DOTTORATO DI RICERCA IN MEDICINA MOLECOLARE

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Diacilglicerolo cinasi alpha
un elemento chiave nella trasduzione del segnale

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INTRODUZIONE

Premessa

Questa tesi è stata scritta ispirandosi a tre articoli pubblicati dal candidato nel corso del dottorato; tali articoli sono inclusi come riferimento sopratutto per quanto riguarda le metodologie utilizzate. I risultati sperimentali ottenuti sono stati analizzati ed eventualmente aggiornati alla luce della letteratura più recente.

Le diacilglicerolo cinasi

Il diacilglicerolo (DAG) e l'acido fosfatidico (PA) sono due lipidi che, seppur poco abbondanti quantitativamente, svolgono un ruolo di primo piano nella fisiologia cellulare facendo da secondi messaggeri regolati da stimoli extracellulari ed intracellulari (Sakane et al. 2007, Fig. 1).

Il DAG è prodotto in seguito a stimolazione della cellula con agonisti quali fattori di crescita, ormoni, neurotrasmettitori in grado di promuovere l'idrolisi dei fosfoinositidi ad opera della fosfolipasi C o dell'azione combinata della fosfolipasi D (principalmente sulla fosfatidil-colina) e della acido fosfatidico fosfatasi. Il DAG regola molte funzioni cellulari legando proteine con domini C1 classici individuati inizialmente nelle proteine cinasi C (PKC) ma presenti in molte altre proteine coinvolte nella trasduzione del segnale quali proteina cinasi D, Unc-13, chimaerin (Rac- GAP) e Ras-GRP. Inoltre il canale cationico permeabile al Ca^{++} TRPC2 è sensibile al DAG pur non avendo domini C1.

Il PA svolge numerosi ruoli nella cellula, infatti, è sia un intermedio nella sintesi dei fosfolipidi di membrana e dei trigliceridi sia un importante secondo messaggero prodotto in risposta a stimoli extracellulari ad opera delle PLD sulla fosfatidilcolina o delle DGK sul diacilglicerolo. PA regola varie proteine chiave nella trasduzione del segnale quali la fosfatidilinositolo-4-fosfato 5-Kinasi, RasGAP, Raf-1, mTOR, le PKC atipiche, $p47^{phox}$, sfingosina cinasi, p21-activated kinase 1, il repressore della trascrizione Opi1p e la proteina fosfatasi-1. Anche se il numero di proteine regolate da PA è in costante crescita non sembra esistere un dominio proteico strutturalmente definito per il legame a questo lipide non consentendo l'individuazione di possibili effettori per via bio-informatica.

Le DGK, consumando DAG per produrre PA, bilanciano la concentrazione di questi due importanti secondi messaggeri e sono quindi protagonisti della trasduzione del segnale nei metazoi; coerentemente con il loro ruolo di modulatori della fisiologia cellulare l'attività delle DGK è strettamente controllata sia temporalmente sia spazialmente. Per permettere una regolazione fine dell'attività DGK nel genoma umano sono presenti dieci geni codificanti diacilglicerolo cinasi, molti

dei quali presentano splicing alternativi portando le isoforme note a 17, spesso con pattern di espressione specifici. Le DGK sono enzimi solubili in grado di associare reversibilmente alle membrane cellulari; tutte le DGK condividono due domini C1 ed un dominio catalitico C terminale estremamente conservato ma presentano domini regolatori N terminali caratteristici che permettono una suddivisione in cinque gruppi (Topham e Prescott 1999, Fig. 2):

- tipo I (DGK α, β e γ) domini EF-hand leganti il calcio e motivi auto-inibitori analoghi alla recoverina, questa classe è il principale bersaglio dell'inibitore farmacologico R59949 (Jiang et al 2000);
- tipo II (DGKs δ, η e κ), domini PH, SAM ed un dominio catalitico bipartito;
- tipo III (DGKε) nessun dominio regolatorio ma elevata specificità per substrati con acidi grassi insaturi in posizione 2;
- tipo IV (DGKs ζ e ι) siti di fosforilazione MARCKS e quattro ankyrin repeats;
- tipo V (DGKθ) tre domini C1, un dominio ricco in glicina e prolina, dominio PH con sovrapposto un dominio di legame a Ras.

