DIACYLGLYCEROL KINASE-α PHOSPHORYLATION BY SRC ON Y_{335} IS REQUIRED FOR ACTIVATION, MEMBRANE RECRUITMENT AND HGF INDUCED CELL MOTILITY

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Running title: Activation and membrane recruitment of Dgk-α

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Abstract

Diacylglycerol kinases (Dgk), which phosphorylate diacylglycerol to generate phosphatidic acid, act as either positive or negative key regulators of cell signaling. We have previously shown that Src mediates growth factors-induced activation of Dgk-α, whose activity is required for cell motility, proliferation and angiogenesis. Inhere we demonstrate that both Hepatocytes Growth Factor (HGF) stimulation and v-Src transformation induce tyrosine phosphorylation of Dgk-α on Y_{335}, through a mechanism requiring the proline-rich C-terminal sequence. Moreover we show that both proline-rich sequence, and phosphorylation of Y_{335} of Dgk-α mediate: *i)* its enzymatic activation, *ii)* its ability to interact respectively with SH3 and SH2 domains of Src, *iii)* its recruitment to the membrane. In addition we show that phosphorylation of Dgk-α on Y_{335} is required for HGF-induced motility, while its constitutive recruitment at the membrane by myristylation is sufficient to trigger spontaneous motility in absence of HGF. Providing the first evidence that tyrosine phosphorylation of Dgk-α is required for growth-factors-induced activation and membrane recruitment, these findings underscore its relevance as a rheostat, whose activation is a threshold to elicit growth factors-induced migratory signaling.

Introduction

Diacylglycerol kinases, which phosphorylate diacylglycerol (DAG) to generate phosphatidic acid (PA), comprise a family of ten distinct enzymes, grouped in five classes each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich C1 domains (Topham and Prescott 1999). Recent evidence showed that α, ζ and θ Dgk isoforms are regulated by extracellular ligands and play a role in signal transduction (reviewed in Luo et al. 2003 and van Blitterswijk and Houssa 2000). Dgk-α is activated by several growth factors: VEGF and HGF in endothelial and epithelial cells (Baldanzi et al. 2004, Cutrupi et al. 2000), and IL-2 in T cells (Cipres et al. 2003, Flores et al. 1999). In T cells Dgk-α and -ζ regulate cell sensitivity to TCR activation by negatively modulating the intensity and the kinetic of DAG-mediated signaling (Jones et al. 2002, Sanjuan et al. 2001, Sanjuan et al. 2003, Zhong et al. 2003). Indeed Dgk-θ and Dgk-ζ, but not Dgk-α, interact with different PKC isoforms, negatively regulating the duration of their activation (Luo et al. 2003, van Baal et al. 2005). In addition in T cells, pharmacological inhibition of Dgk-α enhances the C1-mediated recruitment of both RasGRP and PKC-θ (Carrasco and Merida 2004). Conversely, we previously showed that inhibition of Dgk-α activity, obtained either pharmacologically or by expression of dominant negative mutant or by RNA interference, impairs HGF-, VEGF- and ALK-induced chemotaxis and proliferation in several cell types (Baldanzi et al. 2004, Cutrupi et al. 2000, Bachiocchi et al. 2005), as well as *in vitro* angiogenesis in endothelial cells (Baldanzi et al. 2004). Similarly in T cells, pharmacological inhibition of Dgk-α severely impairs IL-2-induced G1-S phase transition (Flores et al 1999).

Activation of Dgk-α by tyrosine-kinase receptor and IL-2, requires Src-family tyrosine kinase activity and involves association of Dgk-α with either Src or Lck (Baldanzi et al. 2004, Cutrupi et al. 2000, Bachiocchi et al. 2005, Cipress et al. 2003). Furthermore, either pervanadate treatment of endothelial cells or constitutive activation of Lck in T cells result in tyrosine phosphorylation and activation of Dgk-α (Cutrupi et al. 2000, Cipress et al. 2003). Despite these data strongly suggest that Dgk-α is regulated by tyrosine phosphorylation, no tyrosine phosphorylation of Dgk-α had been detected upon stimulation with either HGF, VEGF, IL-2 or upon activation of the ALK receptor in

different cell types (Baldanzi et al. 2004, Cutrupi et al. 2000, Bachiocchi et al. 2005, Cipress et al. 2003).

Several evidences suggest that Dgk-α is activated upon its recruitment to the plasma membrane, through a mechanism requiring multiple steps. For instance, Cipres et al. showed that activation and recruitment of Dgk-α by IL-2 is mediated by binding to PIP_3 and requires the C1 domains of Dgk-α. However, these authors suggested that the lipid binding domain is masked in the three dimensional structure of Dgk-α, and that other molecular events, for instance calcium binding to the EF-hand domain, would unmask it (Cipress et al. 2003, Sanjuan et al. 2001).

Inhere we identify Y_{335} and the proline-rich C-terminal sequence as the molecular determinants of Dgk-α responsible for *i)* its tyrosine phosphorylation and activation upon HGF stimulation or upon oncogenic Src expression, *ii)* its recruitment to the membrane, and *iii)* its ability to transduce HGF chemotactic signaling. These results fully prove the biological relevance of tyrosine phosphorylation of Dgk-α in signaling pathways leading to cell migration elicited by growth factor or oncogenic Src.

Results

Tyrosine 335 and proline-rich C-terminal sequence are required for Src-induced tyrosine phosphorylation of Dgk-α, and for interaction respectively with Src-SH2 and -SH3 domain

We and others have previously shown that Dgk-α is activated by growth factors in a Src-dependent manner, and that it is tyrosine phosphorylated and activated upon co-expression with either Src or Lck (Cutrupi et al. 2000, Cipress et al. 2003). Observing Dgk-α sequence we noted two tyrosine residues featuring isoleucine in the -1 position, FLKIY$_{60}$LEVDN and PPSSIY$_{335}$PSVLA (Fig. 1), suggesting strong substrate selection by Src (Schmitz et al. 1996). In order to verify whether these two tyrosine residues are substrates of Src tyrosine kinase activity, we co-expressed Src in COS cells with myc-tagged Dgk-α, either wt, Y$_{60}$F, or Y$_{335}$F. Tyrosine phosphorylation of Dgk-α was evaluated by anti-phosphotyrosine western blot of anti-myc immunoprecipitates (Fig. 2). Upon co-expression with Src, Myc-Dgk-α-Y$_{335}$F does not feature any detectable tyrosine phosphorylation, while both Myc-Dgk-α wt and Myc-Dgk-α-Y$_{60}$F mutant are tyrosine phosphorylated. Anti-myc and anti-Src western blots confirmed uniform expression of transfected Src and Dgk-α proteins. Thus, this experiment indicates that Y$_{335}$ is the major site of phosphorylation of Dgk-α upon co-expression with Src, suggesting that contribution of Y$_{60}$ is negligible.

As optimal protein-substrate sequences for Src tyrosine kinase activity provide optimal consensus sequences for binding of SH2 domain of Src itself (Songyang et al. 1993, Songyang and Cantley 1995), we decided to investigate the ability of Y$_{335}$ of Dgk-α to mediate interaction with Src-SH2 domain in a *in vitro* pull down assay. Immobilized GST-Src-SH2 fusion protein was incubated with cell lysates obtained from serum cultured COS cells transfected with either empty vector or Myc-Dgk-α wt or mutants. Myc-Dgk-α wt was pulled down by GST-SrcSH2, but not by GST alone, indicating that Dgk-α interacts with Src-SH2 domain (Fig. 3*A*).

Intriguingly, Myc-Dgk-α-Y$_{335}$F, that shows a dramatically reduced phosphorylation upon co-expression with Src, fails to associate with GST-Src-SH2 in the pull down assay. Conversely, Myc-

Dgk-α-Y_{60}F was pulled down by GST-Src-SH2 as well as Myc-Dgk-α wt. These experiments demonstrate that phosphorylation of Y_{335}, but not of Y_{60}, enables Dgk-α to bind Src-SH2.

As several Src substrates, such as p130Cas, become tyrosine phosphorylated upon interaction of their proline-rich motif with Src-SH3 domain (Pellicena and Miller 2001), we verified whether Dgk-α interacts with Src-SH3 domain in a pull down assay. Immobilized GST-Src-SH3 was incubated with cell lysates obtained from serum cultured COS cells transfected with either empty vector or Myc-Dgk-α-wt or mutants. Myc-Dgk-α-wt and Myc-Dgk-α-Y_{335}F were specifically pulled down by immobilized GST-Src-SH3 but not by GST alone (Fig. 3*B*), indicating that indeed Dgk-α interacts with Src-SH3 domain.

Although Dgk-α does not contain a consensus sequence for SH3 interaction (PxxP), it features a highly conserved C-terminal proline-rich sequence (PMLMGPPPR, Fig. 1). Thus, we generated two deletion mutants lacking respectively the entire C-terminal half of Dgk-α (Myc-Dgk-α-STOP) or the last 13 aminoacids PPPRSTNFFGFLS (Myc-Dgk-α-ΔP). We assayed both mutants in the GST-Src-SH3 pull down assay. Fig. 3*B* shows that both Myc-Dgk-α-ΔP and Myc-Dgk-α-STOP mutants, differently from Myc-Dgk-α-wt and Myc-Dgk-α-Y_{335}F, are not pulled down by immobilized GST-Src-SH3 fusion protein. These data indicate that the proline-rich region is required for Dgk-α interaction with Src-SH3 (Fig. 3A).

Based on the model proposed for tyrosine phosphorylation of p130Cas (Pellicena and Miller 2001), we verified whether proline-rich tail of Dgk-α is required for Src-mediated tyrosine phosphorylation. We co-expressed in 293T cells Myc-Dgk-α either wt, ΔP or Y_{335}F with Src-Y_{527}F, an activated form of Src. Tyrosine phosphorylation of Myc-Dgk-α in anti-myc immunoprecipitates was assayed by anti-phosphotyrosine western blot. Fig. 4 shows that Myc-Dgk-α-ΔP and Myc-Dgk-α-Y_{335}F mutants are not tyrosine phosphorylated upon co-expression with Src-Y_{527}F, while Myc-Dgk-α-wt is tyrosine phosphorylated. Anti-myc and anti-Src western blots confirm uniform expression of transfected proteins, either wt or mutant.

Finally these data demonstrate, both in intact cells and *in vitro*, that the proline-rich tail of Dgk-α is required for interaction with Src-SH3 domain as well as for its tyrosine phosphorylation, suggesting that interaction of Dgk-α with Src SH3 domain may precede its tyrosine phosphorylation.

Tyrosine 335 and proline-rich C-terminal sequence are required for HGF- and v-Src-induced enzymatic activation of Dgk-α.

