further confirm this, the level of active RhoA was examined in cells coexpressing PPI-H157A with PC, PC3D or NP. If trapping by PPI-H157A indeed caused impaired RhoGAP function, it was expected that PC3D, which cannot bind

PPI-H157A (see Fig. 1J), should be insensitive to such suppression. Fig. 2G shows that, similar to the effect of full-length Pin1-H157A (Fig. 2F), PPI-H157A effectively blocked the RhoGAP function of PC. However, when PC3D was expressed, such suppression was lost.

These results indicate that Pin1 enhanced the RhoGAP activity, thus leading to reduced levels of active RhoA inside the cells. However, expression of the RhoGAP-inactive mutant BPGAP1-R232A (Shang et al., 2003) in the Pin1-knockdown cells still led to acute Erk activation, similar to that induced by wild-type BPGAP1 (Fig. 2H), implying that changes in RhoA activity did not lead to acute Erk activation. Hence, any modulation of RhoGAP activity by Pin1 is a distinct process from its impact on Erk regulation.

Active Mek2 enhances BPGAP1 and Pin1 interaction

Given that Pin1 can suppress BPGAP1-induced acute Erk activation, we examined whether the interaction between Pin1 and BPGAP1 could be directly regulated by Erk or its upstream regulators, such as c-Raf and Mek. To best identify the specific contribution and functional consequences of the interaction between Pin1 and

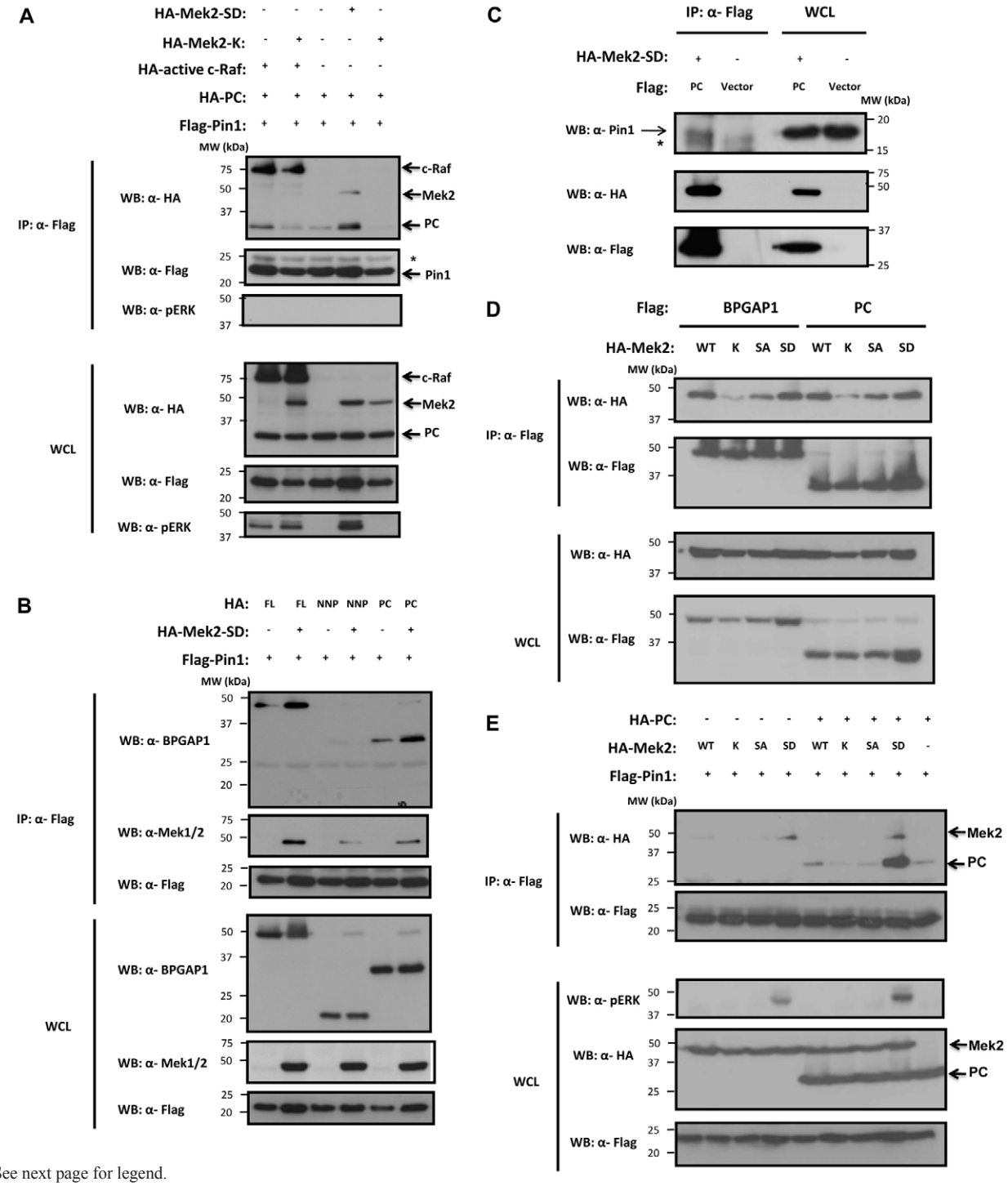

Fig. 3. See next page for legend.

BPGAP1, the PC fragment (C terminus plus PRR of BPGAP1) was primarily used. Cells were transfected with HA-PC and Flag-Pin1 in the absence or presence of active Mek2-S222,226D (Mek2-SD), inactive (or kinase-dead) Mek2-K101A or active c-Raf (Y340D), or both Mek2-K101A and active c-Raf present. Co-immunoprecipitation studies revealed that the basal binding of PC and Pin1 was greatly enhanced by constitutively active Mek2, but not by the kinase-dead mutant (Fig. 3A). When active c-Raf was present, binding of PC to Pin1 was enhanced, probably under the influence of activated endogenous Mek2. However, when kinase-dead Mek2 was used to block the endogenous active Mek2 that was induced by active c-Raf, such activation was abolished to the basal level without affecting the c-Raf and Erk activity, as shown by the presence of phosphorylated Erk1 and Erk2 (Erk1/2) in the lysates (Fig. 3A). Furthermore, active c-Raf was also co-immunoprecipitated with Pin1, whereas phosphorylated Erk1/2 was never detected in the complex between Pin1 and PC. This result indicates that active Mek2 could directly induce the formation of a ternary complex of Pin1-active Mek2-BPGAP1 downstream of active Raf, but independent of active Erk.

