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ORIGINAL ARTICLE

Diacylglycerol kinase-α phosphorylation by Src on Y335 is required for activation, membrane recruitment and Hgf-induced cell motility

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Diacylglycerol (DAG) kinases (Dgk), which phosphorylate DAG to generate phosphatidic acid, act as either positive or negative key regulators of cell signaling. We previously showed that Src mediates growth factors-induced activation of Dgk-α, whose activity is required for cell motility, proliferation and angiogenesis. Here, we demonstrate that both hepatocytes growth factor (HGF) stimulation and v-Src transformation induce tyrosine phosphorylation of Dgk-α on Y335, through a mechanism requiring its proline-rich C-terminal sequence. Moreover, we show that both proline-rich sequence and phosphorylation of Y335 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 Y335 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.
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Introduction

Diacylglycerol (DAG) kinases (Dgk), which phosphorylate DAG to generate phosphatidic acid (PA), comprise a family of 10 distinct enzymes, grouped in 5 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 by van Blitterswijk and Houssa, 2000; Luo *et al*., 2003). Dgk-α is activated by several growth factors: vesicular endothelial growth factor (VEGF) and hepatocytes growth factor (HGF) in endothelial and epithelial cells (Cutrupi *et al*., 2000; Baldanzi *et al*., 2004), and interleukin (IL)-2 in T cells (Flores *et al*., 1999; Cipres *et al*., 2003). Both *in vitro* and *in vivo* experiments in knockout mice, showed that in T cells Dgk-α and -ζ regulate cell sensitivity to T-cell receptor (TCR) activation by negatively modulating the intensity and the kinetic of DAG-mediated recruitment of both RasGRP and protein kinase C (PKC)-θ (Jones *et al*., 2002; Zhong *et al*., 2003; Carrasco and Merida, 2004; Olenchock *et al*., 2006; Zha *et al*., 2006).

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 anaplastic lymphoma kinase (ALK)-induced chemotaxis and proliferation in several cell types (Cutrupi *et al*., 2000; Baldanzi *et al*., 2004; Bacchiocchi *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 (Cutrupi *et al*., 2000; Cipres *et al*., 2003; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005). 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*.,

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2000; Cipres *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 (Cutrupi *et al*., 2000; Cipres *et al*., 2003; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005).

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*. (2003) showed that activation and recruitment of Dgk-α by IL-2 is mediated by binding to phosphatidylinositol (3,4,5)-trisphosphate (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 (Sanjuan *et al*., 2001; Cipres *et al*., 2003).

Here, we identify Y335 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 coexpression with either Src or Lck (Cutrupi *et al*., 2000; Cipres *et al*., 2003). To verify that Dgk-α could be directly phosphorylated by Src tyrosine kinase activity, we incubate partially purified glutathione-*S*-transferase (GST)-Dgk-α with recombinant Src in presence of Mg^{++} and ATP. In these conditions, Src promotes a strong tyrosine phosphorylation of GST-Dgk-α as verified by western blot with anti-phosphotyrosine antibodies (Figure 1a). Observing Dgk-α sequence, we noted two tyrosine residues featuring isoleucine in the −1 position, $FLKIY_{60}LEVDN$ and $PPSSIY_{335}PSVLA$ (Figure 2), suggesting strong substrate selection by Src (Songyang and Cantley, 1995; Schmitz *et al*., 1996). To verify whether these two tyrosine residues are substrates of Src tyrosine kinase activity, we coexpressed Src in COS cells with myc-tagged Dgk-α, either wt, Y60F, or Y335F. Tyrosine phosphorylation of Dgk-α was evaluated by anti-phosphotyrosine western blot of anti-myc immunoprecipitates (Figure 1b). Upon coexpression with Src, Myc-Dgk-α-Y335F does not feature any detectable tyrosine phosphorylation, while both Myc-Dgk-α wt and Myc-Dgk-α-Y60F 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 Y335 is the major site of phosphorylation of Dgk-α upon coexpression with Src, suggesting that contribution of Y60 is negligible.

As optimal protein-substrate sequences for Src tyrosine kinase activity provides 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 Y335 of Dgk-α to mediate interaction with Src-SH2 domain in an *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 (Figure 3a). The interaction between Dgk-α and the SH2 domain is specific, as the GST-Src-SH2 R175L mutant, unable to recognize the phosphorylated tyrosine (Yeo *et al*., 2006), does not interact with Myc-Dgk-α (Table 1). Furthermore, Myc-Dgk-α-Y335F, which shows a dramatically reduced phosphorylation upon coexpression with Src, fails to associate with GST-Src-SH2 in the pull-down assay, while Myc-Dgk-α-Y60F interacts with GST-Src-SH2 as well as Myc-Dgk-α wt. In summary, these experiments demonstrate that Src-SH2 domain interacts selectively with the phosphorylated Y335 of Dgk-α. The interaction of Dgk-α is not limited to Src-SH2 domain, as, at least *in vitro*, Dgk-α interacts also at similar or lower efficiency, with SH2 domains of Bruton's tyrosine kinase (Btk), c-phospholipase C (PLC)γ, Grb2 and Lck, but not with SH2 domains of Abl, n-PLCγ and p85n (Table 1).

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, either control or expressing Myc-Dgk-α-wt or mutants. Myc-Dgk-α-wt and Myc-Dgk-α-Y335F were specifically pulled down by immobilized GST-Src-SH3, but not by GST alone (Figure 3b), indicating that indeed Dgk-α interacts with Src-SH3 domain. The interaction between Dgk-α and the SH3 domain is specific, as the GST-Src-SH3-D99N a SH3 mutant, which is impaired in poly-proline binding (Weng *et al*., 1995), does not interact with Dgk-α. Although Dgk-α does not contain a consensus sequence for SH3 interaction (PxxP), it features a highly conserved C-terminal proline-rich sequence (PMLMGPPPR, Figure 2). Thus, we generated two deletion mutants lacking respectively the entire C-terminal half of Dgk-α (Myc-Dgk-α-STOP) or the last 13 amino acids PPPRSTNFFGFLS (Myc-Dgk-α-ΔP). Both mutants were assayed in the GST-Src-SH3 pull-down assay. Figure 3b shows that both Myc-Dgk-α-ΔP and Myc-Dgk-α-STOP