The data presented so far clearly indicate that Y_{335} and the pro-rich C-terminal sequence of Dgk-α are the major determinants for its Src-mediated tyrosine phosphorylation, and provide the reagents to investigate whether phosphorylation of Y_{335} is required for Src- and HGF-induced enzymatic activation of Dgk-α. Indeed while several evidence have firmly showed that activation of Dgk-α by growth factors depends on Src family tyrosine kinases, the putative role of its tyrosine phosphorylation in growth factor-induced enzymatic activation has been elusive (Baldanzi et al. 2004, Cutrupi et al. 2000, Bachiocchi et al. 2005, Cipress et al. 2003).

The enzymatic activity of Myc-Dgk-α either wt, Y_{335}F or ΔP, were assayed upon co-incubation with Src, in an *in vitro* activation assay performed with crude lysates obtained from either Src or Dgk-α transfected cells. Through this assay we had previously shown that enzymatic activity of Myc-Dgk-α wt is significantly increased upon co-incubation with Src cell lysates (dark column), as compared with control lysates (white columns) (Cutrupi et al. 2000 and Fig. 5). Conversely, the enzymatic activity of either Myc-Dgk-α-Y_{335}F or Myc-Dgk-α-ΔP mutant is not stimulated upon co-incubation with Src in vitro (Fig. 5). This finding provides the first direct demonstration that both Y_{335} and proline-rich sequence are required for activation of Dgk-α *in vitro*.

Next we investigated whether both Y_{335} and proline-rich sequence are also required for HGF-induced activation of Dgk-α in intact cells. We assayed the enzymatic activity of either Myc-Dgk-α-wt, Y_{335}F or ΔP mutant transiently transfected in COS cells, either control or HGF-stimulated (Fig. 6). The enzymatic activity was measured in whole cell lysates, as previously described; under these conditions, the contribution of endogenous diacylglycerol kinases to the total Dgk activity is negligible (Cutrupi et al. 2000 and data not shown). Fig. 6 indicates that, while enzymatic activity of Myc-Dgk-

α-wt is stimulated by HGF, the enzymatic activities of neither Myc-Dgk-α–Y_{335}F nor Myc-Dgk-α–ΔP mutants are stimulated upon HGF cell stimulation. Expression of Myc-Dgk-α-wt and mutants was verified by anti myc western blot (Fig. 6 lower panel).

In order to provide further evidence for the role of Y_{335} and proline-rich sequence as major determinants of Src-mediated activation of Dgk-α in intact cells, we investigated tyrosine phosphorylation and activation of Myc-Dgk-α either wt, Y_{335}F or ΔP in transiently transfected MDCK-*ts*-v-Src epithelial cells (Fig. 7). In these cells, *ts*-v-Src tyrosine kinase activity is impaired at 40 °C, and is activated upon shifting the cell culture to 35 °C (Behrens et al. 1993). Under these conditions, differently from COS and 293T cells, Myc-Dgk-α is not over-expressed, and it does not affect total Dgk activity assayed in whole cell lysates (data not shown). Endogenous canine Dgk-α cannot be detected for lack of specific antibodies.

Shifting MDCK-*ts*-v-Src cells to the permissive temperature results in both tyrosine phosphorylation (Fig. 7*A*) and enzymatic activation (Fig. 6*B*) of Myc-Dgk-α wt, as evaluated respectively by anti-phosphotyrosine western blot of anti-myc immunoprecipitates and *in vitro* Dgk-α assay. Next, we verified whether v-Src induces tyrosine phosphorylation and stimulates enzymatic activity of both Myc-Dgk-α–Y_{335}F and Myc-Dgk-α–ΔP. Activation of *ts*-v-Src fails to induce tyrosine phosphorylation of both Myc-Dgk-α$\tilde{Y}_{335}$F and Myc-Dgk-α–ΔP (Fig. 7A), and fails to stimulate their enzymatic activity (Fig. 7*B*). Expression of both mutants is comparable to the wild type (Fig. 7).

In summary these results, providing the first evidence *in vivo* that Dgk-α is a target of oncogenic Src, demonstrate that Src regulates Dgk-α *in vivo* through phosphorylation of Y_{335}. In addition, as both enzymatic activation and tyrosine phosphorylation of Dgk-α depend on its proline-rich sequence, these data suggest that interaction of Dgk-α proline-rich sequence with Src-SH3 domain is a prerequisite for its phosphorylation and enzymatic activation.

Tyrosine 335 and proline-rich C-terminal sequence are required for HGF-induced membrane recruitment of Dgk-α.

As Dgk-α is a cytosolic enzyme which associates to the plasma membrane upon growth factor stimulation (Flores et al. 1996, Sanjuan et al. 2003), we investigated whether phosphorylation of Dgk-

α on Y_{335} regulates its recruitment to the membrane upon HGF stimulation. In order to address this question, we investigated the subcellular localization of GFP tagged Dgk-α wt, Y_{335}F and ΔP mutants, transiently transfected in MDCK cells. We observed that in most of control transfected cells GFP-Dgk-α wt is localized exclusively in the cytosol, and that upon HGF stimulation it trnaloscates at the plasma membrane in the majority of transfected cells (70%) (Fig. 8A and 9C). In addition, kinase dead mutant (GFP-Dgk-α k-) behaves as the wild type, being diffuse in the cytoplasm in control cells and associated to the plasma membrane in HGF-stimulated cells (Fig. 8B).

To verify whether tyrosine phosphorylation of Dgk-α mediates HGF-induced membrane recruitment of Dgk-α we investigated the subcellular localization of both Y_{335}F and ΔP mutants. Surprisingly, in most of control transfected cells GFP-Dgk-α-Y_{335}F is associated to intracellular vesicles. Similarly GFP-Dgk-α-ΔP is also associated to intracellular vesicles, albeit of different shape and size, in all transfected cells Upon HGF stimulation, none of the two mutants translocate at the plasma membrane, while their vesicular localization is not affected (Fig. 9).

These observations demonstrate that Y_{335} and proline-rich sequence are required for proper localization of Dgk-α and suggest that phosphorylation of Y_{335} is a key event for HGF-induced recruitment to the plasma membrane. In addition, the vesicular localization of both GFP-Dgk-α-Y_{335}F and GFP-Dgk-α-ΔP suggest that the recruitment of Dgk-α to the plasma membrane may occur through vesicular traffic. If this holds true, we should expect that specific inhibition of vesicular traffic between the inner cytosol and the plasma membrane by BFA treatment, would result in accumulation of GFP-Dgk-α-wt in intracellular vesicles (Lippincott et al. 1989). Indeed, upon 15 minutes of treatment with 10μM BFA, even GFP-Dgk-α-wt associates to intracellular vesicles in unstimulated cells, and fails to translocate to the membrane following HGF stimulation (Fig. 10).

These observations strongly suggest that HGF-induced recruitment of Dgk-α to the plasma membrane depends on the integrity of the vesicular transport network, requires phosphorylation of Y_{335}, but does not requires its enzymatic activity.

Membrane recruitment and activation of Dgk-α at the membrane are necessary to transduce HGF migratory signalling and sufficient to induce cell motility.

As we previously showed that activation of Dgk-α is required for HGF- and VEGF-induced cell migration (Baldanzi et al. 2004, Cutrupi et al. 2000), we investigated whether Y_{335} contributes to the transduction of HGF pro-migratory signaling. HGF does not stimulate chemotaxis of COS-7 cells; however, transient over-expression of Myc-Dgk-α-wt makes COS-7 cells able to migrate in response to HGF in a transwell chemotaxis quantitative assay (Fig. 11a). This observation provides a functional assay to verify the requirement for phosphorylation of Y_{335} to transduce HGF-induced migratory signaling. Fig. 10 indicates that the expression of Myc-Dgk-α-Y_{335}F mutant impairs HGF-induced motility of COS cells, as compared with wild type. These data lend further support to the hypothesis that activation and membrane recruitment of Dgk-α, occurring through its phosphorylation on Y_{335}, are required for HGF-induced migratory signaling.

Next we asked whether Dgk-α constitutive recruitment to the plasma membrane provides sufficient signalling to stimulate cell motility. Sanjuan et al. had previously shown that myristylated Dgk-α is constitutively active and associated to the plasma membrane. Transient expression of myr-Dgk-α in COS cells, enhances three fold spontaneous migration of serum starved COS cells in absence of HGF in transwell chemotaxis assay and enhanced spontaneous cell migration in a wound healing assay (Fig 11b and 11c). These observations carried out in two different migration assays, indicate for the first time that constitutive activation of Dgk-α at the cell membrane provides rate limiting intracellular signals, both necessary and sufficient to stimulate cell migration.

Discussion

In recent years, diacylglycerol kinases have been intensively investigated either as negative or positive regulators of cell signaling. For instance activation of Dgk-α is required for growth factors-induced proliferative and chemotactic signaling (Baldanzi et al. 2004, Cutrupi et al. 2000, Cipres et al. 2003), as well as for negative feedback in TCR signaling (Jones et al. 2002, Sanjuan et al. 2001). Activation of Dgk-α by tyrosine-kinase receptors and IL-2, requires Src-family tyrosine kinase activity and involves association of Dgk-α to Src (Baldanzi et al. 2004, Cutrupi et al. 2000, Cipres et al. 2003). Furthermore both pervanadate treatment of endothelial cells and constitutive activation of Lck in T cells, result in tyrosine phosphorylation of Dgk-α and in stimulation of its enzymatic activity (Cutrupi et al. 200, Cipres et al. 2003). However, despite these data strongly suggest that Dgk-α is regulated by tyrosine phosphorylation, no tyrosine phosphorylation of Dgk-α has been detected upon growth factor stimulation. Thus the significance of tyrosine phosphorylation of Dgk-α for its growth factors-induced enzymatic activation, translocation to the plasma membrane, and for its role in growth factors cell signaling, has not proved yet.

Dgk-α contains at least two conserved tyrosine residues, Y_{60} and Y_{335}, both featuring Ile in -1 position, a signature for putative Src substrates (Schmitz et al. 1996). By phenilalanine substitution of either one of the two tyrosines, we showed that Y_{335}, rather than Y_{60}, is the major site of phosphorylation upon co-expression of Dgk-α with Src or v-Src, and is responsible for the association of Dgk-α with Src-SH2 domain. However, our data cannot rule out that upon phosphorylation of Y_{335}, Dgk-α may be then phosphorylated on other sites. The substitution of Y_{60}, differently from Y_{335}, does not affect either Src-induced tyrosine phosphorylation of Dgk-α, either its ability to interact with Src-SH2 domain. These observations suggest either that Y_{60} is not a phosphorylation site of Dgk-α, or that its phosphorylation is secondary to Y_{335} occurring at lower stoichiometry.

The observation that proline-rich sequence of Dgk-α is required for its interaction *in vitro* with Src-SH3, suggests that such interaction may participate in the mechanism leading to its phosphorylation by Src. Indeed, we showed that the proline-rich sequence is required for

phosphorylation and activation (see below) of Dgk-α by Src and HGF, both *in vitro* and in intact cells. These data are highly consistent with the current model for the interaction of Src with its targets, such as p130Cas (Kanemitsu et al. 1997, Scott and Miller 2000). According to this model, Src would first interact with Dgk-α through its SH3 domain, and then it would phosphorylate it on Y_{335}. Subsequently, phosphorylated Y_{335} would become a docking site for Src-SH2 domain, and may lead to the stabilization of the Dgk-α/Src complex, and eventually to phosphorylation of multiple secondary sites, providing additional docking sites for SH2-containing proteins. Alternatively, phosphorylation of Y_{335} itself, may allow interaction of Dgk-α with other SH2-containing proteins.