To recapitulate the earlier observation that active Mek2 could directly enhance the interaction between BPGAP1 and Pin1, the binding of full-length BPGAP1, NNP or PC and Pin1 was further tested in the absence or presence of constitutively active Mek2-S222,226D (Mek2-SD). Interestingly, binding of Pin1 to BPGAP1 or PC was enhanced by Mek2-SD, whereas Mek2-SD itself was co-immunoprecipitated by Pin1 (Fig. 3B). Importantly, the NNP fragment did not bind to Pin1 in the presence of Mek2. Therefore, active Mek2 only selectively promoted the binding of PC and Pin1. To determine the physiological relevance, mildly expressed Flag-tagged PC was immunoprecipitated from cells expressing active Mek2 and endogenous Pin1 was clearly detected in the complex (Fig. 3C), supporting their near-physiological level of interaction. Consistently, an epithelial HeLa cell line that exhibited a higher basal level of Erk activation (supplementary material Fig. S3A) showed enhanced binding between Pin1 and PC, whereas no further augmentation was seen in the presence of Mek2-SD (supplementary material Fig. S3B).

To further examine the significance of Mek2, binding of BPGAP1 or PC to wild-type Mek2, constitutively active Mek2, kinase-dead (K101A) Mek2 or another inactive Mek2 (S222,226A; SA) was compared. Fig. 3D shows that BPGAP1 or PC interacted strongly with the wild type and Mek2-SD, but to a lesser extent with Mek2-SA. However, binding to Mek2-K101A was markedly reduced. By contrast, only active Mek2 interacted with Pin1, and concomitantly the binding of Pin1 to PC was greatly increased, whereas inactive Mek2-K101A and Mek2-SA both showed no effect (Fig. 3E). Despite enhanced binding of PC to Pin1, the level of active Mek2 bound to Pin1 remained unchanged. A similar profile was also observed for Mek1 (supplementary material Fig. S3C). These results indicate that Mek2 possesses differential binding specificity for Pin1 and BPGAP1, raising the interesting prospect of playing a unique role in controlling their interaction.

Fig. 3. Active Mek2 promotes BPGAP1 and Pin1 interaction. (**A**) 293T cells were transfected with various indicated combinations of constructs and the lysates were precipitated with anti-Flag M2 beads. Bound HA-tagged proteins or active Erk in the complex and their expression in whole-cell lysates (WCLs) were detected with anti-HA (first and fourth panels, respectively), anti-phospho-Erk (third and sixth panels, respectively) or anti-Flag (second and fifth panels, respectively). The asterisk indicates the light chain of Flag antibody on the M2 beads. (**B**) 293T cells were transfected with various HA-tagged constructs and Flag-tagged Pin1 in the absence or presence of HA-tagged Mek2-S222D/S226D (Mek2-SD). Lysates were immunoprecipitated with anti-flag M2 beads, and bound proteins detected with anti-BPGAP1 (first panel) and anti-Mek1/2 (second panel). Blot was re-probed with anti-Flag (third panel) as a control for equal levels of precipitated proteins and their expression verified in the WCLs. Anti-BPGAP1 and anti-Mek1/2 were used instead of anti-HA to differentiate full-length BPGAP1 and Mek2, which migrated to the same position in the gel. (**C**) Cells were transfected with vector control or with Flag-PC in the presence of HA-Mek2-SD. Lysates were immunoprecipitated with anti-Flag M2 beads. Endogenous Pin1 or Mek2-SD was detected in the complex with anti-Pin1 (first panel) or anti-HA (second panel), respectively, followed by anti-Flag (third panel) to verify precipitation. The arrow indicates Pin1, whereas the asterisk denotes a non-specific band in the immunocomplex. (**D**) 293T cells were transfected with either HA-tagged wild-type Mek2 (WT), its kinase-dead mutant K101A (Mek2-K), non-active mutant S222A/S226A (Mek2-SA) or constitutively active mutant S222D/S226D (Mek2-SD) in the presence of Flag-tagged full-length BPGAP1 or PC. Lysates were immunoprecipitated with anti-Flag M2 beads. Bound proteins were detected with anti-HA (first panel) and re-probed with anti-Flag (second panel). Protein expression in WCLs was verified with anti-HA (third panel) and anti-Flag (fourth panel). (**E**) 293T cells were transfected with constructs expressing Flag-Pin1 with HA-tagged wild-type Mek2 (WT), its kinase-dead mutant K101A (Mek2-K), non-active mutant S222A/S226A (Mek2-SA) or constitutively active mutant S222D/S226D (Mek2-SD) in the absence or presence of HA-tagged PC. Lysates were immunoprecipitated with anti-Flag M2 beads. Bound proteins or their expression in lysates were detected with anti-HA (first and fourth panels) or anti-Flag (second and fifth panels). The kinase activity of Mek2 was verified by probing for activation of downstream Erk1/2 in the lysates with phospho-Erk1/2 antibody (third panel).