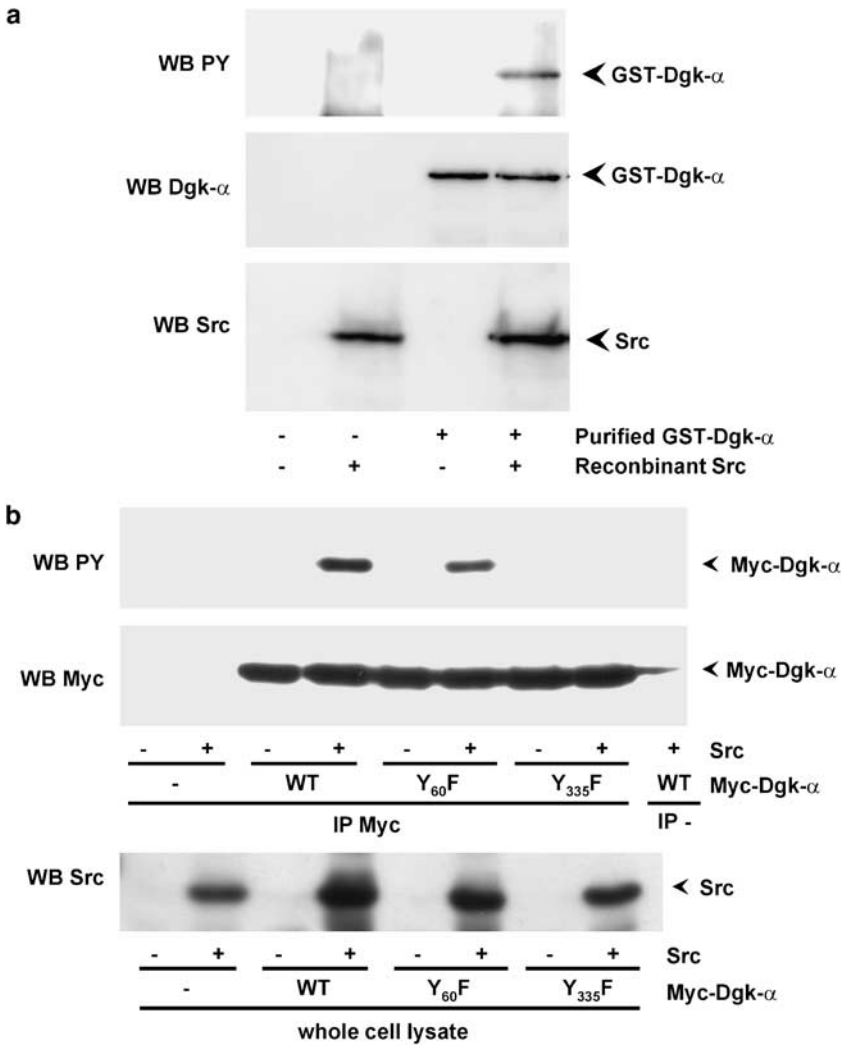

Figure 1 Dgk-α phosphorylation by Src on Y335. (**a**) Partially purified GST-Dgk-α or GST were incubated with or without recombinant Src in kinase buffer for 10 min at 30°C. Samples were split and analysed by western blot with antibodies against phosphotyrosine (upper panel), Dgk-α (middle panel) or Src (lower panel). (**b**) 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 analysed by western blot with anti-phosphotyrosine (upper panel), and anti-myc antibodies (lower panel). Total cell lysates were analysed with anti-Src antibodies (right panel). Dgk, diacylglycerol kinase; GST, glutathione-*S*-transferase.

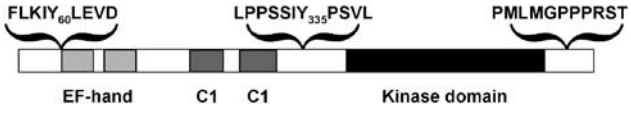

Figure 2 Dgk-α structure. The structure of Dgk-α contains three structurally defined domains: 2 EF-hand motifs, a double atypical C1 domain and a catalytic domain. Y60 lies upstream of the EF-hand motif, Y335 between the second C1 domain and the catalytic domain and the proline-rich region at the C-terminal end. Dgk, diacylglycerol kinase.

mutants, different from Myc-Dgk-α-wt and Myc-Dgk-α-Y335F, 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 (Figure 3a). The interaction of Dgk-α is not limited to the SH3 domain of Src, but SH3 domains of both Lck and Abl interact as well with Dgk-α (Table 1).

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 coexpressed in 293 T cells Myc-Dgk-α either wt, ΔP or Y335F with Src-Y527F, an activated form of Src. Tyrosine phosphorylation of Myc-Dgk-α in anti-myc immunoprecipitates was assayed by anti-phosphotyrosine western blot. Figure 4 shows that Myc-Dgk-α-ΔP and Myc-Dgk-α-Y335F mutants are not tyrosine phosphorylated upon coexpression with Src-Y527F, 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