Le DGK sembrano essere caratteristiche degli organismi multicellulari, infatti in eucarioti unicellulari quali i lieviti non è stata identificata un'attività DGK né un omologo diretto mentre la DGK di *Escherichia coli* mostra un'ampia selettività di substrato e condivide solo una limitata omologia di sequenza con le DGK eucariotiche. Recentemente sono stati caratterizzati e cristallizzati geni procariotici che condividono una certa omologia con le DGK eucariotiche quali la fosfatidilglicerolo cinasi YegS di *Escherichia coli* (Bakali et al. 2007, Fig. 3)

Si stanno accumulando evidenze che le diverse isoforme di Dgk agendo in maniera regolata spazialmente e temporalmente regolino effettori specifici (tabella I e II). Le diverse isoforme agendo in contesti differenziati risultano coinvolte in numerosi eventi fisiologici e patologici; in questa discussione viene approfondita la regolazione ed il ruolo biologico dell'isoforma α che è stata ampiamente caratterizzata nel laboratorio del candidato.

Dgk-α nella trasduzione del segnale

La DGK-α è stata la prima isoforma clonata (Shaap et al. 1990) e come le altre isoforme di classe I presenta due EF-hand, due domini C1 atipici non in grado di legare il diacilglicerolo, ed una regione N terminale di omologia alle recoverine, nell'insieme questa parte dell'enzima agisce come una regione auto-inibitoria dove il legame con Ca^{++} promuove la transizione ad una conformazione aperta ed in grado di interagire con le membrane e di fosforilare il substrato (Yamada et al 1997).

La DGK-α è espressa ad alti livelli in linfociti ed oligodendrociti, mentre presenta livelli di espressione minori in epiteli ed endoteli. Questo dato e l'osservazione che i principali difetti del topo DGK-α -/- sono a carico dei linfociti T hanno fatto sì che la maggior parte degli studi funzionali presenti in letteratura riguardino i linfociti T. In questo sistema cellulare I.Merida ha riportato che DGK-α è traslocata dal citoplasma alla membrana plasmatica in seguito alla stimolazione del T cell receptor (TCR) metabolizzando il DAG e bloccando la traslocazione di RasGRP1 e PKC-θ (Sanjuan et al. 2001 e 2003, Carasco e Merida 2004). Questa traslocazione alla membrana plasmatica è mediata dal Ca^{2+} liberato a seguito dell'attivazione della PLC e richiede inoltre la fosforilazione della tirosina 335 ad opera di LCK (Merino et al 2007). A conferma del ruolo di DGK-α come regolatore negativo del signalling del TCR studi nel topo DGK-α $KO^{-/-}$ (Olenchock et al. 2006) e su linfociti T sovraesprimenti DGK-α (Zha et al. 2007) indicano che DGK-α consumando il DAG promuove l'instaurarsi ed il mantenimento di uno stato di irresponsività a lungo termine (anergia).

Questa inibizione del signalling di Ras e della conseguente proliferazione indotta dal TCR è in contrasto con l'osservazione che DGKα è richiesta per la proliferazione indotta da IL-2 promuovendo il passaggio dalla fase G1 alla fase S del ciclo cellulare (Flores et al. 1999). DGK-α sembra quindi essere un enzima bifronte: da un lato è un regolatore negativo dell'attivazione linfocitaria (Olenchock et al. 2006) ed allo stesso tempo è un effettore positivo del signalling di citochine e fattori di crescita (Flores et al. 1999, Bachiocchi et al. 2006). Una possibile spiegazione per questa contraddizione è la differenza nelle vie di trasduzione del segnale del TCR e del recettore di IL-2 che non attiva PLC e non induce rilevante produzione di DAG e liberazione di Ca^{2+} promuovendo la traslocazione di DGK-α ad una regione perinucleare in maniera Ca^{2+}-indipendente (Flores et al 1996), con concomitante produzione di PA derivato dalla fosforilazione di un pool pre-esistente di 1-alchil-2-acilglicerolo (Jones et al. 1999). In seguito a stimolazione con IL-2 l'attivazione di DGK-α richiede sia l'attività di LCK sia l'attivazione di PI3K i cui prodotti PI 3,4-bisfosfato e PI 3,4,5-trisfosfato attivano DGK-α *in vitro* (Cipres et al. 2003)