Active Mek2 as a dynamic regulatory scaffold for the BPGAP1 and Pin1 interaction

Next, we examined how active Mek2 could induce the binding of BPGAP1 and Pin1. Could Mek2-SD activate PC and Pin1 through their phosphorylation and/or could active Mek2 provide a structural platform that brings PC and Pin1 together? To examine the first possibility, immunoprecipitates of Pin1 or Pin1-H157A were treated extensively with alkaline phosphatase. However, such treatment had no effect on the binding of PC to Pin1 (supplementary material Fig. S4A) or to Pin1-H157A (supplementary material Fig. S4B), implying that Mek2-induced binding did not involve phosphorylation. Instead, Mek2 could act as a physical scaffold to bridge BPGAP1 and Pin1. It is worth noting that, despite HA-tagged Mek2 and PC being present at the same levels, the amount of PC bound to Pin1 was always much greater than that of Mek2, suggesting that a trimeric Pin-Mek-PC complex was very transient, but was essential to promote the formation of a more stable Pin1-PC interaction. To explore such a dynamic event, immunoprecipitation experiments were performed using different amounts of transfected plasmids for wild-type or mutant Mek2 titrated against a fixed amount of HA-PC and Flag-Pin1. Strikingly, with increasing concentration of active Mek2 (Mek2-SD), the binding of PC and Pin1 started to decrease, despite increased binding of Mek2 to Pin1 (Fig. 4A). These results indicate that the appropriate concentration of active Mek2 could greatly enhance the binding of PC and Pin1. However, when active Mek2 was present in excess, it could inhibit the formation of the PC-Pin1 complex, possibly by sequestering them as separate Mek2-PC and Mek2-Pin1 complexes instead. By strong contrast to the dynamic role of active Mek2, wild-type Mek2 could only moderately stimulate PC-Pin1 binding, whereas Mek-K101A completely abolished their interaction in a dominant-negative manner (Fig. 4B,C). Coupled with earlier observations that active Mek2 stimulated PC and Pin1 binding independently of its kinase activity and target phosphorylation

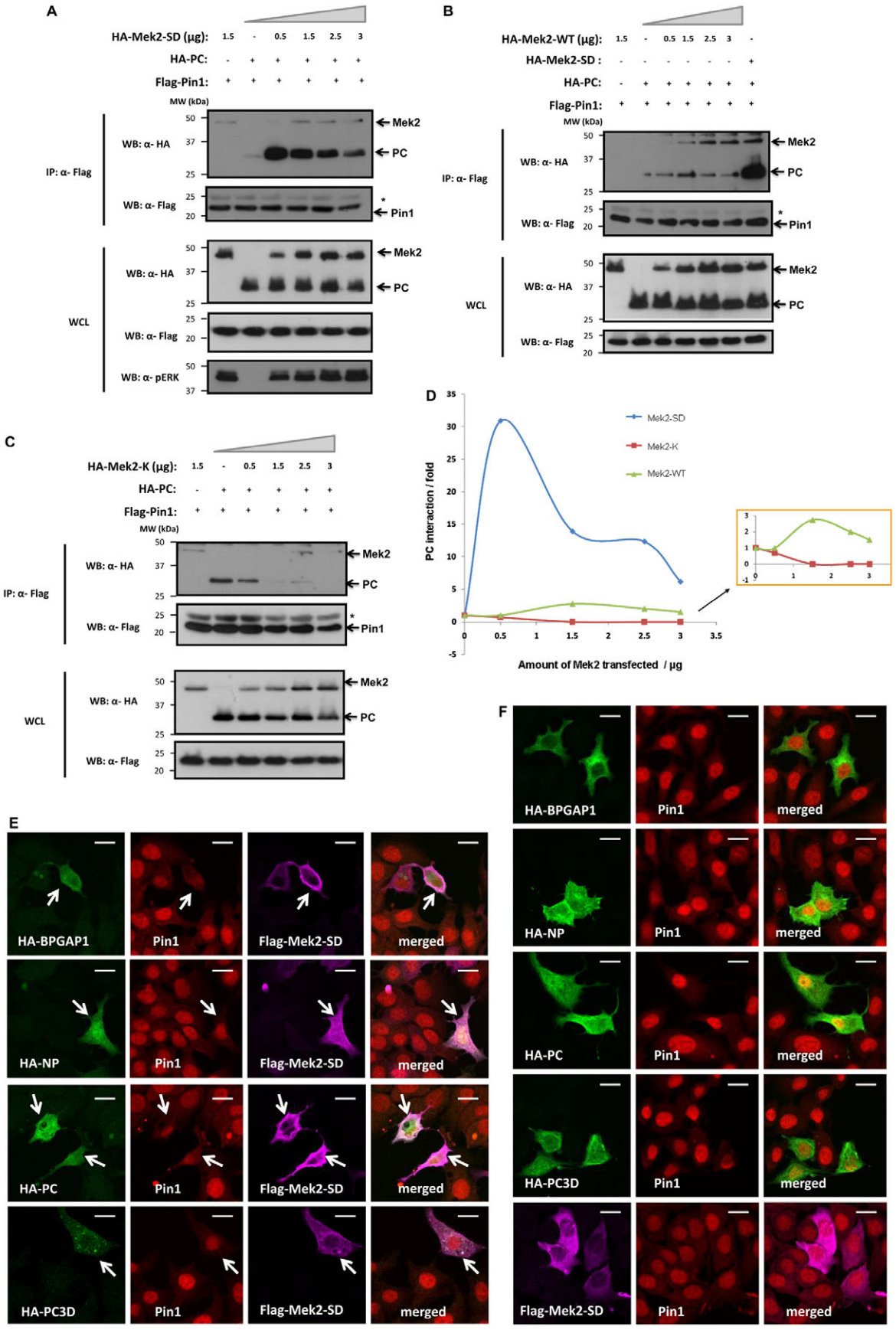

Fig. 4. See next page for legend.

(supplementary material Fig. S4A,B), these results therefore unveil an unexpected function of Mek2 as a novel scaffold protein when present in an active conformation (Fig. 4D).

We next examined how such scaffolding effects could have consequential impact on cellular localisation. Pin1 is known to be predominantly localised in the nucleus, but can also be present in the cytoplasm (Ryo et al., 2001; Liou et al., 2002; Li et al., 2008). When BPGAP1 or PC was coexpressed with Mek2-SD, less endogenous Pin1 was retained in the nucleus. By contrast, most Pin1 remained in the nucleus when the non-interactive PC-3D mutant or the negative control NP fragment was coexpressed with Mek2-SD (Fig. 4E). Moreover, expression of BPGAP1, PC or Mek2-SD alone failed to elicit the re-distribution of Pin1 (Fig. 4F), further supporting the notion that Pin1 distribution to the cytosol requires the presence of a functional complex comprising PC and active Mek2.