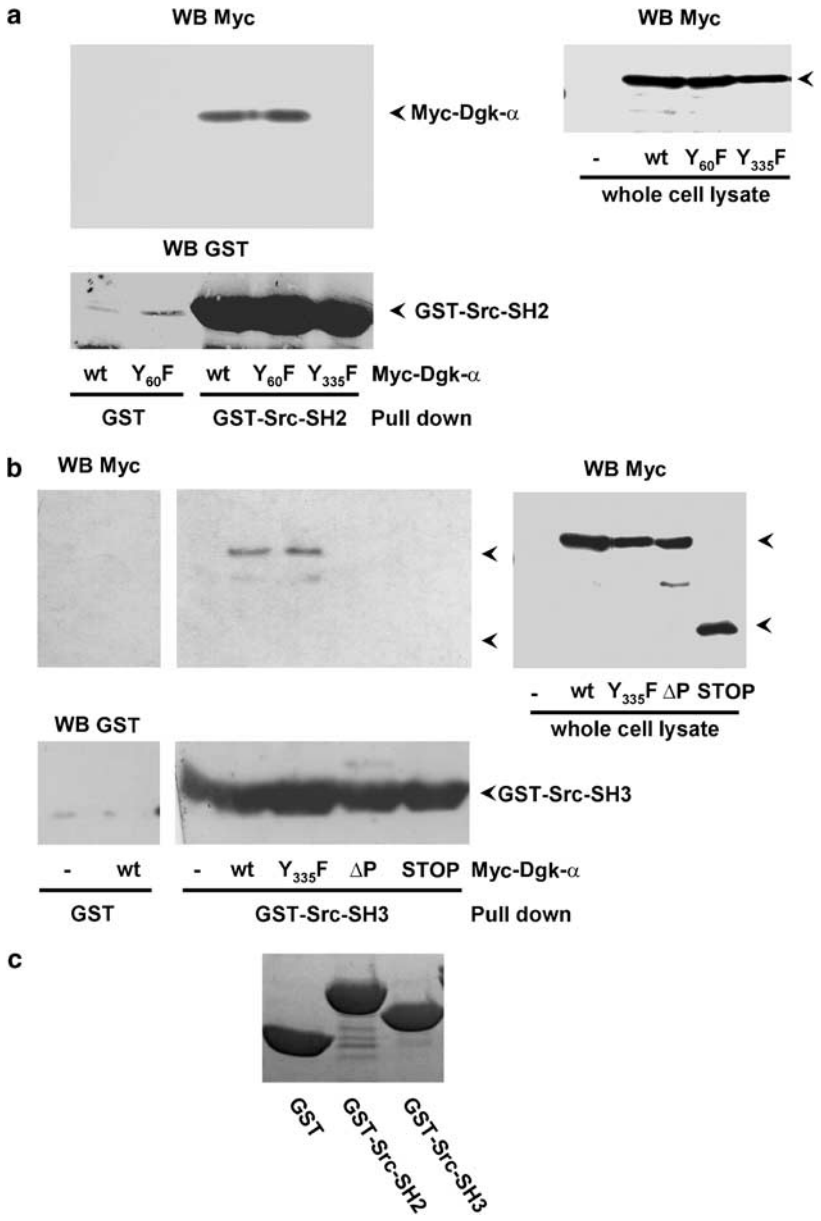

Figure 3 Dgk-α interaction with Src-SH2 and Src-SH3 domains. (**a**) Growing COS-7 cells, transfected with indicated Myc-Dgk-α constructs, were lysed in buffer A. Cell lysates were incubated with agarose-bound purified GST or GST-Src-SH2 for a pull-down assay. Pulled down Myc-Dgk-α (left panel) and Myc-Dgk-α expression in 1% of total cell lysates input (right panel) were detected by anti-myc western blot, loaded GST-Src-SH2 was detected by anti-GST western blot (lower panel). (**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 1% of total cell lysates input (right panel) were detected by anti-myc western blot, loaded GST-Src-SH3 was detected by anti-GST western blot (lower panel). (**c**) Expression and purity of GST, GST-Src-SH2 and GST-Src-SH3 used as bait, was determined by 15% SDS–PAGE and Comassie Blue staining. Dgk, diacylglycerol kinase; GST, glutathione-*S*-transferase; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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 Y335 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 Y335 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 (Cutrupi *et al*., 2000; Cipres

Table 1 Binding of Myc-Dgk-α to GST-SH2 and GST-SH3 domains of different proteins

Bait for pool-down	*Binding (++ strong, + weak, − no)*
GST	−
GST-Src-SH2	++
GST-Src-SH2-R175L	−
GST-Btk-SH2	++
GST-PLCg-cSH2	++
GST-PLCg-nSH2	−
GST-Abl-SH2	−
GST-Grb2-SH2	+
GST-Lck-SH2	+
GST-p85-nSH2	−
GST-Src-SH3	+
GST-Src-SH3-D99N	−
GST-Fyn-SH3	+
GST-Abl-SH3	+

Abbreviations: Btk, Bruton's tyrosine kinase; GST, glutathione-*S*-transferase; PLC, phospholipase C.

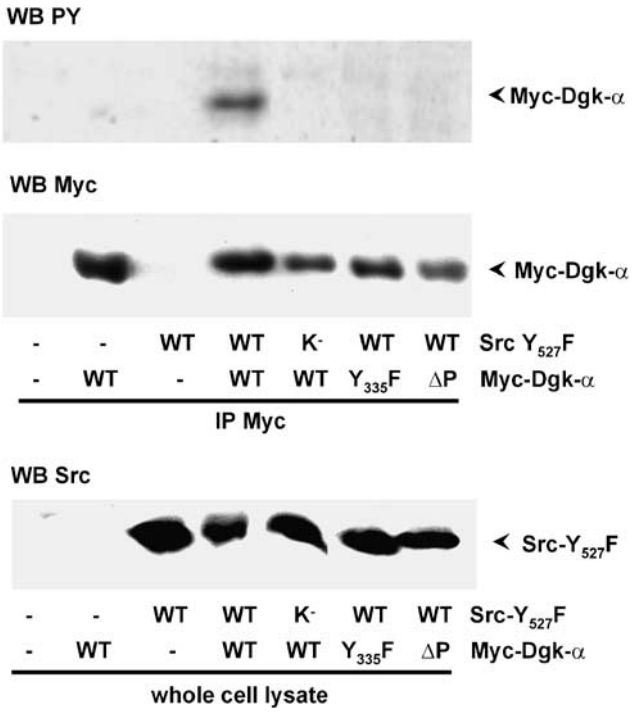

Figure 4 Dgk-α phosphorylation by Src requires Y335- and proline-rich C-terminal sequence. Growing HEK 293T co-transfected with the indicated Myc-Dgk-α and Src-Y527F constructs were lysed in detergent-containing buffer A. Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed by western blot with anti-phosphotyrosine (upper panel), and anti-myc antibodies (middle panel). Total cell lysates were analysed with anti-Src antibodies (lower panel). Dgk, diacylglycerol kinase.

et al., 2003; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005).

The enzymatic activity of Myc-Dgk-α either wt, Y335F 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; Figure 5). Conversely, the enzymatic activities of either Myc-Dgk-α–Y335F or Myc-Dgk-α–ΔP mutant are not significantly stimulated upon co-incubation with Src *in vitro* (Figure 5). This finding provides the first direct demonstration that both Y335 and proline-rich sequence are required for activation of Dgk-α *in vitro*.

Next, we investigated whether both Y335 and proline-rich sequence are also required for HGF-induced activation of Dgk-α in intact cells. We assayed the enzymatic activity of Myc-Dgk-α-wt, Y335F or ΔP mutant (Figure 6a), transiently transfected in COS cells, either control or HGF-stimulated. The enzymatic activity was measured in whole-cell lysates, as described previously; under these conditions, the contribution of endogenous Dgk to the total Dgk activity is negligible (Cutrupi *et al*., 2000; and data not shown). Figure 6a indicates that, while enzymatic activity of Myc-Dgk-α-wt is stimulated by HGF, the enzymatic activities of either Myc-Dgk-α–Y335F or Myc-Dgk-α–ΔP mutants are not stimulated on HGF cell stimulation. Expression of Myc-Dgk-α-wt and mutants was verified by anti-myc western blot (Figure 6a, lower panel).

Consistently, the enzymatic activity of the double mutant Myc-Dgk-α–Y335F–ΔP, featuring a lower basal activity, is not further activated upon HGF stimulation, as assayed in anti-myc immunoprecipitates (Figure 6b). The expression of Myc-Dgk-α-wt and Myc-Dgk-α–Y335F–ΔP was verified by anti-myc western blot (Figure 6b, lower panel).

To provide further evidence for the role of Y335 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, Y335F or ΔP in transiently transfected Madin–Darby canine kidney (MDCK)-*ts*-v-Src epithelial cells (Figure 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 expressed at low level, and it does not significantly affect total Dgk activity assayed in whole-cell lysates (Figure 7b).

Shifting MDCK-*ts*-v-Src cells to the permissive temperature results in both tyrosine phosphorylation (Figure 7a) and enzymatic activation (Figure 7b) 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–Y335F and Myc-Dgk-α–ΔP. Activation of *ts*-v-Src fails to induce tyrosine phosphorylation of both Myc-Dgk-α−Y335F and Myc-Dgk-α–ΔP (Figure 7a), and fails to stimulate their enzymatic activity (Figure 7b). Expression of both mutants is comparable to the wild type (Figure 7).