Phenylalanine substitution of Y_{335} abrogates both HGF- and v-Src-induced activation of Dgk-α in intact cells, while it does not affect its basal activity. In addition, even deletion of proline-rich sequence of Dgk-α, which impairs its tyrosine phosphorylation, significantly reduces enzymatic activation without affecting its basal activity. Both these observations support our conclusion that phosphorylation of Y_{335} dictates the ability of Dgk-α to be stimulated by both growth factors and v-Src activation.

Y_{335} lies in a linker sequence between the second C1 and the kinase domain, which according to the surface exposition plot (http://scansite.mit.edu/), features high surface accessibility. We may speculate that phosphorylation of Y_{335} acts as a molecular switch, which inhibits an intra-molecular interactionand and shift Dgk-α toward an open active configuration and/or a configuration able to interact with an activator. A similar model has been demonstrated for growth factors-induced activation of Raf-1, whose activity is stimulated by Src-mediated phosphorylation of Y_{340}, a residue placed, like Y_{335} of Dgk-α, in a linker region between the C1 domain and the catalytic domain (Tran and Frost 2003, Mason et al. 1999).

Activation of soluble enzymes acting on lipid substrates is tightly coupled to their recruitment to the membrane, where they encounter their substrates as well as their regulators. PI 3-kinase, PLC-γ, PI4P 5-kinase and PLD are mostly cytosolic proteins which are recruited to the membrane through their ability to interact with tyrosine phosphorylated receptors. membrane bound small GTPases, lipids and other membrane associated scaffolding proteins (Santarius et al. 2006 ...). The data presented inhere clearly demonstrate that both Y_{335} and proline-rich sequence are major determinants for both

membrane recruitment and activation of Dgk-α upon HGF cell treatment. These results are highly consistent with recently reported data showing that phosphorylation of Y_{335} of murine Dgk-α is required for vitamin E-induced membrane recruitment and for its enzymatic activation (Fukunaga 2005). Thus we may speculate that phosphorylation of Y_{335} may displace an intra-molecular interaction, opening the access to a membrane-binding sequence. This event may regulate the interaction of Dgk-α with DG, its lipid substrate, and with a putative activator such as PIP3 or with a membrane associated protein. Intriguingly atypical C1 domains, featured by most diacylglycerol kinase isforms, which differently from typical C1 domains, does not bind DG and phorbol esters, has been suggested to interact with small GTPases (Hurley et al. 1997). Thus it is highly suggestive to speculate that tyrosine phosphorylation may enable Dgk-α to interact with a protein-bound small GTPase. On the other hand Cipres et al. showed that direct interaction with PIP_3 determines membrane recruitment and activation of Dgk-α upon IL-2 stimulation, and that PI 3-kinase is required for Dgk-α activation upon active Lck over-expression (Cipres et al. 2003). However upon HGF stimulation of epithelial cells, membrane recruitment of Dgk-α does not require PI 3-kinase, while it is dependent on PLC-gamma and Src activity (data not shown).

Finally the observation that phosphorylation of Y_{335} is required for membrane recruitment and for enzymatic activation of Dgk-α, but becomes undetectable when the protein is still active, suggest that transient phosphorylation of Y_{335} would act as a switch allowing the direct interaction of atypical C1 domain with either a membrane protein or lipid. This model is consistent with our finding that activation of Dgk-α *in vitro* by Src requires the presence of intact membranes, as does not occur by co-incubation of purified proteins (data not shown). Moreover according to this model, activation of Dgk-α would generate a coincidence signal derived from time- and space-coincidence of two independent signals, activated Src and a still unidentified membrane signal, either lipidic or proteic.

Alternatively, the surprising observation that both Myc-Dgk-α-Y_{335}F and Myc-Dgk-α-ΔP mutants are associated to intracellular vesicles rather than being diffuse in the cytosol, may suggest that the failure of both mutants to be phosphorylated in intact cells, depends on their mislocalization and segregation from Src. However both mutants, differently from the wild type, are not activated by Src in the *in vitro* assay with cell extracts (Fig. 3), and are not tyrosine phosphorylated by Src in an *in vitro*

assay with purified recombinant proteins (data not shown). Moreover in intact cells Src and Dgk-α mutants do not appear to be segregated from each other, as observed in immunofluorescence (data not shown). These observations make unlikely that the defective phosphorylation and activation of both mutants may depend on their mislocalization.

The inability of both Dgk-α mutants to be activated and/or tyrosine phosphorylated by Src, and their vesicular localization, might also depend on their putative misfolding. However as both protein mutants feature the same basal enzymatic activity as the wild type (data not shown), it is unlikely that their inability to be activated by Src, depends on their putative misfolding. Moreover even wild type Dgk-α localize to similar vesicles upon cell treatment with low doses of Brefeldin A (Fig. 10) (see below).

The surprising observation that both Myc-Dgk-α-Y_{335}F and Myc-Dgk-α-ΔP are associated to intracellular vesicles rather than being diffuse in the cytosol, suggest that phosphorylation of Y_{335} by Src may be required to couple Dgk-α to vesicular transport from the inner cytoplasm to the plasma membrane. Consistently with this hypothesis BFA treatment results in the accumulation of both wt Dgk-α and Src on intracellular vesicles (Kaplan et al 1992 and Fig. 9).

Intriguingly upon growth factor stimulation, Src itself is recruited from the perinuclear area to the plasma membrane through Rab11-dependent endosomal traffic (Sandilands et al. 2004). Based on these observations we may speculate that SH3- and SH2-mediated interaction with Src, may couple Dgk-α to the endosomal traffic machinery responsible for Src targeting from the perinuclear region to the plasma membrane. This speculation is consistent with previous data reporting arachidonate-induced association of Dgk-α to the Golgi in CHO cells (Shirai et al. 2000), and that Dgk-α associates with the trans Golgi network and late endosomal compartments, regulating the secretion of FAS-L bearing lethal exosomes (Alonso et al 2005). In addition over-expression of Dgk-δ, bearing distinct regulatory domains from alpha isoform, suppresses ER to Golgi traffic, and inhibits Golgi reassembly following BFA treatment and washing (Nagaya et al. 2002). However, the investigation of the role of Dgk-α in endosomal traffic and the characterization of intracellular vesicles associated to Myc-Dgk-α-Y_{335}F and Myc-Dgk-α-ΔP mutant are beyond the scope of this communication.

We and others had previously shown that activation of Dgk-α is required for growth factors-induced cell migration and proliferation (Baldanzi et al. 2004, Cutrupi et al. 2000, Bachiocchi et al. 2005, Flores et al 1999). The biological relevance of Dgk-α activation and membrane recruitment in conveying growth factors-induced migratory signal is underscored by the findings that in COS cells, HGF-induced motility strictly depends on the extent of expression of Dgk-α and on the presence of Y335. Such relevance is further enhanced by the demonstration that constitutive recruitment of Dgk-α at the membrane provides intracellular signalling sufficient to trigger spontaneous cell motility, even in un-stimulated cells.

This finding demonstrates that phosphorylation of Y_{335} is indeed required to transduce HGF chemotactic signaling and suggests that activation of Dgk-α may finely tune threshold signals coordinating the function of downstream targets.

The specific signaling pathways regulated by activation of Dgk-α still await elucidation. Activation of Dgk-α, by both terminating DG-mediated signaling and activating PA-mediated signaling, may finely coordinate the function of downstream targets of both lipid second messengers. Although a specific PA binding domain has not been clearly identified, PA binds and regulates several signaling proteins, including PI(4)P 5-kinase, mTor, PKC-ε, Raf and NADPH oxidase complex (Topham and Prescott 1999), which are involved in tyrosine kinase receptor signaling. Alternatively, as the ratio between PA and its metabolite LPA has been shown to regulate membrane curvature during membrane fission in endocytosis (Ohashi et al. 1995, Kooijman et al. 2003), activation of Dgk-α may be involved in the regulation of either plasma and endosomal membrane shape and dynamics.

Materials and Methods

Cells Culture - COS-7 and HEK 293T cells were obtained from ATCC, MDCK and MDCK-ts-v-Src (Behrens et al. 1993) are a kind gift of W. Birchmeier (Berlin). COS-7, HEK 293T, MDCK and MDCK-ts-v-Src were cultured in high glucose DMEM (Sigma), supplemented with glutamine, 10% fetal calf serum (Gibco) and antibiotic-antimycotic solution (Sigma).

Reagents - Recombinant HGF was from Peprotech, anti phosphotyrosine 4G10 and anti-Myc 9E10 antibodies were from Upstate Biotechnology. Src-2 anti-Src antibodies were from Santa Cruz. Secondary antibodies anti-mouse and anti-rabbit IgG HRP-labeled were from NEN (PerkinElmer life sciences). Alexa Fluor 456 Falloidin was from Molecular Probes.

Construction of expression vectors and site directed mutagenesis - Myc tagged Dgk-α c-DNA cloned into pMT2 expression vector was previously described (Cutrupi et al. 2000). GFP-Dgk-α wt was obtained by cloning Dgk-α wt in pcDNA-DEST53 (Invitrogen) using the Gateway kit (Invitrogen). GST-Dgk-α wt was obtained by cloning Dgk-α wt in pcDEST-27 (Invitrogen) using the Gateway kit (Invitrogen). Detailed information and protocols on the Gateway technology are available on www.invitrogen.com. Point mutations on Dgk-α were obtained using QuikChange Site-Directed Mutagenesis Kit (Stratagene); mutating oligonucleotides were:

$Y_{60}F$ GGGAACATTATCCACTTCGAGGAAGATTTTCAGGAATTGCTG and CAGCAATTCCTGAAAATCTTCCTCGAAGTGGATAATGTTCCC;

$Y_{335}F$ GGCCAGGACACTGGGAAAGATGGAAGATGGAGG and CCTCCATCTTCCATCTTTCCCAGTGTCCTGGCC;

STOP GATGATTTAAATTAGAGCACCTCTGAGGCT and AGCCTCAGAGGTGCTCTAATTTAAATCATC;

K- CGGATTGGTGTGTGGTGACGACGGCACAGTAGGC and GCCTACTGTGCCGTCGTCACCACACACCAAAATCCG.

Dgk-α-ΔP was obtained by insertion of the annealing product of the oligonucleotide CATAACTGCAGTTATGGGCC at the ApaI site at position 2168 of Dgk-α wt cDNA. All mutants

used have been verified by direct sequencing (MWG biotech or C.R.I.B.I.-BMR). Plasmid encoding Src wt, $SrcY_{527}F$ and $SrcY_{527}F$ K- were a kind gift of G. Superti-Furga.