Active Mek2 releases an autoinhibited PRR to promote the concerted binding of WW and PPI domains to the PRR and RhoGAP domain of BPGAP1, respectively

To elucidate how active Mek2 could promote the interaction between PC and Pin1, the roles of the WW and PPI domains of Pin1 and various subregions of the BPGAP1 RhoGAP domain were further examined under the influence of active Mek2. Indeed, binding of the WW domain or full-length Pin1 to PC was greatly enhanced by active Mek2 (Fig. 5A) in the absence of phosphorylation (supplementary material Fig. S4A,C). In comparison, binding of the PPI domain exhibited only a marginal increase under the same conditions (Fig. 5A). This result indicates that the WW and PPI domains of Pin1 target different sites of PC. Although the PRR was not involved under unstimulated conditions (see Fig. 1B,D), we postulate that the presence of active Mek2 could help expose the PRR, rendering it accessible to the proline-directed WW domain. To test this hypothesis, different deletion or point mutants of the PRR were generated (Fig. 5B) and their binding tested in the presence of Mek2-SD. Fig. 5C shows that both P1 (without the entire PRR) and P2 (retaining only PPPT of the PRR) did not bind to the WW domain. However, P3 (lacking only PPPT of the PRR) retained the strong interaction with WW, indicating that the 180-KTPPPRPPLP-189 region of the PRR contains a putative WW-binding site. This region was further dissected using substitution mutants PP (substitution of prolines at 184 and 186), TPPP (181-TPPP/AAAA-184) and PPLP (186-PPLP/AAAA-189). Fig. 5D shows that, similar to the PC control, the PP and TPPP mutants still interacted strongly with the WW domain. However, PPLP completely failed to bind to the WW domain. Therefore, 186-PPLP-189 of the PRR represents a crucial binding motif in mediating the Mek2-induced interaction between BPGAP1 and the WW domain of Pin1.

We then set out to examine whether the entire PRR or part of it, in particular 186-PPLP-189, was masked in the unstimulated state. We hypothesize that, under such autoinhibition, removing all or part of the PRR should unmask the motif, enhancing the interaction between PC and full-length Pin1 even when active Mek2 is not present. Fig. 5E shows that P1, which lacks the entire PRR, and P2, without the 180-KTPPPRPPLP-189 region, readily interacted with full-length Pin1 in the absence of active Mek2. However, no further increase in binding was observed in the presence of active Mek2, because the inhibition was already fully relieved. Furthermore, binding to P2 was not mediated by the WW domain, as the PPLP motif was not present in the mutant. It was probably mediated by the PPI domain of Pin1 gaining more access to the 256-DDYGD-260 motif of the RhoGAP domain. Consistent with its autoinhibition, P3 (with 180-KTPPPRPPLP-189) did not bind Pin1 unless Mek2-SD was present.

Our further studies showed that loss of prolines 184 and 186 in PP or removal of the 186-PPLP-189 motif in PPLP led to increased binding of PC and Pin1 in the absence of active Mek2. However, loss of the 181-TPPP-184 motif (which includes 184-Pro of PP) in TPPP did not result in autonomous binding unless active Mek2 was present (Fig. 5F). Consistently, PC12 (see diagram in Fig. 1E), which harbours the PRR and 256-DDYGD-260 (the PPI-binding motif), interacted with Pin1 only in the presence of active Mek2. However, PC23, which harbours only the PPI-binding motif, readily interacted with Pin1 without the need for active Mek2 (supplementary material Fig. S5). Conversely, loss of PPI recognition by deletion of the 256-DDYGD-260 motif of PC (PC3D) led to loss of enhanced binding even when active Mek2 was present to unmask the 186-PPLP-189 motif (Fig. 5G). This was not due to the loss of PC3D binding to active Mek2, because lack of this motif did not affect its binding preference for Mek2 mutants (Fig. 5H). Therefore, both WW and PPI domains are necessary for the optimal binding of Pin1 to PC.

Taken together, these results strongly suggest that active Mek2 could help release autoinhibition at 186-PPLP-189 of BPGAP1, therefore providing enhanced access for concerted binding of the WW and PPI domains of Pin1 to their respective non-canonical motifs – 186-PPLP-189 in the PRR and 256-DDYGD-260 in the RhoGAP domain of BPGAP1. Because this autoinhibited motif did not contain serine or threonine, and loss of the preceding 181-TPPP-184 sequence still allowed Mek2-dependent activation, such regulation was consistent with a phosphorylation-independent event.

Pin1 suppresses cell motility induced by BPGAP1 and active Mek2

All the results presented so far have revealed a novel mechanism for crosstalk in the signalling pathway between BPGAP1, Pin1 and active Mek2-Erk, for which the precise modes of their interaction

Fig. 4. Active Mek2 promotes BPGAP1 and Pin1 binding by acting as a regulatory scaffold. (**A-C**) 293T cells were co-transfected with the indicated amounts of plasmids encoding HA-tagged PC and Flag-tagged Pin1, in the absence or presence of fixed or varying amounts of plasmids encoding either constitutively active HA-Mek2-SD (A), wild-type HA-Mek2 (B) or kinase-dead HA-Mek2-K (C). Lysates were then immunoprecipitated with anti-Flag M2 beads, and bound proteins in the precipitates, their expression in WCLs and activation of Erk1/2 were analyzed with anti-HA (for PC and Mek2) and anti-phospho-Erk, and re-probed with anti-Flag (for Pin1), as indicated. The asterisk indicates the light chain of the Flag antibody. (**D**) The amounts of PC bound to Pin1 under the influence of constitutively active Mek2-SD (A), wild-type Mek2 (B) or kinase-dead Mek2-K (C) were quantified by densitometric analyses, normalized against the amount of Pin1 precipitated, and then expressed as the fold over the PC signal detected in the absence of Mek2 within the same set of experiments (to control internal gel variations and film exposure). The fold induction was plotted against varying concentrations of Mek2 used in each experiment. The inset reveals differences in the binding as a result of either wild-type Mek2 or the kinase-dead mutant of Mek2. (**E**,**F**) MCF7 cells were transfected with Flag-tagged Mek2-SD alone (F) or with HA-tagged BPGAP1, NNP, PC or PC3D in the absence (F) or presence (E) of Flag-tagged Mek2-SD. Cells were then permeabilized, stained with anti-HA, anti-Flag and anti-Pin1, followed by secondary antibodies conjugated with Alexa Fluor 488 (green), Alexa Fluor 633 (pink) and Alexa Fluor 555 (red), respectively. Images were visualized under confocal fluorescent microscopy as described in the Materials and Methods section. Scale bars: 20 μm.