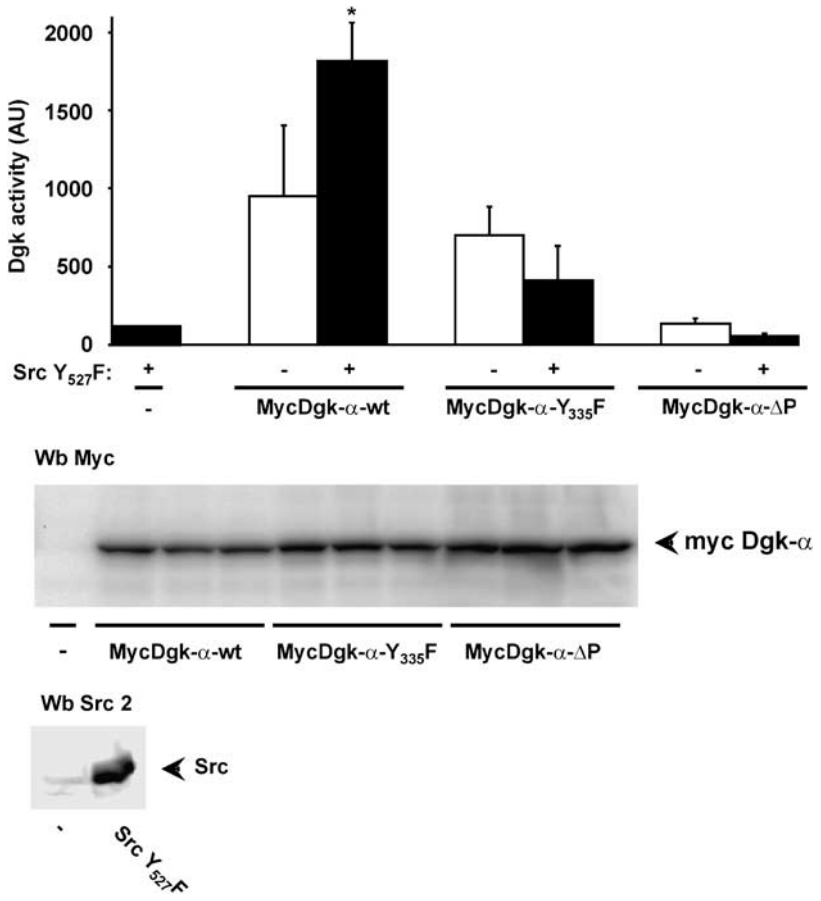

Figure 5 Dgk-α activation by c-Src *in vitro* requires Y335- and proline-rich C-terminal sequence. COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP or Src were homogenized with buffer B in absence of detergent. Cell extracts were mixed as indicated in presence of 1 mM ATP for 15 min, and analysed for Dgk activity (upper panel). Values are mean ± s.e.m. of triplicates (*t-test, $P<0.05$). Myc-Dgk-α and Src protein expression were verified by anti-myc and anti-src western blot (lower panel). Dgk, diacylglycerol kinase.

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 Y335. 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.

Y335 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; Sanjuán *et al*., 2003), we investigated whether phosphorylation of Dgk-α on Y335 regulates its recruitment to the membrane upon HGF stimulation. To address this question, we investigated the subcellular localization of GFP tagged Dgk-α wt, Y335F 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 translocates at the plasma membrane in the majority of transfected cells (70%) (Figures 8a and 9c). In addition, the kinase dead mutant (GFP-Dgk-α-k−) behaves as the wild type, being diffuse in the cytoplasm in control cells and associates to the plasma membrane in HGF-stimulated cells (Figure 8b). HGF-induced membrane recruitment was dependent on Src activity, as it was reduced of 50% by pharmacological inhibition of Src with 10 μM PP2 (Figure 8).

To verify whether tyrosine phosphorylation of Dgk-α mediates HGF-induced membrane recruitment of Dgk-α, we investigated the subcellular localization of both Y335F and ΔP mutants. Surprisingly, in most of control-transfected cells, GFP-Dgk-α-Y335F 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, neither mutant translocates at the plasma membrane, while their vesicular localization is not affected (Figure 9).

These observations demonstrate that Y335 and proline-rich sequence are required for proper localization of Dgk-α, and suggest that phosphorylation of Y335 is a key event for HGF-induced recruitment to the plasma membrane. In addition, the vesicular localization of both GFP-Dgk-α-Y335F and GFP-Dgk-α-ΔP suggest