Transfection with plasmid vectors and stimulation - COS-7 AND HEK 293T cells were transiently transfected with Cell-Phect Transfection kit (Amersham-Pharmacia) using respectively DEAE-dextrane or calcium phosphate method. MDCK and MDCK-ts-v-Src were transfected by lipofectamine 2000 (Invitrogen). Cells were lysed after 48 hours from transfection and expression of transfected protein verified by western blot. For HGF stimulation experiments, cells were serum starved for 16 hours and then stimulated for 15 minutes with recombinant HGF (100 ng/ml). MDCK-ts-v-Src were made quiescent by culturing in 0.1 % serum at the non-permissive temperature of 40 °C for 16 hours and then switched to the permissive temperature of 35°C for 1 hour. When inhibitors were used, they were added 15 minutes before stimulation and controls were treated with equal amounts of vehicle (DMSO).

Preparation of Cell Lysates, Homogenates, Immunoprecipitation, Western Blotting - Cells were lysed in buffer A (25mM Hepes pH 8, 1% NP-40, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM ammonium molibdate, 10 mM NaF, 1mM sodium orthovanadate and protease inhibitor cocktail (Sigma)) (18). Cells homogenates (Fig. 5 and Sup. mat. 1) were prepared by collecting the cells with a rubber scraper in buffer B (buffer A without NP-40), homogenizing them with a 23 G syringe (Sigma) and by spinning at 500 x g for 15 minutes. Protein concentration was determined by the BCA method (Pierce) and equalized for each point using buffer. Immunoprecipitation, SDS-PAGE and western blots were performed as previously described (18). Western blot results were acquired and quantified with Versadoc system (BIORAD).

Dgk-α assay – Dgk-α activity in cell homogenates (25 µl) was assayed by measuring initials velocities (5 minutes at 30°C) in presence of saturating substrates concentration (1 mg/ml diolein (Fluka), 5 mM ATP, 3 µCi/µl [α^{32}P]-ATP (Amersham), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM EGTA in 25 mM Hepes pH 8, final reaction volume 50 µl). Lipids were extracted as described (Graziani et al. 1991), and PA was separated by TLC in chloroform: methanol: water: 25% ammonium hydroxide (60:47:11:4). TLC plates had been previously coated with (potassium oxalate 1.3%, EDTA 5mM):(methanol) 3:2 and desiccated. [^{32}P]-PA was identified by co-migration with non radioactive

PA standards stained by incubation in iodine chamber. Radioactive signals were detected and quantified by GS-250 Molecular Imager and Phosphor Analyst Software (BIO-RAD). The experiments of activation *in vitro* were carried out by co-incubating the homogenates (10 μg protein) for 15 min at 15°C in presence of 1mM ATP and 5mM MgCl2, as previously reported (Cutrupi et al. 2000).

Purification of GST fusion proteins - pGEX, pGEX-Src-SH2 and pGEX-Src-SH3 were a gift from L. Cantley. GST-Src-SH3, GST-Src-SH2 and GST were expressed in E. coli and purified according to standard protocol. In brief protein synthesis was induced with 1 mM IPTG and cells were harvested 4 h later by centrifugation. Pellet were resuspended in buffer G (50 mM Tris-HCl, 100 mM NaCl, 5% glycerol, pH 8) and cells disrupted by sonication (Branson). Supernatants were collected by centrifugation (15 minutes at 12000 x g) and purified on glutathione-sepharose column (Amerscham Pharmacia). The matrix with the attached proteins was removed from the column and used for the subsequent pull down experiments. Purity and quantity of proteins were determined by SDS page and Coomassie-blue staining, usually purity was ≥ 80%.

In vitro pull-down with GST-fusion proteins - 50 μg of the fusion protein immobilized on glutatione-sepharose resin was incubated for 1 h at 4°C with the indicated lysate (500 μg protein), and washed as for immunoprecipitations. Pulled down proteins were solubilized in Laemmli buffer and analyzed by western blot.

Cell staining and confocal microscopy - MDCK cells were seeded on glass coverlips (Marienfeld) settled at the bottom of the wells of 24-well cell culture plates, cultured to appropriate confluence and then transfected. Before stimulation, cells were serum starved overnight in DMEM and then stimulated with HGF 50 ng/ml. Where indicated cells were pre-treated with 10 μM PP2 for 15 min. After stimulation, cells were washed twice in PBS and fixed with fixing solution (3% paraformaldeyde-4% sucrose in PBS) for 5 minutes at room temperature. After 2 washes in PBS, cells were permeabilized with a HEPES-Triton Buffer (20mM HEPES pH 7.4, 300 mM sucrose, 50 mM NaCl, 3mM $MgCl_2$, 0.5% Triton X-100) for 5 minutes at 4°C. Cells were then washed three times with PBS containing 0.2% BSA and incubated for 15 minutes with PBS containing 2% BSA. TRITC-falloidin (1:100 in PBS-2% BSA) was added directly onto the glass plates in the humidified chamber

for 30 minutes and the excess was washed away by three wash with PBS-0.2% BSA. Each glass coverlips was washed briefly in water and blocked onto a glass microscope slide with Mowiol (20% Mowiol 4-88 in PBS 1X pH 7.4). Images were acquired with a 63 X objective using a Leica TCS SP2 Confocal Microscope.

Cell migration assay –

Cos cells, transfected and serum starved as indicated, were seeded (10^7 cells/ml in 200 µl suspension 0,1% FCS) in 8 µm pore size transwell (Corning-Constar). The lower chamber was filled with 0,1% FCS medium with or without HGF (50 U/ml) and incubated at 37°C in air with 5% CO2 for 8 hours. Cells remaining in the insert were then mechanically removed and the lower surface of filters stained with crystal violet and counted at the inverted microscope.

Statistical analysis - Statistical test used is two tails t test. Data on graph are shown as mean ± SEM.

Acknowledgements

We thank Cecilia Deantonio and Marianna Notario for their helpful work throughout the project.

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Titles and legends to figures

Fig. 1 Dgk-α structure

The structure of Dgk-α contains three structurally defined domains: 2 E-F hand motifs, a double C1 domain and a catalytic domain. Y_{60} lies upstream of the EF-hand motif, Y_{335} between the second C1 domain and the catalytic domain, and the proline rich region at the C-terminal end.

Fig. 2 Dgk-α phosphorylation by Src on tyrosine 335

Growing COS-7 cells co-transfected with the indicated Myc-Dgk-α and Src constructs were lysed in detergent-containing buffer A. Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analyzed by western blot with anti phosphotyrosine (upper panel), and anti-myc antibodies (lower panel). Total cell lysates were analyzed with anti Src antibodies (right panel).

Fig. 3 Dgk-α interaction with Src-SH2 and Src-SH3 domains

(A) Growing COS-7 cells, transfected with indicated Myc-Dgk-α constructs, were lysed in buffer A. Cell lysates were incubated with agarose-bound purified GST or GST-Src-SH2 for a pull-down assay. Pulled-down Myc-Dgk-α (left panel) and Myc-Dgk-α expression in total cell lysates (right panel) were detected by anti-myc western blot.

(B) Growing COS-7 cells, transfected with indicated Myc-Dgk-α constructs, were lysed in buffer A. Cell lysates were incubated with agarose-bound purified GST or GST-Src-SH3 for a pull-down assay. Pulled-down Myc-Dgk-α (left panel) and Myc-Dgk-α expression in total cell lysates (right panel) were detected by anti-myc western blot.

Fig. 4 Dgk-α phosphorylation by Src requires tyrosine 335 and proline rich C-terminal sequence

Growing HEK 293T co-transfected with the indicated Myc-Dgk-α and Src-Y_{527}F constructs were lysed in detergent-containing buffer A. Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analyzed by western blot with anti phosphotyrosine (upper panel), and anti-myc antibodies (middle panel). Total cell lysates were analyzed with anti Src antibodies (lower panel).

Fig. 5 Dgk-α activation by c-Src in vitro requires tyrosine 335 and proline rich C-terminal sequence

COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-$Y_{335}F$, Myc-Dgk-α-ΔP or Src were homogenized with buffer B in absence of detergent. Cell extracts were mixed as indicated in presence of 1mM ATP for 15 minutes, and analyzed for Dgk activity (upper panel). Values are mean ± SEM of six independent experiments normalized for sample incubated with control lysate (* t-test P = 0.05). Myc-Dgk-α protein expression in a typical experiment was verified by anti-myc western blot (lower panel).

Fig. 6 Dgk-α activation by HGF in vivo requires tyrosine 335 and proline rich C-terminal sequence

COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-$Y_{335}F$, Myc-Dgk-α-ΔP were stimulated with HGF (100 μg/ml, 15 minutes), homogenized with buffer B in absence of detergent and analyzed for Dgk activity (upper panel). Values are mean ± SE of triplicates normalized for unstimulated samples (b * t-test P = 0.01). Myc-Dgk-α protein expression was verified by anti-myc western blot (lower panel).

Fig. 7 Dgk-α phosphorylation and activation by v-Src requires tyrosine 335 and proline rich C-terminal sequence

Ts-v-Src/MDCK cells transfected with the indicated Myc-Dgk-α constructs, were cultured at non permissive temperature (40°C), and, where indicated, shifted at the permissive temperature (35°C) for 1 hour.

(A) After lysis in detergent-containing buffer A, myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analyzed by western blot with anti-phosphotyrosine antibodies (upper panel), and anti-myc antibodies (lower panel).

(B) Cells were homogenized in buffer B, not containing detergent, and homogenates were assayed for Dgk activity (upper panel). Values are mean ± SE of triplicates normalized for activity at the non

permissive temperature (* t-test, P = 0.01). Myc-Dgk-α protein expression was verified by anti-myc western blot (lower panel).

Fig. 8 Dgk-α is recruited at cell membrane upon HGF treatment

MDCK cells transfected with GFP-Dgk-α wt or GFP-Dgk-α-K- were treated with HGF (50 ng/ml, 15 minutes). Cells were stained with falloidin-TRITC and images acquired by confocal microscopy (scale bar 16 μm).

Fig. 9 Recruitment of Dgk-α at cell membrane requires tyrosine 335 and proline rich C-terminal sequence.

MDCK cells tranfected with either GFP-Dgk-α-wt, GFP-Dgk-α-ΔP (A) or GFP-Dgk-α-Y_{335}F (B) were stimulated with HGF (50 ng/ml, 15 minutes). Cells were stained with falloidin-TRITC and images acquired by confocal microscopy (scale bar 16 μm).

(C) For each point, more than an hundred cells were scored for Dgk-α localization: membrane (filled bars), cytoplasm (empty bars), vescicles (dashed bars).