and regulation have been identified. To further address the physiological relevance of such a mechanism and to establish how Erk activation and cell migration are linked and regulated by this crosstalk, we set out to determine how Pin1 and Pin1-H157A might regulate PC-induced cell motility in response to active Mek2. In Pin1-knockdown 293T cells, overexpressing PC, Pin1 and Mek2-K101A or active Mek2 alone did not affect the basal level of cell migration (supplementary material Fig. S6). Interestingly, when active Mek2 was coexpressed with PC, there was a synergistic effect that resulted in enhanced cell motility (P=0.002), whereas coexpressing kinase-dead Mek2-K101A with PC did not potentiate such effect. However, coexpression of Pin1 with PC and active Mek2 completely attenuated this enhanced cell migration (P=0.002; Fig. 6), indicating that Pin1 also acts as a suppressor of BPGAP1 function in cell motility. By contrast, coexpression of Pin1-H157A, which failed to suppress acute Erk signalling (see Fig. 2D) but maintained overall high Rho activity in cells by blocking the RhoGAP activity of PC (see Fig. 2F,G), also failed to inhibit the enhanced cell migration. All these results therefore confirm that Pin1 downregulates BPGAP1-induced acute Erk activation and cell motility by directly targeting the unique motifs of BPGAP1 through the concerted action of its WW and PPI domains.

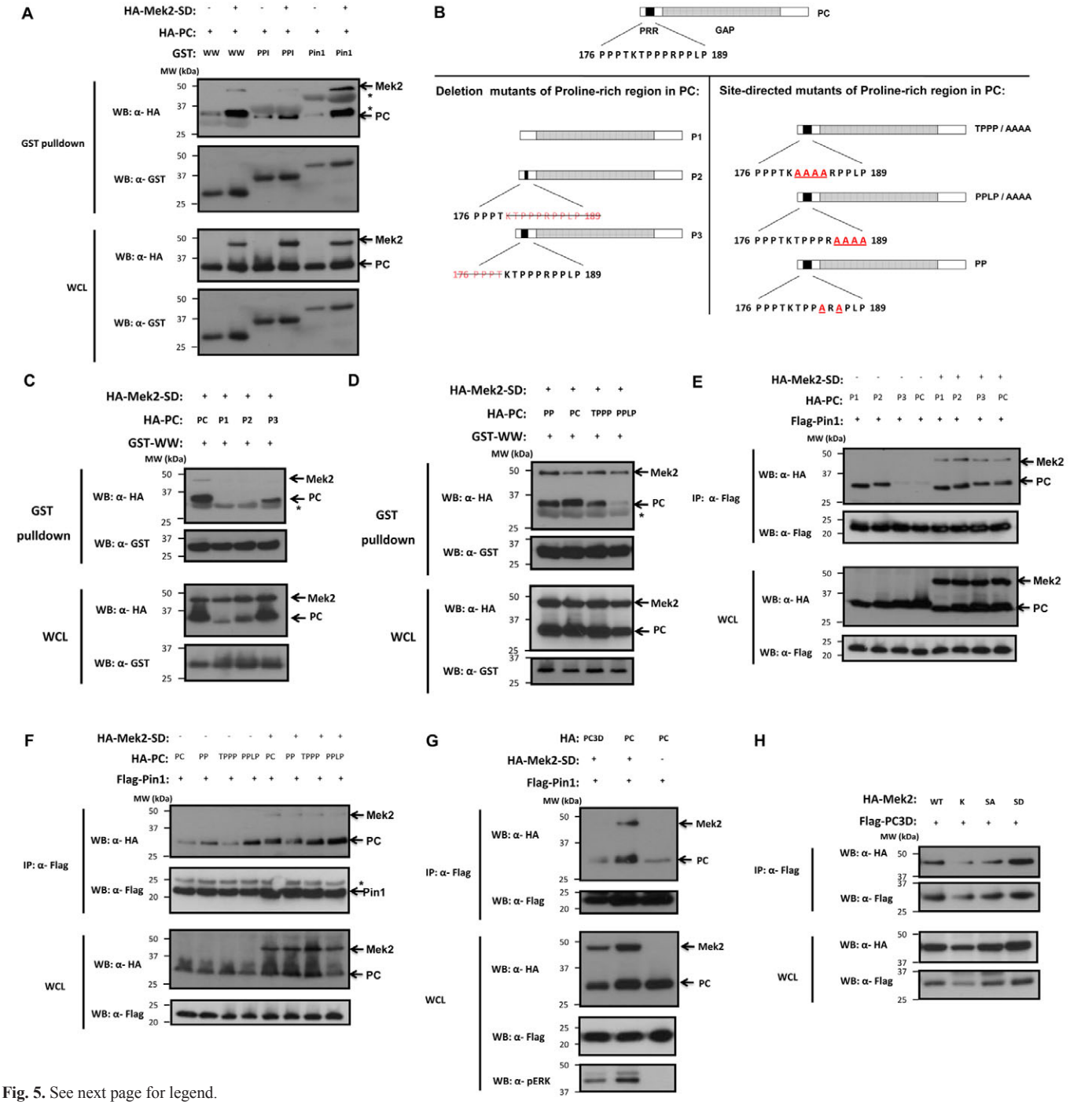

Fig. 5. See next page for legend.

Discussion

New functions of the PRR and RhoGAP domain of BPGAP1

The long PRR (176-PPPTKTPPPRPPLP-189) provides a rich repertoire for BPGAP1 to target proteins that carry SH3 or WW domains. Against the general belief that the Pin1 WW domain recognizes phospho-S/T-Pro motifs, we show that it resembles instead the type II WW domains, which recognize PPxP motifs (Sudol and Hunter, 2000). This raises the prospect of Pin1 being able to target an even wider spectrum of cellular targets.