Il ruolo di DGK-α come regolatore positivo della proliferazione e del movimento cellulare indotto da fattori di crescita è stato approfondito nel laboratorio del candidato, con particolare attenzione ad epiteli ed endoteli in cui DGK-α è espressa a livelli consistenti. In particolare abbiamo dimostrato che in cellule endoteliali l'attivazione di Dgk-α genera un segnale indispensabile alle risposte proliferative e migratorie a fattori di crescita quali HGF (Cutrupi et al. 2000) e VEGF (Baldanzi et al. 2004, discusso successivamente). L'attività tirosina cinasica di Src è richiesta per l'attivazione di DGK-α e le due proteine associano in un complesso in cellule stimolate (Cutrupi et al.

2000). Src fosforila la tirosina 335 di DGK-α promuovendone l'attivazione e questa fosforilazione è necessaria insieme al rilascio di Ca^{+2} per la traslocazione al nascente lamellipodio in cellule epiteliali ed alla membrana plasmatica in linfociti T (Baldanzi et al. 2007 discusso in seguito, Merino et al. 2007).

Proliferazione ed apoptosi sono eventi strettamente interconnessi, non stupisce quindi che DGK-α oltre a promuovere la proliferazione abbia anche effetti antiapoptotici. Usando l'inibitore farmacologico R59949 è stato suggerito che DGKα sia necessaria per la sopravvivenza dei linfociti $CD4^{+}CD8^{+}$ doppi positivi durante la selezione timica (Outram et al. 2002) anche se Olenchock et al. non hanno osservato differenze nel numero o nella differenziazione dei linfociti nel topo DGKα -/- (Olenchock et al. 2006). A questo proposito è interessante notare che Alonso et al. dimostrano che DGK-α attenua la morte cellulare indotta da attivazione, un fenomeno rilevante per la limitazione della risposta cellulo mediata (Alonso et al. 2005). Questo effetto protettivo di Dgk-α è realizzato bloccando la secrezione di exosomi presentanti FAS in forma attiva associata alle membrane; la secrezione di tali vescicole coinvolge la loro formazione a livello del trans-Golgi network e la loro fusione con la plasma membrana. E' rilevante che la vescicolazione dal post-trans-Golgi network dipende dal pool di DAG e DGK-α è principalmente associata con il trans-Golgi network in linfociti T. Quindi verosimilmente l'attenuazione dell'accumulo di DAG ad opera di DGK-α causa il blocco del trasporto di proteine (compreso FAS) dal trans-Golgi network alla superficie cellulare; consistentemente ci sono indicazioni che Dgk-α si localizza all'apparato di Golgi in CHO stimolate con acido arachidonico (Shirai et al. 2000).