Fig. 10 Brefeldin causes accumulation of Dgk-α on cytoplasmic vesicles

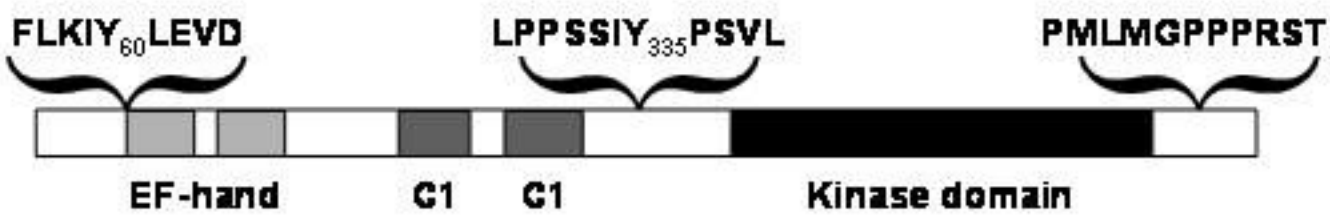
MDCK cells transfected with GFP- Dgk-α wt were treated with HGF (50 ng/ml, 15 minutes). Where indicated cells were pre-treated with BFA (10 μM BFA for 15 minutes). Cells were stained with falloidin-TRITC and images acquired by confocal microscopy (scale bar 16 μm).

Fig. 11 Membrane recruitment of Dgk-α is necessary and sufficient for cell motility

(a) COS cells transfected with either Dgk-α wt, or Y335 mutant or empty vector were stimulated to migrate by HGF (100 ng/ml) in a transwell chemotaxis assay. Data are expressed as fold increase over control, values are mean ± SE of four independent experiments (* paired t-test, P 0,07).

(b) Spontaneous cell migration of COS cells transiently transfected with myr-Dgk-α in a transwell chemotaxis assay. Data are expressed as fold increase over control, values are mean ± SE of four independent experiments (* paired t-test, P 0,06)

(c) Spontaneous cell motility of COS cells transiently transfected myr-with Dgk-α was assayed in a wound healing assay. A representative field of multiple experiments is shown.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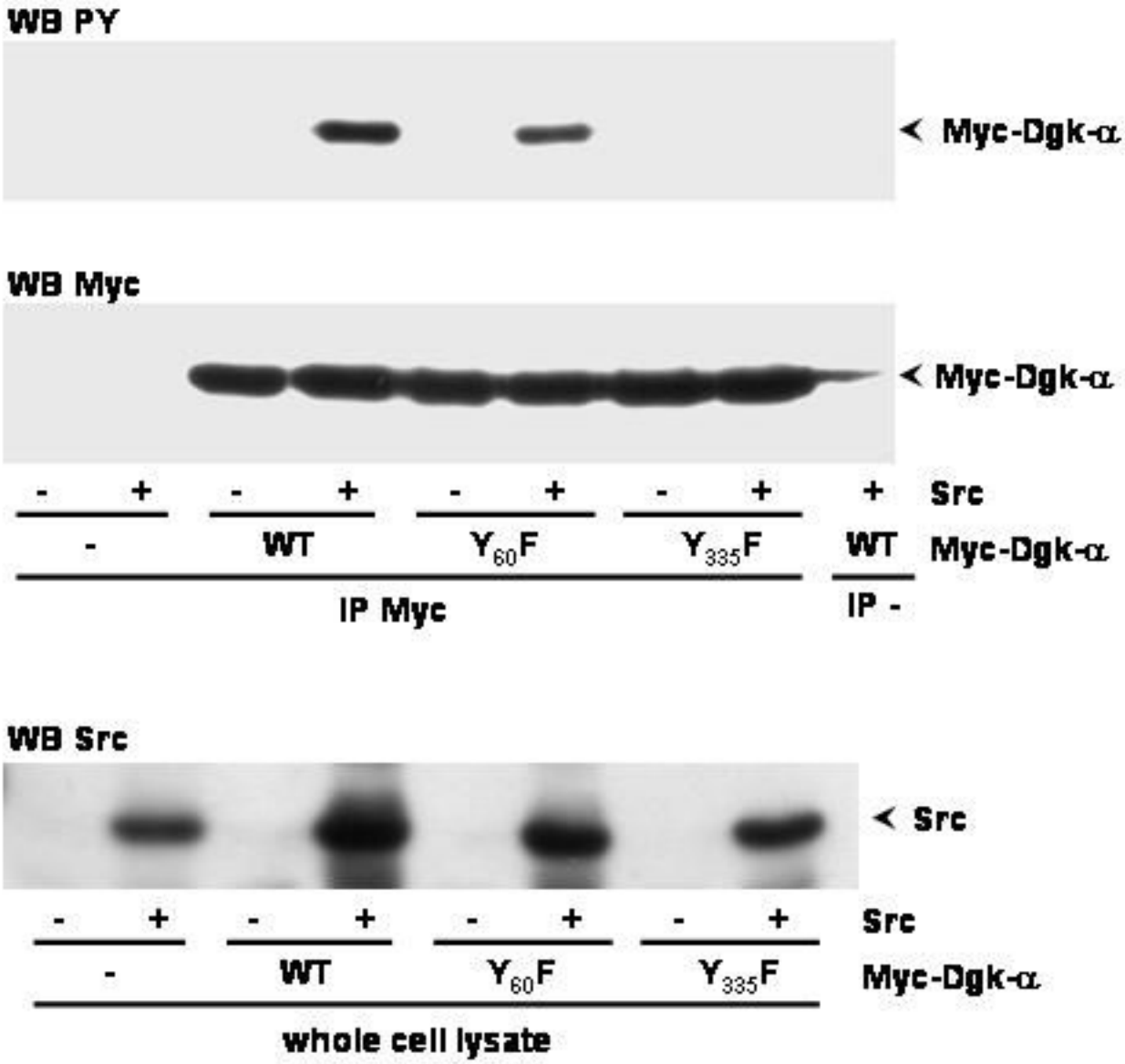
EF-hand
C1
C1
Kinase domain

Fig. 1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IP Myc
IP -
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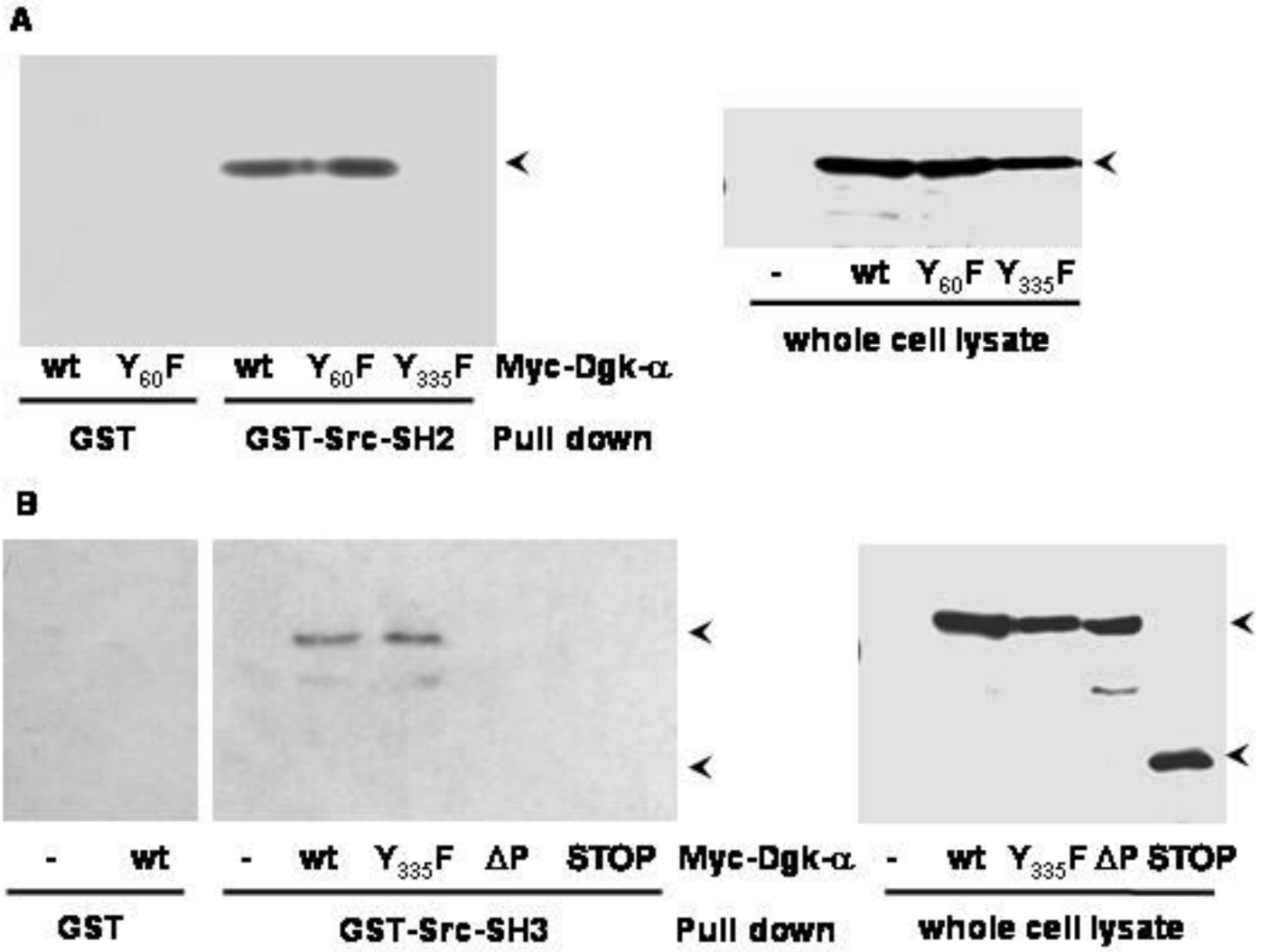
whole cell lysate

Fig. 2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whole cell lysate
B
-
wt
-
wt
Y335F
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whole cell lysate