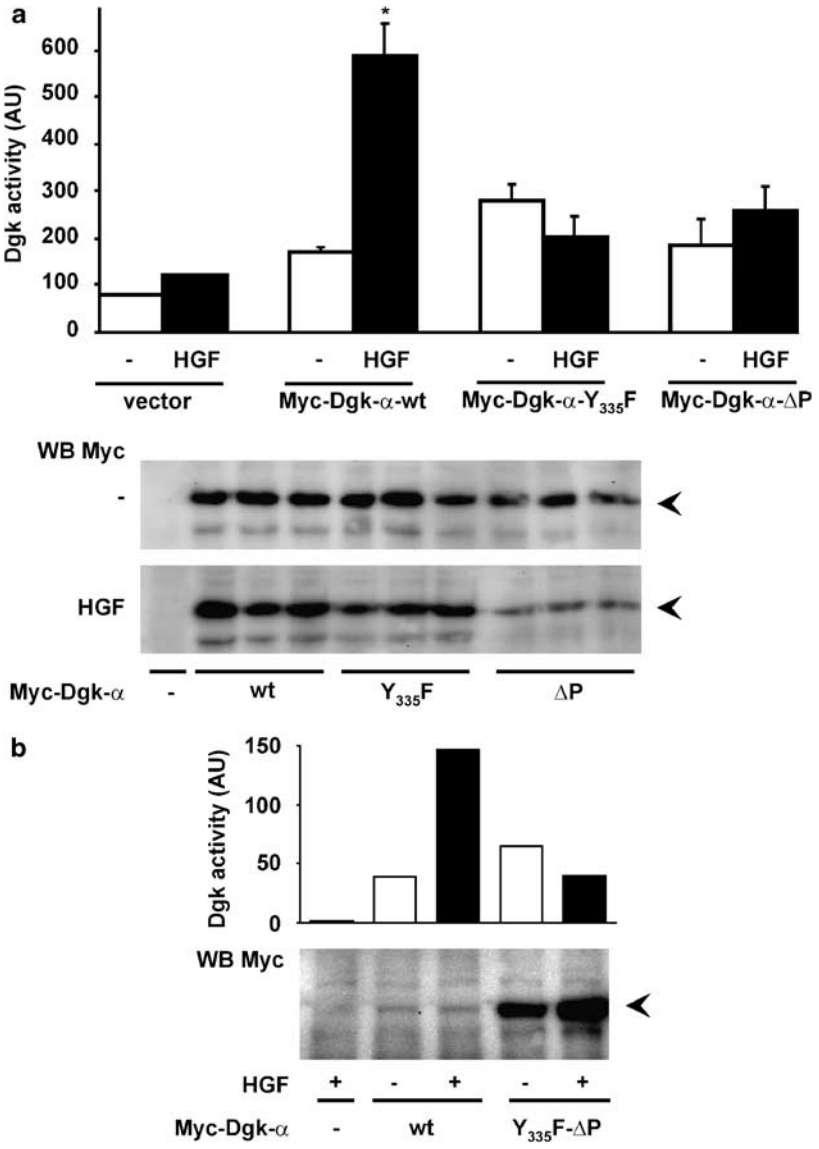

Figure 6 Dgk-α activation by HGF *in vivo* requires Y335- and proline-rich C-terminal sequence. (**a**) COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP were stimulated with HGF (100 μg/ml, 15 min), homogenized with buffer B in absence of detergent and analysed for Dgk activity (upper panel). Values are mean ± s.e.m. of triplicates (*t-test $P<0.05$). Myc-Dgk-α protein expression was verified by anti-myc western blot (lower panel). Myc-Dgk-α protein expression was verified by anti-myc western blot (lower panel). (**b**) COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F-ΔP were stimulated with HGF (200 μg/ml, 15 min), lysed and Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed for Dgk activity (upper panel). Dgk-α protein expression was verified by anti-myc western blot (lower panel). Dgk, diacylglycerol kinase; HGF, hepatocytes growth factor.

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 Brefeldin A (BFA) treatment, would result in accumulation of GFP-Dgk-α-wt in intracellular vesicles (Lippincott-Schwartz *et al*., 1989). Indeed, upon 15 min of treatment with 10 μM BFA, even GFP-Dgk-α-wt associates to intracellular vesicles in unstimulated cells and fails to translocate to the membrane following HGF stimulation (Figure 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 Y335 by Src, but does not require its enzymatic activity.

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

As we previously showed that activation of Dgk-α is required for HGF- and VEGF-induced cell migration (Cutrupi *et al*., 2000; Baldanzi *et al*., 2004), we investigated whether Y335 contributes to the transduction of HGF pro-migratory signaling. Although HGF

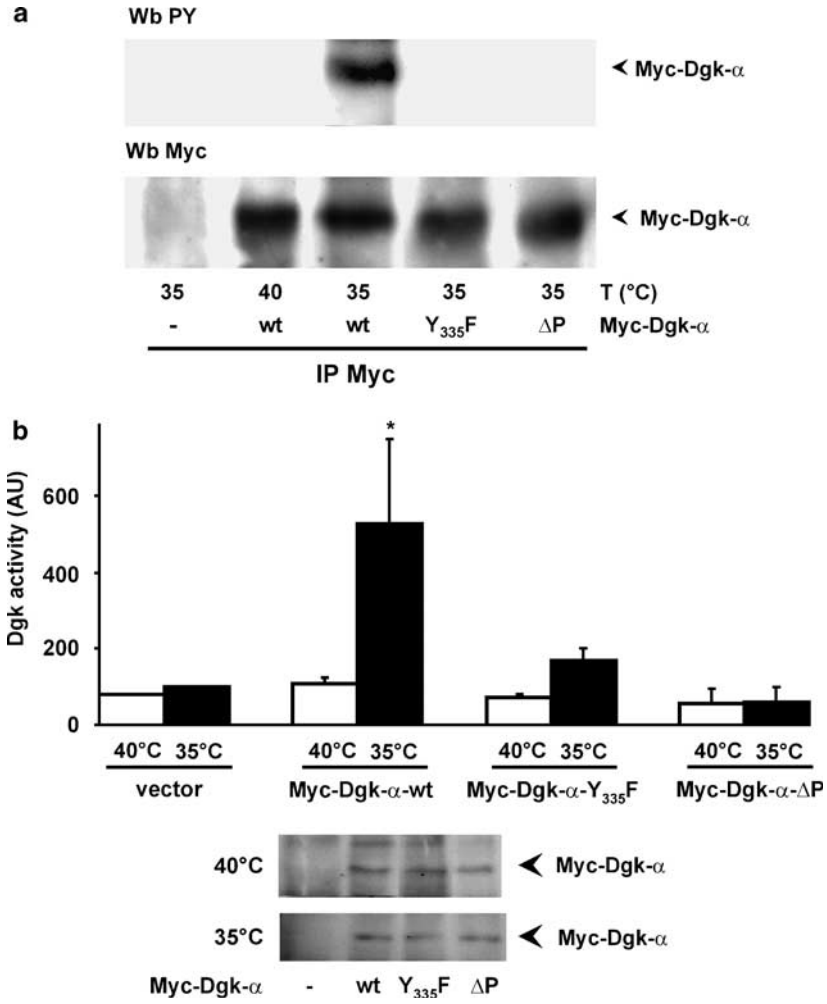

Figure 7 Dgk-α phosphorylation and activation by v-Src requires Y335- and proline-rich C-terminal sequence. Ts-v-Src/MDCK cells transfected with the indicated Myc-Dgk-α constructs, were cultured at nonpermissive temperature (40°C), and, where indicated, shifted at the permissive temperature (35°C) for 1 h. (**a**) After lysis in detergent-containing buffer A, myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed 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 ± s.e. of triplicates (*t-test, $P<0.05$). Myc-Dgk-α protein expression was verified by anti-myc western blot (lower panel). Dgk, diacylglycerol kinase; MDCK, Madin–Darby canine kidney.

does not stimulate chemotaxis of COS-7 cells, transient overexpression of Myc-Dgk-α-wt makes COS-7 cells able to migrate in response to HGF in a transwell chemotaxis quantitative assay (Figure 11a). This observation provides a functional assay to verify the requirement for phosphorylation of Y335 to transduce HGF-induced migratory signaling. Figure 11 indicates that the expression of Myc-Dgk-α-Y335F 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 Y335, are required for HGF-induced migratory signaling.