Although it is not known how autoinhibition is mediated by the PRR and RhoGAP domain, binding of active Mek2 is likely to result in conformational changes that lead to the unmasking of the 186-PPLP-189 motif that is crucial to the binding of the WW domain. Using single-domain binding studies, we showed that PPI specifically targets the 256-DDYGD-260 motif of the RhoGAP domain, whereas the WW domain recognizes only the 186-PPLP-189 motif of the PRR. Thus, the WW domain could target Pin1 to BPGAP1 upon Mek2 activation and allow the PPI domain to act on the 256-DDYGD-260 site of the RhoGAP domain (Fig. 7). Further to the requirement for such bipartite motifs, binding of full-length Pin1 involves concerted action of both domains, because loss of PPI binding to 256-DDYGD-260 does not lead to a stable complex, despite the presence of active Mek2 and the WW domain to unmask the PPLP motif. Consequently, introducing Pin1-H157A (non-functional PPI) or removing 256-DDYGD-260 (preventing PPI binding) alone was sufficient to abolish the ability of Pin1 to suppress BPGAP1-induced acute Erk activation, RhoGAP activity towards Rho, and BPGAP1- and Mek2-coinduced cell migration.

Intriguingly, the 256-DDYGD-260 motif does not contain any proline residues. Such non-canonical binding by Pin1 has also been observed with BNIP-H (or caytaxin) (Buschdorf et al., 2008), A3G cytidine deaminase (Watashi et al., 2008) and the Pro-X-Thr-Pro recognition motif of phosphatase inhibitor-2, but independent of phosphorylation (Li et al., 2008). In addition, Pin1 can bind to the pThr-Gly motif of cyclin E (Yeh et al., 2005). These results strongly support the versatility of Pin1 in regulating a wide spectrum of cellular targets and processes.

Mek2 as a dynamic regulatory scaffold for BPGAP1 and Pin1

We have showed that only active Mek2 can promote the interaction between BPGAP1 and Pin1, acting in a highly dynamic scaffold manner. This is possible if active Mek2 undergoes high turnover from the stable Pin1-BPGAP1 complex once it is achieved. At higher concentrations, active Mek2 fails to bridge the PC-Pin1 interaction, because of the self-competition that favours separate pools of Mek2-Pin1 and Mek2-PC complexes instead. This mechanism, unlike most passive scaffolds in signalling cascades, should offer a more dynamic regime to regulate the localization and specificity of cellular functions of BPGAP1. Interestingly, the active scaffold Ste5 has recently been shown to catalytically unlock and activate Fus3 mitogen-activated protein kinase (MAPK) (Good et al., 2009), whereas the PAK1 kinase domain serves as a scaffold that acts independently of its kinase activity to facilitate Akt stimulation by PDK1 (Higuchi et al., 2008). It remains unclear how active Mek2 could exert its scaffolding effect independent of phosphorylation.

BPGAP1 and Pin1 as novel modulators of Mek and Erk signalling and cell motility

Ras-MAPK signalling is tightly controlled by various modulators, for example, their kinases and phosphatases (which provide the on and off switch), compartment-specific scaffolds that augment signal amplification and signal crosstalk, the adaptors that control their spatial distribution, and the inhibitors that suppress their signals (Kolch, 2005; Roberts and Der, 2007; Ebisuya et al., 2005). Our current findings show that Pin1 specifically modulates BPGAP1-induced acute Erk signaling by directly binding to two unique motifs of the PRR and RhoGAP domain of BPGAP1, in a process that is facilitated by active Mek2 acting as a scaffold. In addition, we have demonstrated that Pin1 enhances the RhoGAP activity of BPGAP1 towards Rho, but suppresses BPGAP1- and Mek2-coinduced cell migration.

Erk has been shown to regulate cell motility through the phosphorylation of MLCK and FAK (Zhou et al., 2008; Klemke et al., 1997; Totsukawa et al., 2004; Huang et al., 2004), which function in parallel to and also downstream of Rho signaling (Burridge and Wennerberg, 2004). The enhanced cell motility resulting from the synergism of BPGAP1 and Mek2 could result from the convergence of Rho and Erk signalling pathways. In particular, both MLCK and the effector of Rho, Rho kinase (ROCK), can phosphorylate the myosin-light chain (MLC), leading to regulation of membrane protrusions, stress fibres and focal adhesions. It is therefore likely that Pin1 suppresses BPGAP1- and Mek2-coinduced cell motility through the simultaneous downregulation of these two converging pathways of Erk and Rho. Interestingly, Pin1 can be recruited to focal adhesions in response to Ras-initiated Fgd1-cdc42-PAK1-

Fig. 5. Active Mek2 releases autoinhibited PRR to promote the concerted binding of WW and PPI domains to the PRR and RhoGAP domain of BPGAP1. (**A**) Lysates from 293T cells that expressed HA-tagged PC and GST-tagged WW, PPI or full-length Pin1, with or without HA-tagged Mek2-SD, were incubated with glutathione beads. Bound proteins and their expression were analyzed by western blots with anti-HA (first and third panels, respectively) and re-probed with anti-GST (second and fourth panels, respectively). The asterisk indicates the shadow staining to GST fusion proteins. (**B**) Schematic diagram of various mutants of the PRR of the PC fragment of BPGAP1: PC-P1 (devoid of the entire PRR, 176-PPPTKTPPPRPPLP-189), PC-P2 (devoid of 180-KTPPPRPPLP-189), PC-P3 (devoid of 176-PPPT-179), PC-TPPP/AAAA (residues 181-TPPP-184 are replaced with alanines), PC-PPLP/AAAA (residues 186-PPLP-189 are replaced with alanines) and PC-PP (proline residues at position 184 and 186 are replaced with alanines). (**C**,**D**) Lysates from 293T cells that expressed HA-tagged PC or the indicated mutants, together with HA-tagged Mek2-SD and GST-tagged WW, were incubated with glutathione beads and bound HA-tagged proteins. WCLs were analyzed by western blot with anti-HA (first and third panels) and re-probed with anti-GST (second and fourth panels). The asterisk indicates the shadow staining to GST fusion proteins. (**E**,**F**) Lysates from cells that expressed the indicated HA-tagged PC mutants depicted in B and Flag-tagged Pin1, with or without the HA-tagged Mek2-SD, were immunoprecipitated with anti-Flag M2 beads. Bound proteins and their expression were analyzed by western blot with anti-HA (first and third panels, respectively) and re-probed with anti-Flag (second and fourth panels, respectively). The asterisk indicates the light chain of the Flag antibody. (**G**) Lysates expressing HA-tagged PC or PC3D (devoid of the 256-DDYGD-260 motif), together with both HA-tagged Mek2-SD and Flag-tagged Pin1, were immunoprecipitated with anti-Flag M2 beads. Bound proteins and their expression were analyzed by western blot with anti-HA (first and third panels, respectively), anti-Flag (second and fourth panels, respectively) or anti-phospho-Erk (fifth panel). (**H**) 293T cells that expressed Flag-PC3D and either HA-tagged Mek2 or its mutants were lysed and immunoprecipitated with anti-Flag M2 beads. Bound proteins and their expression were analyzed by western blot using anti-HA (first and third panels, respectively) and anti-Flag (second and fourth panels, respectively).