Fig. 3

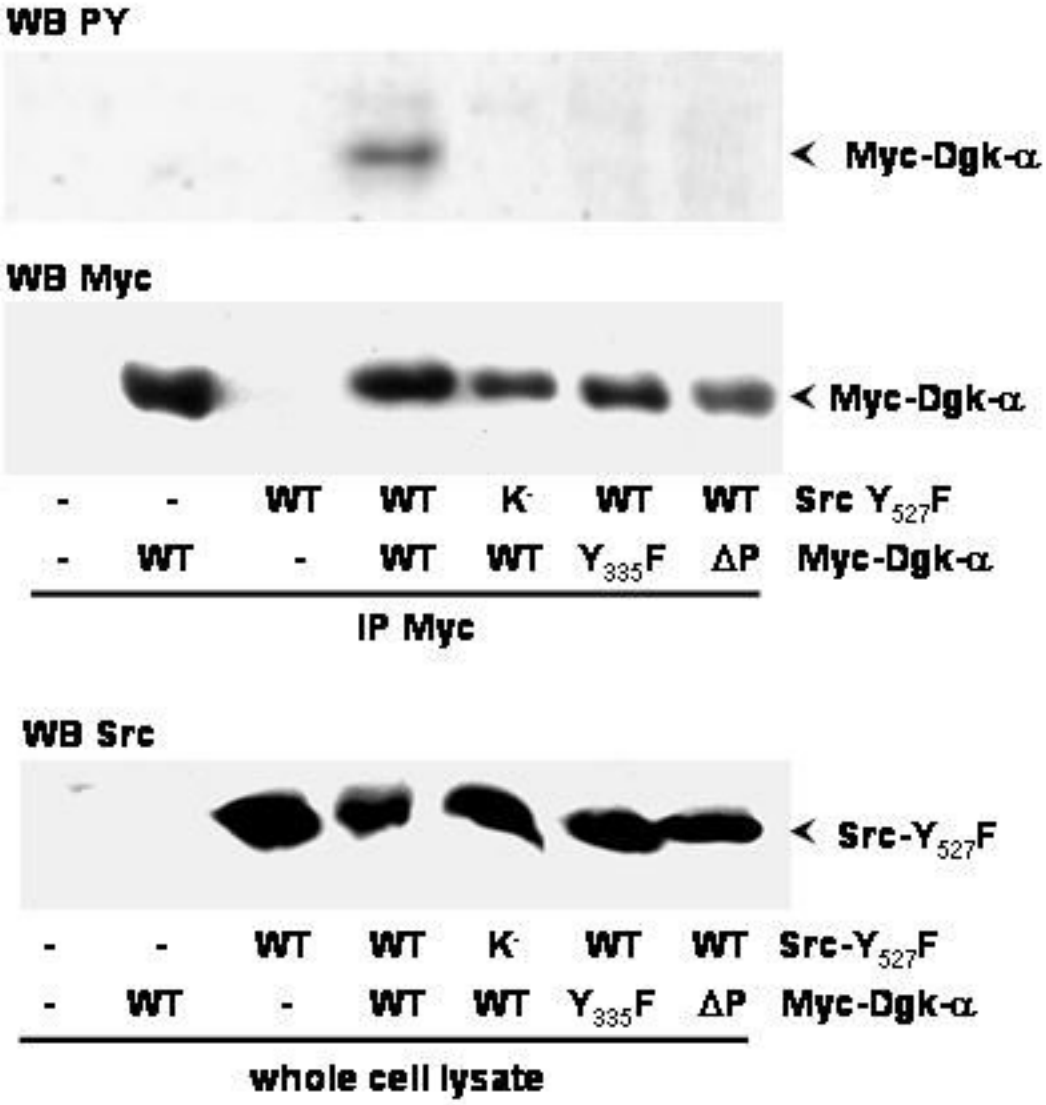
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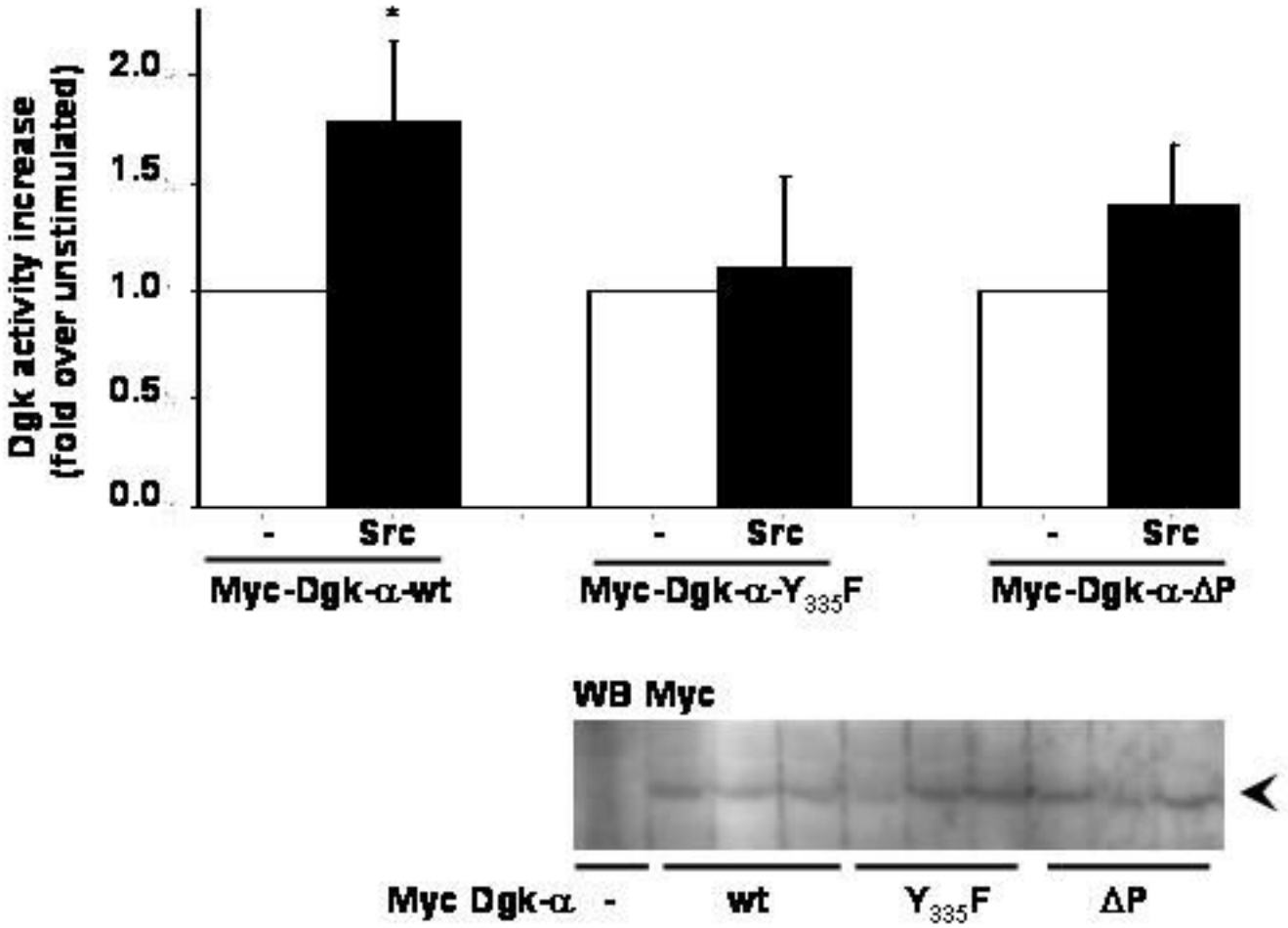
Fig. 4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1.5
1.0
0.5
0.0
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Src
-
Src
-
Src
Myc-Dgk-α-wt
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Myc Dgk-α
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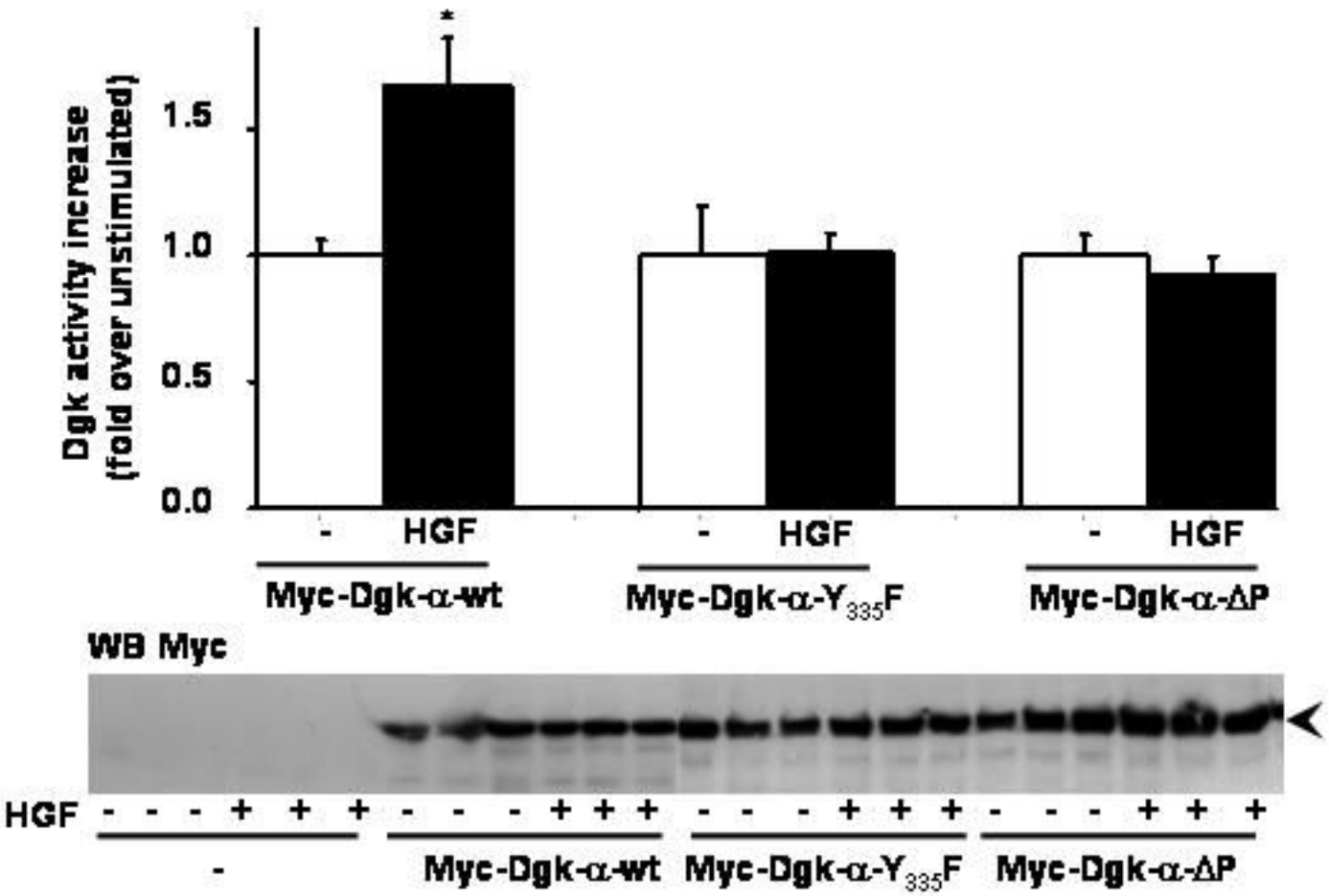
wt
Y335F
ΔP