Next, we asked whether Dgk-α constitutive recruitment to the plasma membrane provides sufficient signaling to stimulate cell motility. Sanjuan *et al.* (2001) 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 threefold 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 (Figures 11b and c). 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

An increasing body of evidence from our laboratory and others showed that Dgk-α is activated by growth factors

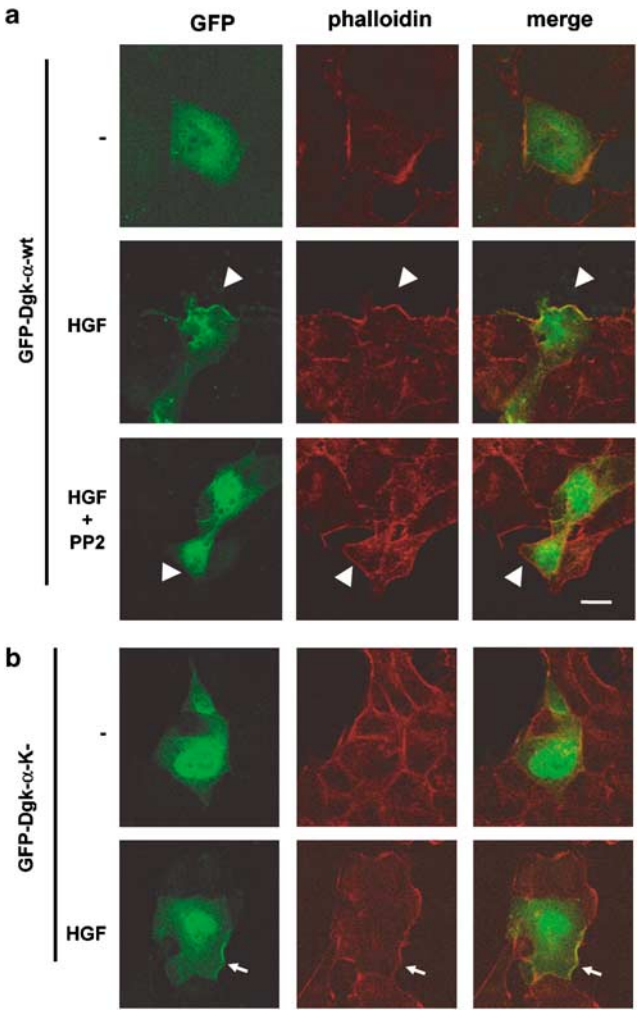

Figure 8 Dgk-α is recruited at cell membrane upon HGF treatment. MDCK cells transfected with GFP-Dgk-α wt or GFP-Dgk-α-K− were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with PP2 (10 μM PP2 for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal microscopy (scale bar 16 μm). Dgk, diacylglycerol kinase; GFP, green fluorescent protein; HGF, hepatocytes growth factor; MDCK, Madin–Darby canine kidney.

through Src-family tyrosine kinases, although the significance of tyrosine phosphorylation 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, Y60 and Y335, both featuring Ile in −1 position, a signature for putative Src substrates (Schmitz *et al*., 1996). By phenylalanine substitution of either one of the two tyrosines, we showed that Y335, rather than Y60, is the major site of phosphorylation upon coexpression 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 Y335, Dgk-α may be then phosphorylated on other sites. The substitution of Y60, differently from Y335, 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 Y60 is not a phosphorylation site of Dgk-α, or that its phosphorylation is secondary to Y335 occurring at lower stoichiometry.

The observation that C-terminal 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 Y335. Subsequently, phosphorylated Y335 would become a docking site for Src-SH2 domain, and may lead to the stabilization of the Dgk-α/Src complex, and eventually to the phosphorylation of multiple secondary sites, providing additional docking sites for SH2-containing proteins. Alternatively, phosphorylation of Y335 itself, may allow interaction of Dgk-α with other SH2-containing proteins.

Phenylalanine substitution of Y335 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 Y335 dictates the ability of Dgk-α to be stimulated by both growth factors and v-Src activation.

Y335 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 Y335 acts as a molecular switch, which by unfolding an intramolecular interaction, shifts 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 Y340, which, similar to Y335of Dgk-α, is placed in a linker region between the C1 domain and the catalytic domain (Mason *et al*., 1999; Tran and Frost, 2003).

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. Phosphatidylinositol (PI) 3-kinase, PLC-γ, PI4P 5-kinase and phospholipase D 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 here clearly demonstrate that both Y335- and proline-rich

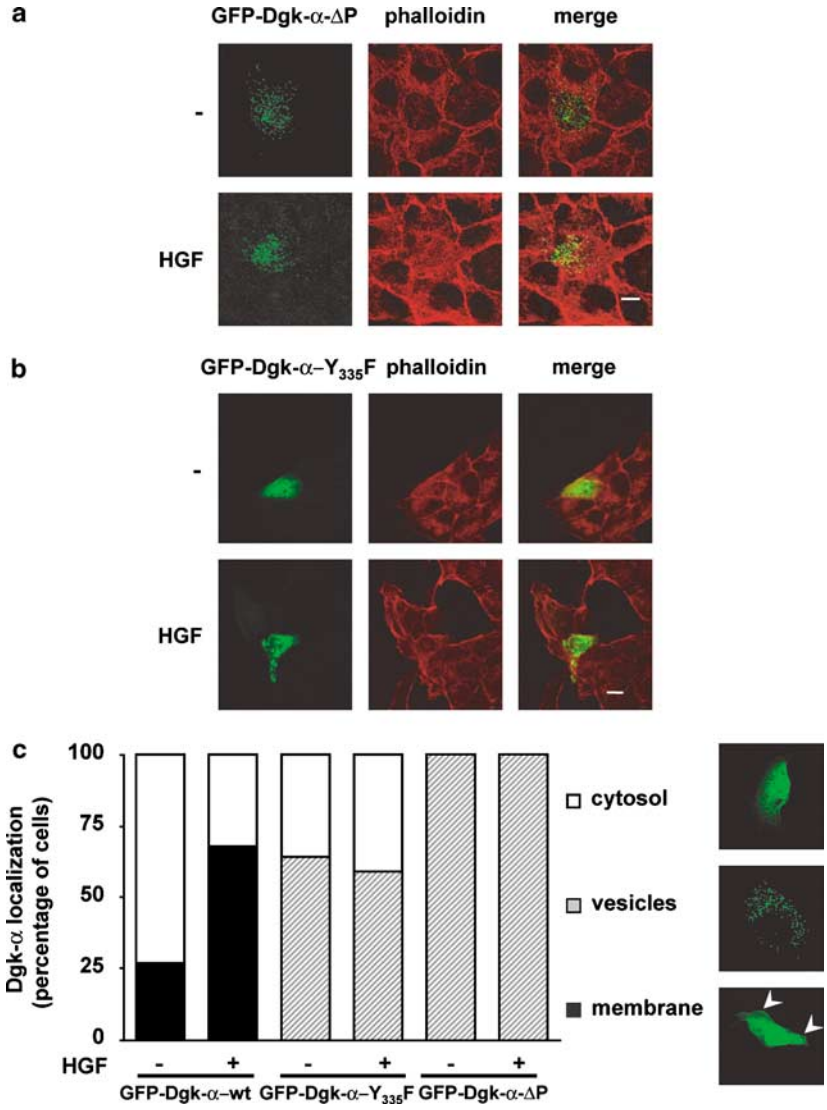

Figure 9 Recruitment of Dgk-α at cell membrane requires Y335- and proline-rich C-terminal sequence. MDCK cells tranfected with either GFP-Dgk-α-wt, GFP-Dgk-α-ΔP (**a**) or GFP-Dgk-α-Y335F (**b**) were stimulated with HGF (50 ng/ml, 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal microscopy (scale bar 16 μm). (**c**) For each point, more than 100 cells were scored for Dgk-α localization: membrane (filled bars), cytoplasm (empty bars), vesicles (dashed bars), examples of each class are shown on the right. Dgk, diacylglycerol kinase; GFP, green fluorescent protein; HGF, hepatocytes growth factor.