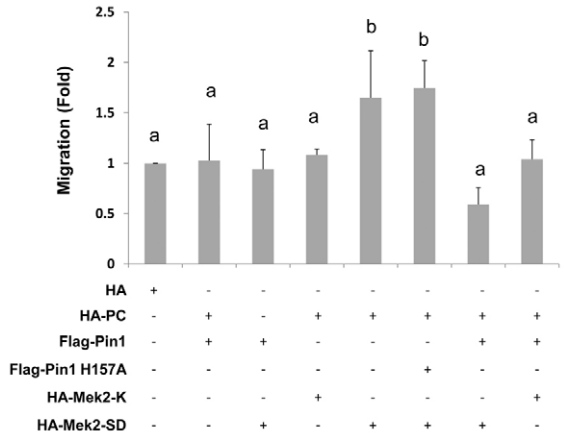

Fig. 6. Pin1 suppresses cell motility induced by BPGAP1 and active Mek2. Pin1-knockdown 293T cells were transfected with vector control or plasmids in various combinations as indicated, and cell migration was assayed as described in the Materials and Methods section. Data are means ± standard deviations of three independent experiments. Data sharing different letters are statistically significant at $P<0.005$, as tested by ANOVA. Results are expressed as fold stimulation over the vector control.

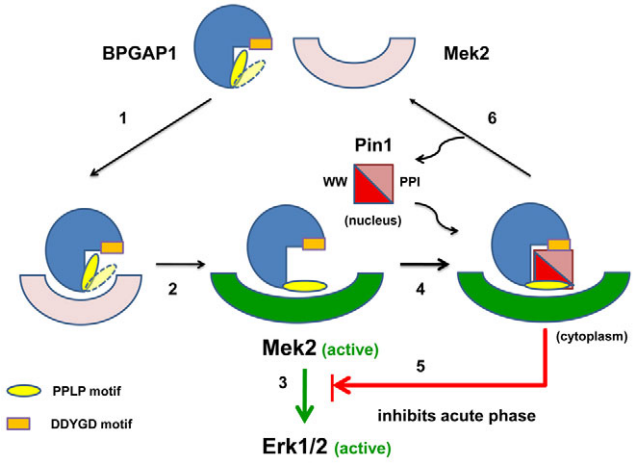

Fig. 7. Pin1 and Mek2 are two newly identified modulators of BPGAP1 function. A model depicting how Mek2 acts as a regulatory scaffold to promote Pin1 binding to BPGAP1 and suppress BPGAP1-induced acute Erk signalling. Regulation of the PRR and the C-terminal RhoGAP domain of BPGAP1 (PC) in the basal and Mek2-stimulated states. Step 1. Mek2 binds to PC independently of its active state, because non-active Mek2 and constitutively active Mek2 bind PC equally well. However, kinase-dead Mek2-K101A fails to interact with PC and also impairs the binding of Pin1 and PC. The PRR is mainly autoinhibited and is not recognized by the WW domain, whereas binding of the PPI domain can only be observed after trapping it with the PPI-H157A mutant at the 256-DDYGD-260 motif. Step 2. Upon Mek2 activation, the PRR is exposed as a result of a conformational change that is not linked to phosphorylation and is independent of its RhoGAP activity. Step 3. How such super-induction is triggered remains unknown, although Pin1 plays a crucial role in directly suppressing this effect. Step 4. After release of the PRR, active Mek2 becomes a target of Pin1, because Pin1 does not recognize other forms of Mek2. This promotes concerted binding of the Pin1 WW domain to the exposed PPLP motif and the PPI domain to the 256-DDYGD-260 motif of the RhoGAP domain. Step 5. With active Mek2 acting as an atypical scaffold, this interaction provides a feedback loop that ensures that acute Mek2 and Erk activation is negatively regulated. This is supported by the loss of BPGAP1-induced super-induction of Erk and cell motility when key PC-Pin1 binding motifs become non-functional. Step 6. This dynamic system helps ensure that Mek2 is recycled to promote the formation of more PC-Pin1 complexes.

Mek1-Erk signalling, leading to FAK inhibition and enhanced cell invasion (Zheng et al., 2009). Although this and an earlier study from Ryo et al., (Ryo et al., 2005) support the pro-metastatic potential of Pin1 in malignant cells, our findings in turn uncovered an unexpected role for Pin1 in suppressing cell migration in 293T cells. In addition, recent studies have shown that the effector of the Ras and Erk pathway, p90 ribosomal S6 kinase (RSK1), could promote $p27^{Kip1}$ phosphorylation and increased cell motility (Larrea et al., 2009), through $p27^{Kip1}$-RhoA interaction, while preventing RhoGEF binding (Besson et al., 2004). It is worth noting that the stability of $p27^{Kip1}$ is enhanced by Pin1 (Zhou et al., 2009), further demonstrating the complex yet important crosstalk between Erk, Rho, RhoGAP and Pin1 signalling in regulating at least cell migration.

We therefore propose a 'GMP signalome' model based on the principle of BPGAP1 as an inducer, Mek2 as a scaffold and Pin1 as a suppressor (see supplementary material Fig. S7). The concerted action of Pin1 in enhancing the inhibitory activity of BPGAP1 towards Rho and attenuating acute Erk signalling could converge to regulate MLCK- and FAK-mediated cell motility. Together with the known BPGAP1 functions involving endophilin-II (Lua and Low, 2005a) and cortactin (Lua and Low, 2004), it is believed that BPGAP1, Pin1 and these regulators could form a complex network of regulation for Ras-MAPK signalling through Raf-PP2A (Dougherty et al., 2005), Erk-FAK (Zheng et al., 2009) and/or BPGAP1-Mek-Erk nodes under various cellular conditions.