Fig. 5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Myc-Dgk-α-ΔP
WB Myc
HGF - - - + + + - - - + + + - - - + + + - - - + + +
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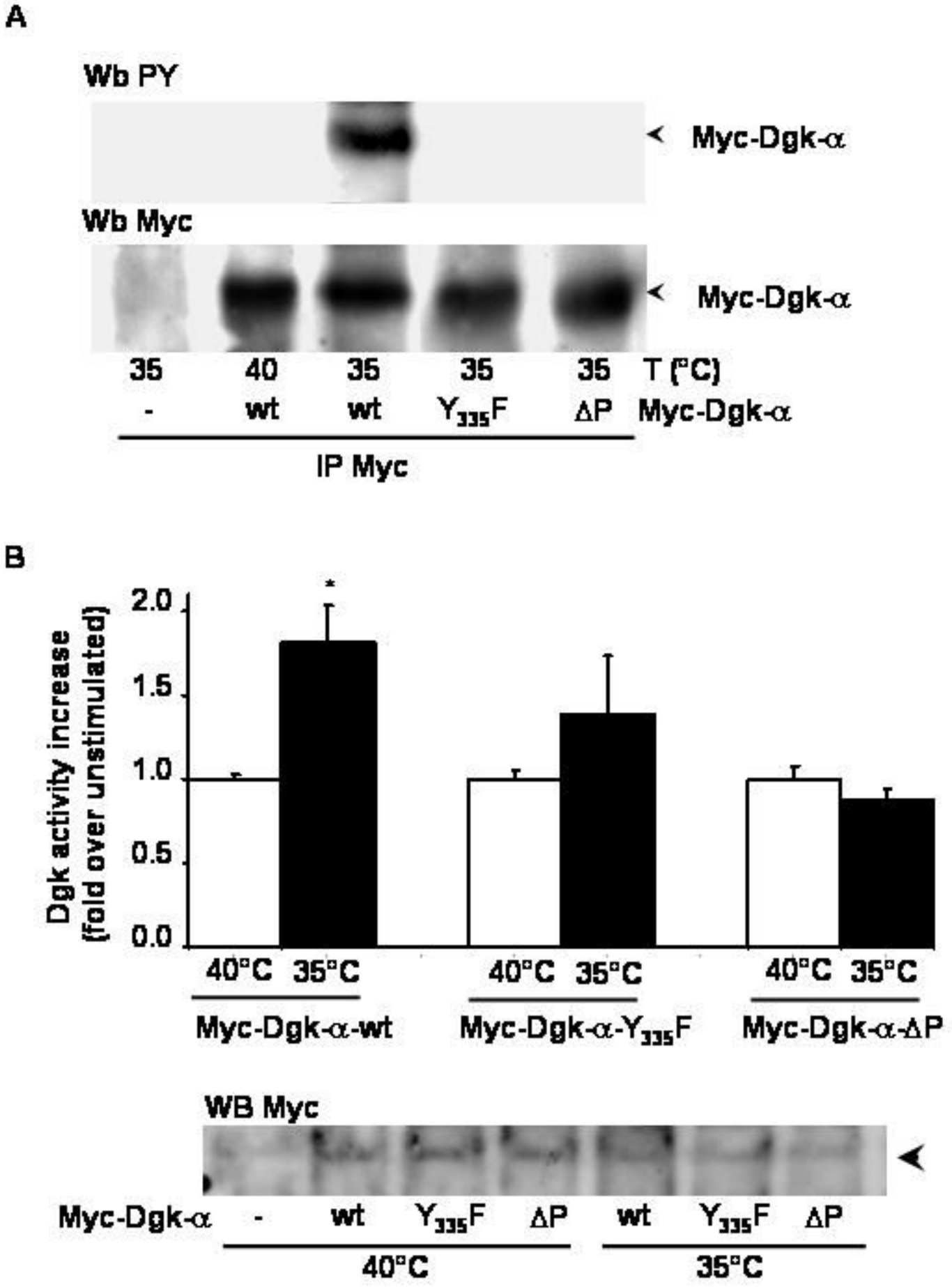
Fig. 5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Wb Myc
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IP Myc
B
Dgk activity increase (fold over unstimulated)
2.0
1.5
1.0
0.5
0.0
*
40°C 35°C
40°C 35°C
40°C 35°C
Myc-Dgk-α-wt
Myc-Dgk-α-Y335F
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40°C
35°C

Fig. 7

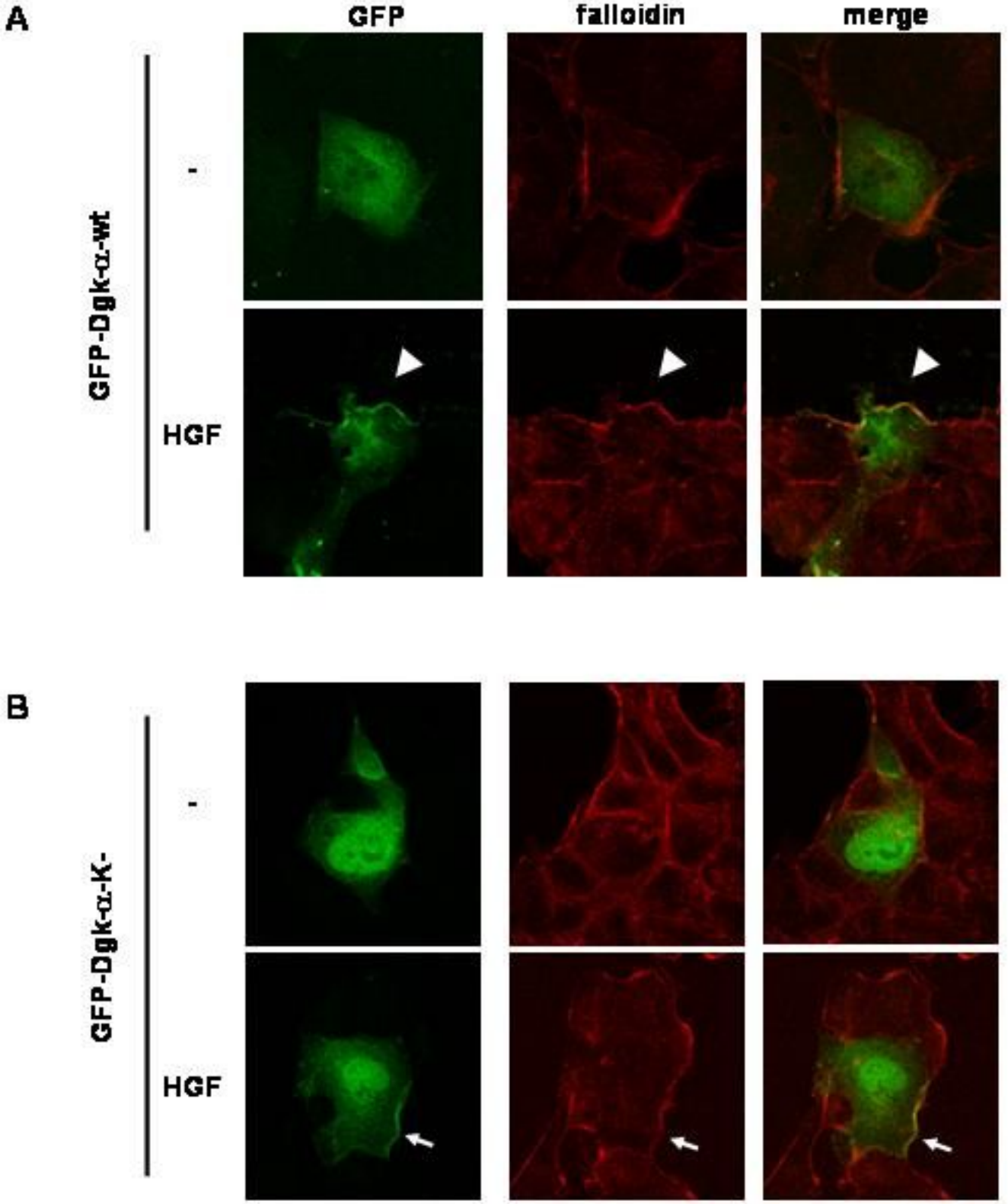
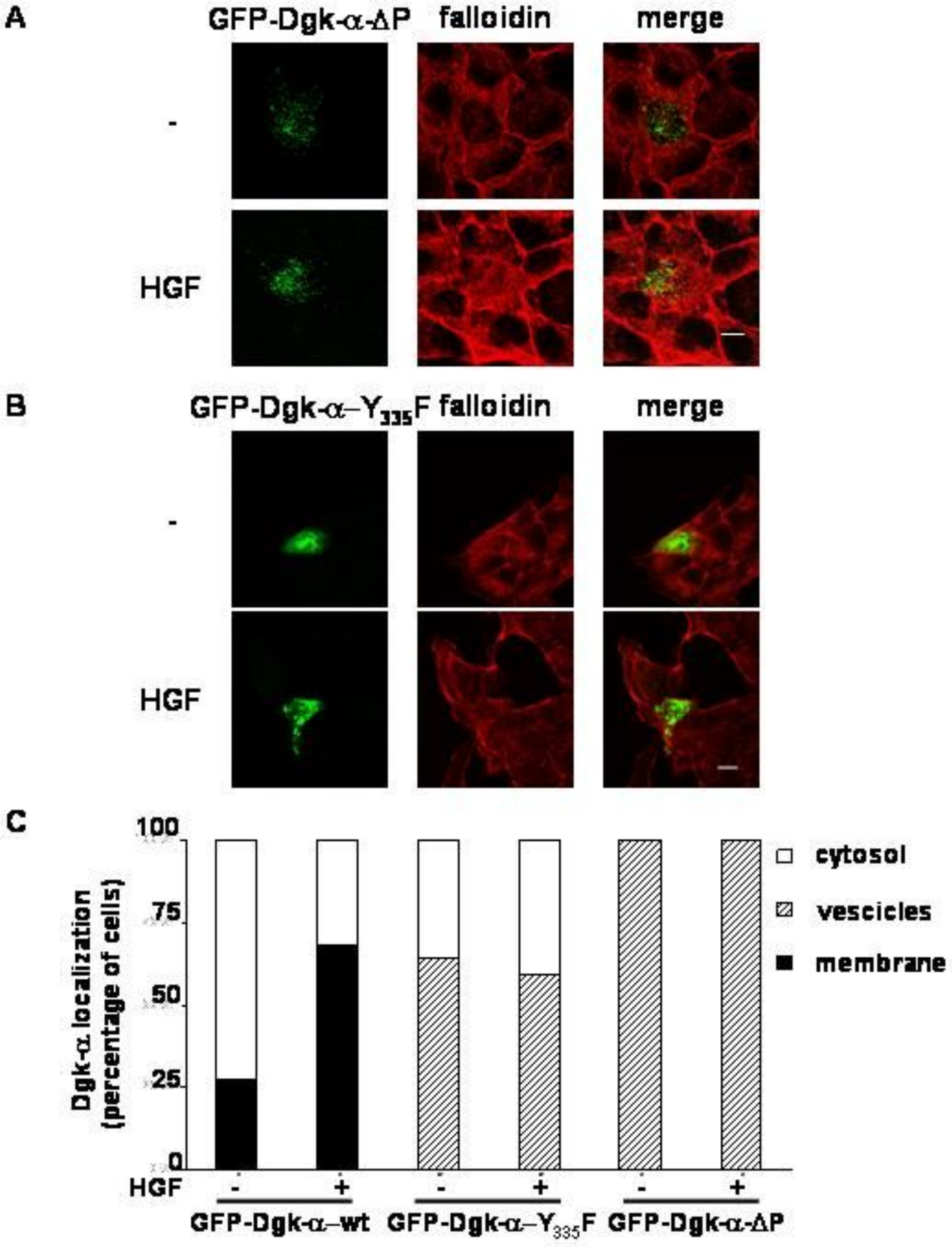