C-terminal sequence are major determinants for both membrane recruitment and activation of Dgk-α on HGF cell treatment. These results are highly consistent with recently reported data showing that phosphorylation of Y335 of murine Dgk-α is required for vitamin E-induced membrane recruitment and for its enzymatic activation (Fukunaga-Takenaka *et al*., 2005). Thus, we may speculate that phosphorylation of Y335 may unfold an intramolecular 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 membrane-bound activator, yet to be identified. The atypical C1 domains of Dgk-α are incapable of binding to phorbol esters and Dgk-α is not recruited to the membrane on cell stimulation with phorbol esters, suggesting that DG does not regulate its membrane recruitment (Ahmed *et al*., 1991; Shirai *et al*., 2000). Conversely, atypical C1 domains have been suggested to interact with small GTPases (Hurley *et al*., 1997), leading to the speculation that tyrosine phosphorylation may enable Dgk-α to interact with a protein-bound small GTPase. In addition, direct interaction of Dgk-α with PIP_3 has been reported to determine its membrane recruitment and activation upon IL-2 stimulation (Cipres *et al*., 2003). However, in epithelial cells, PI 3-kinase is not required for HGF-induced membrane recruitment of Dgk-α, which, conversely, is dependent on PLC-γ and Src activity (data not shown; Figure 8).

Finally, the observation that phosphorylation of Y335 is required for membrane recruitment and for enzymatic activation of Dgk-α, but becomes undetectable

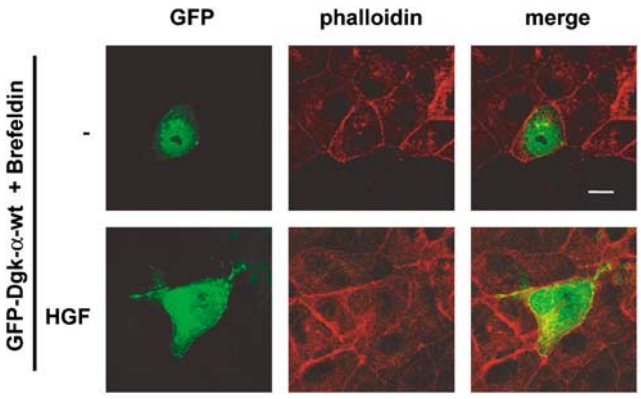

Figure 10 Brefeldin causes accumulation of Dgk-α on cytoplasmic vesicles. MDCK cells transfected with GFP- Dgk-α wt were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with BFA (10 μM BFA for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal microscopy (scale bar 16 μm). BFA, brefeldin A; Dgk, diacylglycerol kinase; GFP, green fluorescent protein; HGF, hepatocytes growth factor; MDCK, Madin–Darby canine kidney.

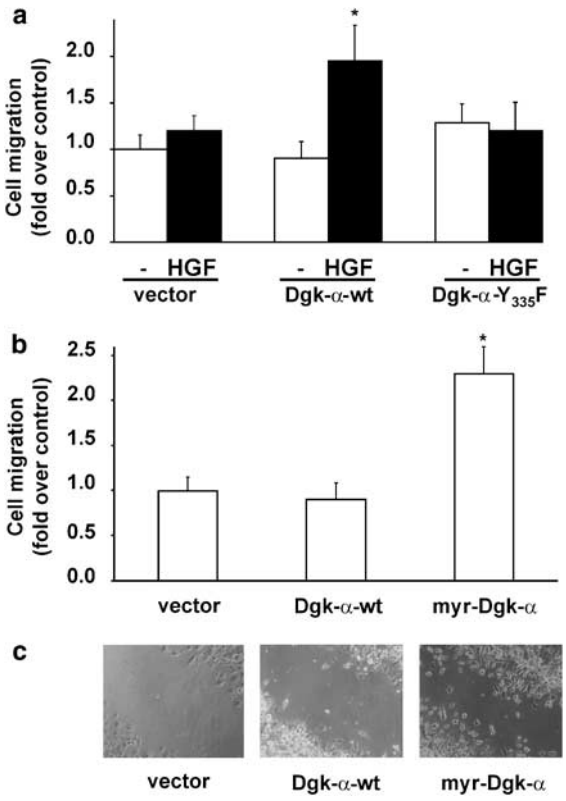

Figure 11 Membrane recruitment of Dgk-α is necessary and sufficient for cell motility. (**a**) COS cells transfected with either Dgk-α wt, or Y335 mutant or empty vector were stimulated to migrate by HGF (100 ng/ml) in a transwell chemotaxis assay. Data are expressed as fold increase over control, values are mean ± s.e. 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 ± s.e. 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. Dgk, diacylglycerol kinase; HGF, hepatocytes growth factor.

when the protein is still active, suggest that transient phosphorylation of Y335 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 it does not occur by co-incubating the two purified proteins (data not shown). Moreover, according to this model, activation of Dgk-α would generate a coincidence signal derived from time- and space-co-incidence of two independent signals, Src activation and a still unidentified membrane signal, either lipidic or proteic.

Alternatively, the surprising observation that both Myc-Dgk-α-Y335F and Myc-Dgk-α-ΔP mutants are associated to intracellular vesicles rather than being diffuse in the cytosol, may suggest that they are mislocalized and segregated from Src, resulting in defective tyrosine phosphorylation. However, different from the wild type, neither mutant is activated by Src in the *in vitro* assay with whole-cell extracts (Figure 3), and becomes tyrosine phosphorylated upon co-incubation with 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 either mutant 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 a basal enzymatic activity, 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 BFA (Figure 10; see below).

The surprising observation that both Myc-Dgk-α-Y335F and Myc-Dgk-α-ΔP are associated to intracellular vesicles rather than being diffuse in the cytosol, suggest that phosphorylation of Y335 by Src may be required to couple Dgk-α to vesicular transport from the inner cytoplasm to the plasma membrane. Consistent with this hypothesis, BFA treatment results in the accumulation of both wt Dgk-α and Src on intracellular vesicles (Figure 9; Kaplan *et al*., 1992).

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, overexpression of Dgk-δ, bearing distinct regulatory domains from α-isoform, suppresses endoplasmic reticulum (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-Y335F 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 (Flores *et al*., 1999; Cutrupi *et al*., 2000; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005). 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 signaling sufficient to trigger spontaneous cell motility, even in unstimulated cells.

This finding demonstrates that phosphorylation of Y335 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 lysophosphatidic acid 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 293 T, MDCK and MDCK-ts-v-Src were cultured in high glucose DMEM (Sigma, Milan, Italy), supplemented with glutamine, 10% fetal calf serum (Gibco, Milan, Italy) and antibiotic-antimycotic solution (Sigma).