In summary, our findings provide the first evidence that BPGAP1 induces acute Erk signaling in response to EGF. This process is kept in check by a feedback mechanism involving Mek2 acting as a scaffold to recruit Pin1 to the BPGAP1-Mek2 complex. Pin1 binding to the two unorthodox motifs of the PRR and RhoGAP domain is essential to regulating the acute enhancement of Erk, RhoGAP activity and consequently cell motility. This new signalling node provides an additional checkpoint for Mek-Erk signalling while opening up new avenues for probing the detailed functional coupling between Pin1, BPGAP1-Rho and Mek-Erk signalling. This will not only enable us to determine the final outcome of cell motility, but will also improve our future understanding on the coordination of cell spreading and cell adhesion, which are linked to cell motility (Huveneers and Danen, 2009). This should further help define the crucial steps in motility control by the GMP signalome during normal and pathophysiological conditions.

Materials and Methods

Cell culture

293T cells were maintained in RPMI-1640 (Hyclone) with 10% (v/v) fetal bovine serum (FBS; from PPA), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone), whereas MCF7 cells were grown in high-glucose DMEM (Hyclone). All cells were grown at 37°C, 5% CO_2. 293T and MCF7 cells were transfected using Mirus (TransIT). To assay for activated ERK1/2, cells were starved for 18 hours in serum-free medium before treatment with 100 ng/ml EGF (Sigma) for the times indicated.

Plasmid construction, expression of mutants, functional studies and rescue analysis

All human BPGAP1, Pin1, c-Raf and mouse Mek1/2 constructs were cloned in Flag-, GST- or HA-tagged PXJ40 vectors (gift from Ed Manser, IMCB, Singapore) and propagated in *Escherichia coli* strains XL1-Blue and DH5α. c-Raf and Mek1/2 plasmids were a generous gift from Graeme Guy, IMCB, Singapore.

To provide a detailed mechanistic understanding of how the interaction of BPGAP1 with Pin1 and active Mek2 could impact both Erk activation and cell motility, it is important that the specific binding motifs and functional consequences of Pin1 binding to the PRR and RhoGAP domain of BPGAP1 be identified. To achieve this, the PC fragment harbouring these two domains was primarily used and their properties were verified in parallel with full-length BPGAP1. These include their binding to Mek and Mek mutants, their binding to Pin1 with or without Mek, their impact on ERK and their colocalization. Based on such data, the putative binding motifs were then subtly mutated in the backbone of full-length BPGAP1 and subsequently used to delineate the actual impact arising from their faithful interaction or loss of their interactions.

Co-immunoprecipitation, pull-down and western blot analyses

Transfected 293T cells were lysed with RIPA buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.3, 0.25 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 50 mM NaF, 5 mM sodium orthovanadate, protease inhibitors (Roche Applied Science)] and directly analyzed as whole-cell lysates or aliquots used for pull-down with various GST fusion proteins (20 μg) or anti-Flag M2 affinity gel (Sigma), as previously described (Low et al., 2000). Samples were run in SDS-PAGE gels followed by western blot analyses using the Pierce Pico ECL (Thermo Scientific). Polyclonal anti-Flag (Sigma), polyclonal anti-HA (Zymed), monoclonal anti-Pin1 (Santa Cruz), polyclonal anti-RhoA (Santa Cruz), polyclonal Mek1/2 (Cell Signaling Technology), monoclonal phospho-ERK (Sigma), monoclonal pan-Erk1/2 (BD Transduction Laboratories) and polyclonal BPGAP1 were used.

Phosphatase treatment

Beads with precipitation complex were washed twice with NEBuffer 3 and incubated with 20 U of calf intestine alkaline phosphatase (NEB) for the times indicated at 37°C. After three more washes, proteins were eluted from the beads by boiling in Laemmli buffer and analyzed by western blot.

Generating Pin1 knockdown lines

Retroviruses for stable control and Pin1 knockdown in 293T cells were prepared as previously described (Ryo et al., 2005) using Pin1 and control small interfering (si)RNA constructs (a generous gift from Akihide Ryo, University School of Medicine, Yokohama, Japan). Lines were selected upon repeated culture on selection media and the efficacy of knockdown was validated by western blot analyses with anti-Pin1.

Immunofluorescence

Twenty-four hours post-transfection, MCF7 cells on glass coverslips were fixed with formaldehyde (PBS; 15 minutes, room temperature). Fixed cells were washed three times with PBS, permeabilised with 0.2% Triton X-100 in PBS (15 minutes, room temperature), and blocked with 2% BSA and 7% FBS in PBS for 1 hour. Cells were incubated with 40 μl blocking buffer containing 0.4 μg chicken HA, mono-Pin1 and poly-Flag for 1 hour at 37°C. Samples were washed three times with PBS containing 0.1% Triton X-100 and incubated with Alexa Fluor 488-conjugated goat anti-chicken IgG, Alexa Fluor 555-conjugated donkey anti-mouse IgG, Alexa Fluor 633-conjugated donkey anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. This was followed by three more washes before mounting with FluorSave (Calbiochem) and examination under a LSM510 Carl Zeiss confocal microscope.

Cell migration assay

5×10^4 transfected 293T cells were placed on Boyden chambers (24-well Transwell plates, 8 μm pore size; Corning Costar) coated with 10 μg/ml fibronectin (Invitrogen) with the lower chamber filled with 600 μl medium with 10% FBS. Other aliquots were seeded on 6-well plates for validating protein expression. After 16 hours, cells that did not penetrate the filters were removed by cotton swab. Migrated cells were fixed with 3.7% paraformaldehyde in PBS, stained with Giemsa solution and evaluated in five random fields. Statistical significance was analyzed using ANOVA and the Student-Newman-Kuels multiple range test (StatsDirect). Data are means ± standard deviation for at least three independent samples ($P<0.005$) and expressed as fold stimulation over the vector control.

RhoA activation assay

Control and transfected 293T cells were lysed in RIPA buffer, and 300 μg lysates were incubated with 20 μg of GST-Rhotekin-RBD (from Simone Schoenwaelder, Monash University, Australia) for 30 minutes at 4°C. The beads were washed three times with lysis buffer and bound proteins analyzed by western blot with anti-RhoA (Santa Cruz).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/6/903/DC1

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