Fig. 8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C
Dgk-α localization (percentage of cells)
100
75
50
25
0
cytosol
vescicles
membrane
HGF
-
+
-
+
-
+
GFP-Dgk-α–wt
GFP-Dgk-α–Y335F
GFP-Dgk-α-ΔP

Fig. 9

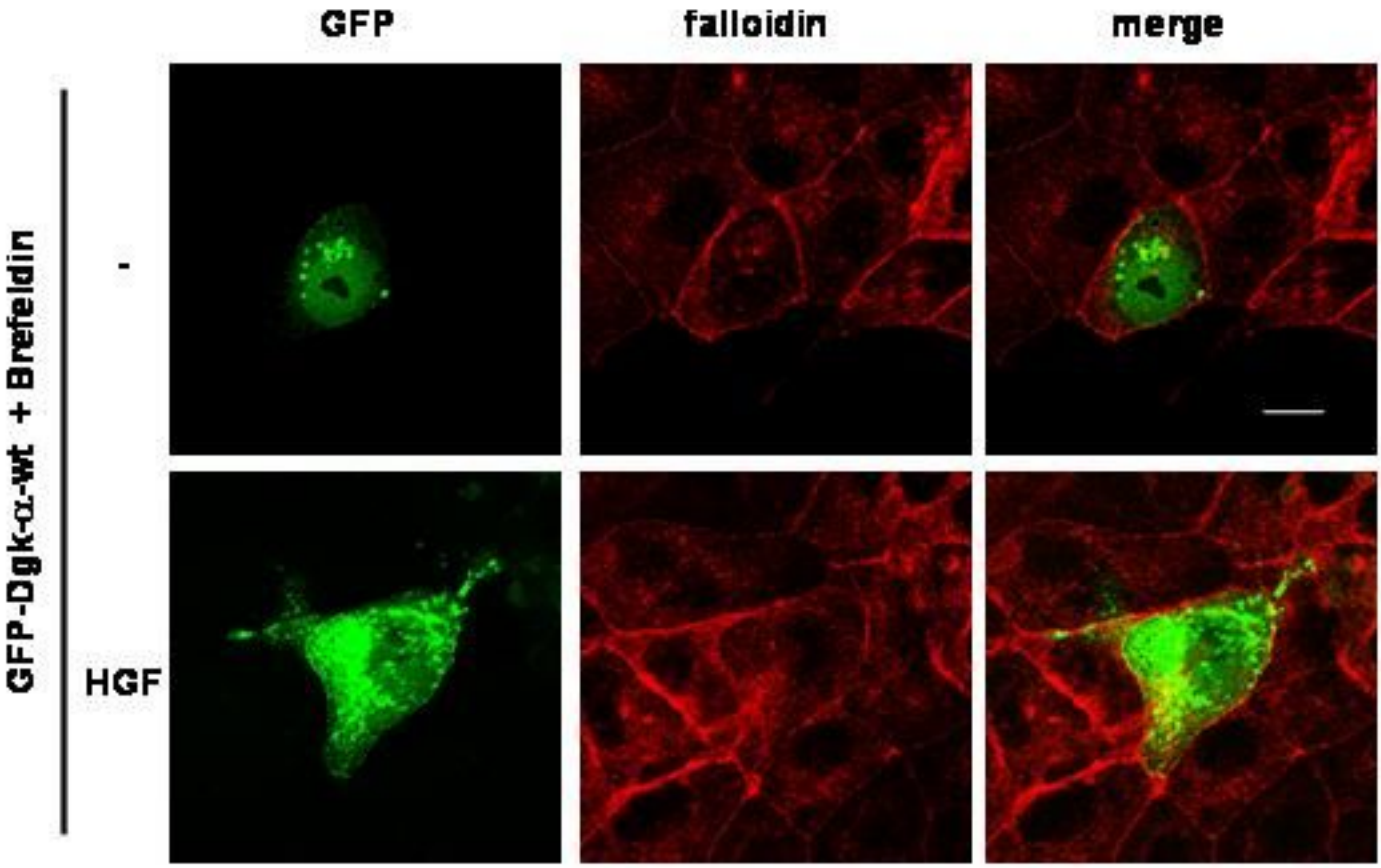
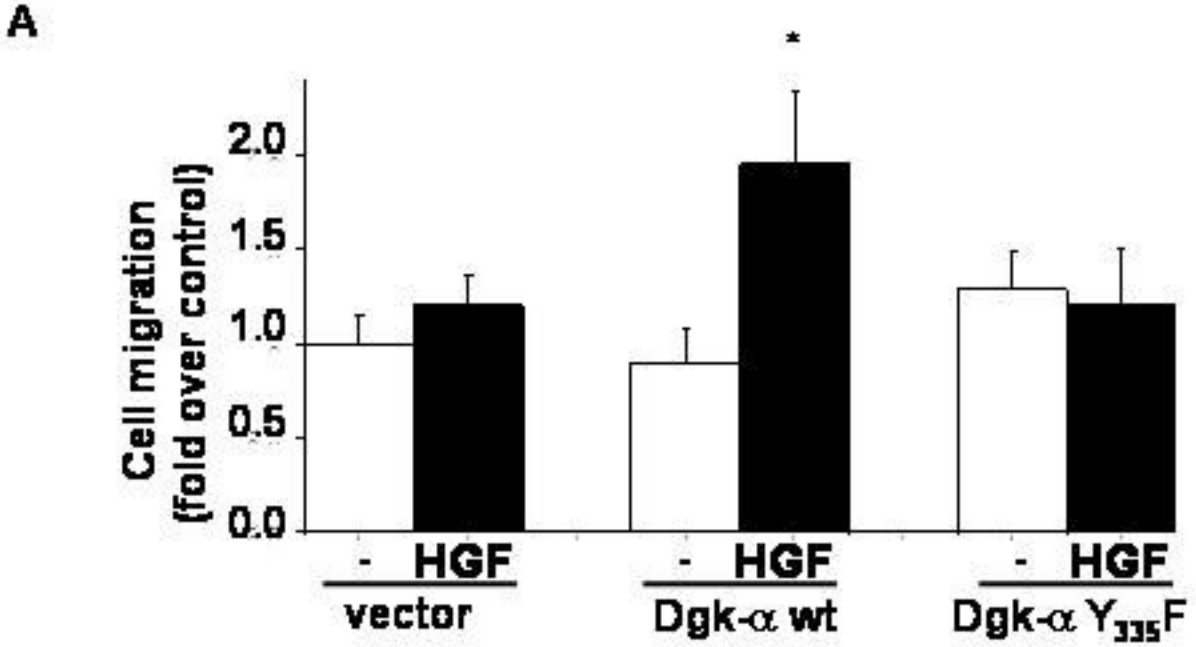


Fig. 10


A
Cell migration (fold over control)
2.0
1.5
1.0
0.5
0.0
*
- HGF
- HGF
- HGF
vector
Dgk-α wt
Dgk-α Y335F

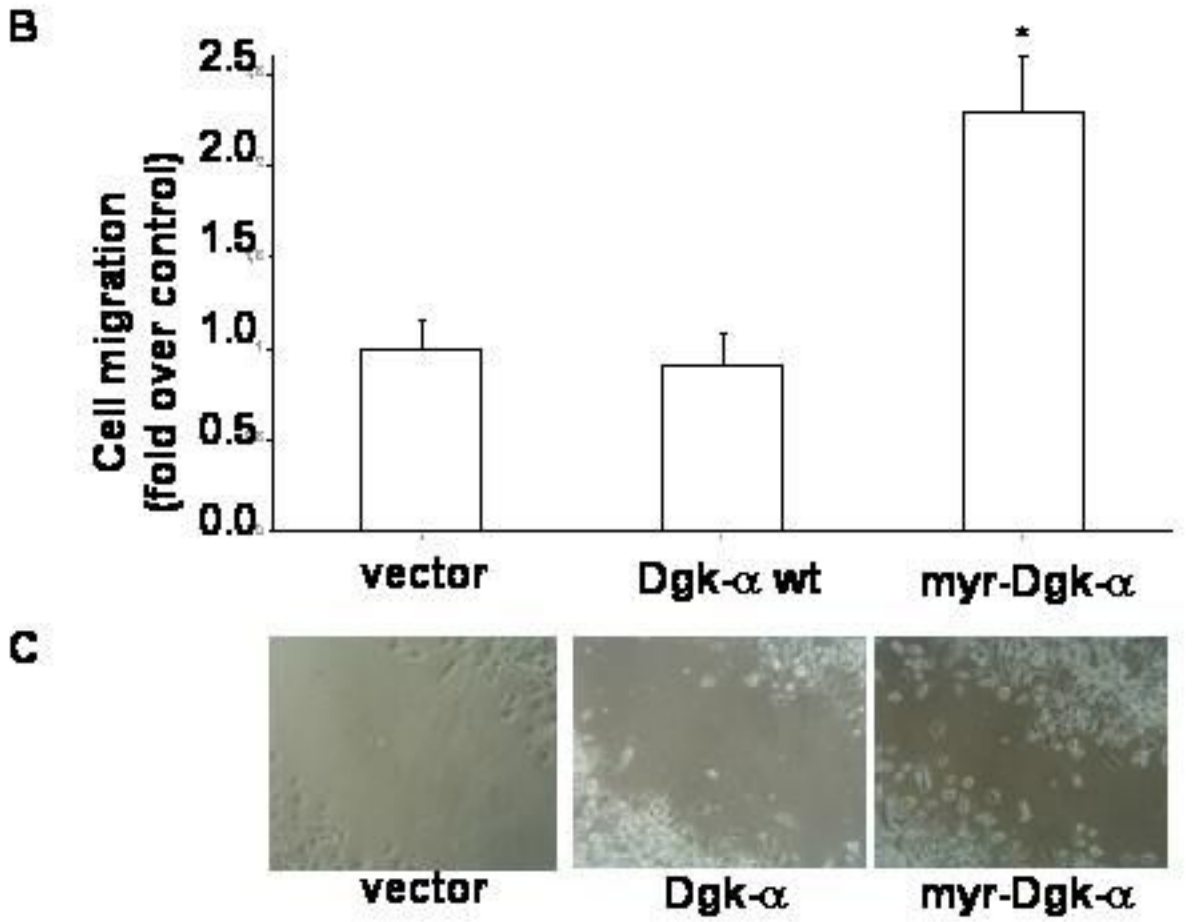

B
Cell migration (fold over control)
2.5
2.0
1.5
1.0
0.5
0.0
*
vector
Dgk-α wt
myr-Dgk-α
C
vector
Dgk-α
myr-Dgk-α

Fig. 11