Reagents

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

Construction of expression vectors and site-directed mutagenesis

Myc tagged Dgk-α c-DNA cloned into pMT2 expression vector was described previously (Cutrupi *et al*., 2000). GFP-Dgk-α wt was obtained by cloning Dgk-α wt in pcDNA-DEST53 (Invitrogen, Milan, Italy) 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, Amsterdam, The Netherlands); mutating oligonucleotides were:

Y60F GGGAACATTATCCACTTCGAGGAAGATTTT CAGGAATTGCTG and CAGCAATTCCTGAAAATCTTC CTCGAAGTGGATAATGTTCCC;

Y335F GGCCAGGACACTGGGAAAGATGGAAGATG GAGG and CCTCCATCTTCCATCTTTCCCAGTGTCCT GGCC;

STOP GATGATTTAAATTAGAGCACCTCTGAGGCT and AGCCTCAGAGGTGCTCTAATTTAAATCATC;

K− CGGATTGGTGTGTGGTGACGACGGCACAGTA GGC and GCCTACTGTGCCGTCGTCACCACACACCAA AATCCG.

Dgk-α-ΔP was obtained by insertion of the annealing product of the oligonucleotide CATAACTGCAGTTATGG GCC at the *Apa*I site at position 2168 of Dgk-α wt cDNA. All mutants used have been verified by direct sequencing (MWG biotech (Milan, Italy) or C.R.I.B.I.-BMR (Padua, Italy)). Plasmid encoding Src wt, SrcY527F and SrcY527F K− were a kind gift from G Superti-Furga and Sara Courtneidge. pGEX, pGEX-Src-SH2, pGEX-Src-SH3, pGEX-BTK-SH2, pGEX-PLCγ-cSH2, pGEX-PLCγ-nSH2, pGEX-ABL-SH2, pGEX-GRB2-SH2, pGEX-LCK-SH2, pGEX-p85-nSH2, pGEX-FYN-SH3 pGEX-ABL-SH3 were a gift from LC Cantley. pGEX-Src-SH2-R175L and pGEX-Src-SH3-D99N were obtained using QuikChange Site-Directed Mutagenesis Kit (Stratagene); mutating oligonucleotides were:

D99N AGTCCCGGACTGAAACGAACTTGTCCTTCA AGAAA and TTTCTTGAAGGACAAGTTCGTTTCAGTC CGGGACT.

R175L GAACCTTCTTGGTCCTGGAGAGCGAGACGA and GTCGTCTCGCTCTCCAGGACCAAGAAGGTT.

Transfection with plasmid vectors and stimulation

COS-7 AND HEK 293T cells were transiently transfected with Cell-Phect Transfection kit (Amersham-Pharmacia, Milan, Italy) 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 h from transfection and expression of transfected protein verified by western blot. For HGF stimulation experiments, cells were serum starved for 16 h and then stimulated for 15 min with recombinant HGF (100 ng/ml). MDCK-*ts*-v-Src were made quiescent by culturing in 0.1% serum at the nonpermissive temperature of 40°C for 16 h and then switched to the permissive temperature of 35°C for 1 h. When inhibitors were

used, they were added 15 min before stimulation and controls were treated with equal amounts of vehicle (dimethyl sulfoxide).

Preparation of cell lysates, homogenates, immunoprecipitation, western blotting
Cells were lysed in buffer A (25 mM Hepes (pH 8), 1% NP-40, 10% glycerol, 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 2 mM ethylene glycol-bis(beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM $ZnCl_2$, 50 mM ammonium molibdate, 10 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma)) (Lippincott-Schwartz *et al*., 1989). Cells homogenates 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 *g* for 15 min. Protein concentration was determined by the bicinchoninic acid method (Pierce, Milan, Italy) and equalized for each point using buffer. Immunoprecipitation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blots were performed as described previously (Lippincott-Schwartz *et al*., 1989). Western blot results were acquired and quantified with Versadoc system (Bio-rad, Milan, Italy).

Dgk-α assay
Dgk-α activity in cell homogenates (25 μl) was assayed by measuring initial velocities (5 min at 30°C) in presence of saturating substrates concentration (1 mg/ml diolein (Fluka, Milan, Italy), 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 previously (Graziani *et al*., 1991), and PA was separated by thin layer chromatography (TLC) in chloroform:methanol:water:25% ammonium hydroxide (60:47:11:4). TLC plates had been previously coated with (potassium oxalate 1.3%, EDTA 5 mM):(methanol) 3:2 and desiccated. [^{32}P]-PA was identified by co-migration with nonradioactive 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 1 mM ATP and 5 mM $MgCl_2$, as reported previously (Cutrupi *et al*., 2000).

Purification of GST fusion proteins
SH3 and SH2 domains fused to GST were expressed in *Escherichia coli* and purified according to standard protocol. In brief, protein synthesis was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside and cells were harvested 4 h later by centrifugation. Pellets were resuspended in buffer G (50 mM Tris–HCl, 100 mM NaCl, 5% glycerol, pH 8) and cells disrupted by sonication (Branson, Danbury, CT, USA). Supernatants were collected by centrifugation (15 min at 12 000 *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 $\geqslant$80%.

GST-Dgk-α-wt was transfected in COS-7 and 48 h after transfection cells were lysed in buffer G supplemented with 1% NP40 and centrifuged (15 min at 12 000 × *g*). Recombinant GST-Dgk-α-wt was partially purified from supernatants by glutathione-sepharose column affinity purification (Amerscham Pharmacia) usually purity is $\geqslant$30%. The matrix with the attached proteins was removed from the column and used for the subsequent *in vitro* phosphorylation experiments.

In vitro *Dgk-α phosphorylation*
Partially purified GST-Dgk-α was incubated in 100 μl of reaction buffer (protein tyrosine kinase buffer Sigma) with or without 3 U of recombinant purified Src (Upstate) 10 min at 30°C. Reaction was halted by washing four times with buffer A and solubilizing in Laemmli buffer.

In vitro *pull down with GST-fusion proteins*
A 50 μg portion 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 analysed by western blot.

Cell staining and confocal microscopy
MDCK cells were seeded on glass coverslips (Marienfeld, Germany) 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 phosphate-buffered saline (PBS) and fixed with fixing solution (3% paraformaldehyde-4% sucrose in PBS) for 5 min at room temperature. After two washes in PBS, cells were permeabilized with a Hepes-Triton Buffer (20 mM Hepes pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) for 5 min at 4°C. Cells were then washed three times with PBS containing 0.2% BSA and incubated for 15 min with PBS containing 2% BSA. TRITC-phalloidin (1:100 in PBS-2% BSA) was added directly onto the glass plates in the humidified chamber for 30 min and the excess was washed away by three wash with PBS-0.2% BSA. Each glass coverslip was washed briefly in water and blocked onto a glass microscope slide with Mowiol (20% Mowiol 4-88 in PBS 1 × pH 7.4). Images were acquired with a × 63 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, Milan, Italy). 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% CO_2 for 8 h. 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 ± s.e.m.

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