Questa serie di dati hanno suggerito al gruppo di ricerca del candidato un possibile ruolo di DGK-α nel controllo della proliferazione e della resistenza all'apoptosi di cellule tumorali. Come discusso successivamente abbiamo osservato che Dgk-α è constitutivamente attivata e sostiene la proliferazione di cellule trasformate dall'oncogene NPM/ALK. Questo oncogene deriva da un riarrangiamento inappropriato caratteristico dei linfomi anaplastici a grandi cellule che da origine ad una proteina di fusione tra la nucleofosmina e la tirosina kinasi ALK, realizzando una tirosina kinasi citoplasmatica constitutivamente attiva. NPM/ALK è in grado di attivare Dgk-α tramite Src, inoltre l'inibizione di Dgk-α con R59949 o con siRNA specifici riduce significativamente la proliferazione di cellule trasformate da NPM/ALK (Bachiocchi et al. 2005). Il ruolo di Dgk-α nella trasformazione tumorale non sembra essere esclusivo dei linfomi in quanto in cellule MDA-MB-231, un modello di carcinoma mammario ER negativo, l'inibitore R59949 è in grado di ridurre la crescita in assenza di

ancoraggio e l'invasione della matrice (Filigheddu et al. 2008). Analogamente Sakane e colleghi hanno recentemente pubblicato che Dgk-α è espressa ad alti livelli in numerose linee cellulari derivate da melanomi umani e che la sua sovraespressione sopprime l'apoptosi indotta da TNF-α attivando NF-κB (Yanagisawa et al 2007).

RISULTATI E DISCUSSIONE

Dgk-α nella trasduzione del segnale angiogenetico di VEGF.

La neo-angiogenesi è un evento fondamentale in numerosi eventi sia fisiologici quali il ciclo mestruale sia patologici come la rivascolarizzazione di tessuti ischemici, la crescita tumorale e la metastatizzazione. Nell'adulto la formazione di nuovi vasi sanguigni avviene per gemmazione e ramificazione da vasi pre-esistenti (Folkman, 1972; Carmeliet and Jain, 2000). La neoangiogenesi è indotta da fattori angiogenetici secreti da tessuti ipossici o danneggiati, quali VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor) e HGF (hepatocytes growth factor) (Bussolino et al.1992; Matsumoto and Claesson-Welsh, 2001). Un ruolo centrale in vivo è svolto in particolare da VEGF-A i cui recettori sono espressi principalmente in cellule endoteliali (Folkman, 1972; Carmeliet e Jain, 2000; Matsumoto e Claesson-Welsh, 2001).

Dato che l'inibizione dell'angiogenesi è considerata un approccio promettente per la cura dei tumori solidi vi è un cospicuo interesse nell'identificazione di nuove proteine necessarie per la trasduzione del segnale angiogenico; tali proteine rappresentano, infatti, potenziali bersagli biochimici per terapie antiangiogeniche (Folkman, 1972; Carmeliet e Jain, 2000; Matsumoto e Claesson-Welsh, 2001).

VEGF-A promuove l'angiogenesi attivando in maniera coordinata ed ordinata nel tempo numerose vie di segnalazione intracellulare che portano alla migrazione, proliferazione ed organizzazione in strutture tubulari (vedi Matsumoto e Claesson-Welsh, 2001). Tra queste vie di trasduzione del segnale notiamo i secondi messaggeri derivanti dal $PI_{4,5}P2$; l'angiogenesi indotta da VEGF-A richiede sia la fosforilazione ad opera della PI3K sia l'idrolisi ad opera della PLCγ. Mentre $PI_{3,4,5}P3$ è richiesto per l'attivazione di AKT e la sopravvivenza dell'endotelio (Gerber et al. 1998), DG media il signaling proliferativo e chemiotattico di VEGF-A ed è richiesto per l'attivazione di PKC-α (Wellner et al. 1999; Matsumoto e Claesson-Welsh, 2001).

Ci siamo interessati all'angiogenesi in quanto avevamo precedentemente osservato che, in una linea cellulare derivata da endotelio aortico porcino (PAE), la stimolazione con HGF promuoveva la formazione di un complesso con la tirosina cinasi Src e l'attivazione di Dgk-α. Inoltre l'inibizione di Dgk-α, sia tramite l'espressione di un mutante dominante negativo sia con l'inibitore farmacologico R59949, bloccavano la chemiotassi indotta da HGF in cellule endoteliali (Cutrupi et al. 2000). Confortati anche dal fatto che la proliferazione e la motilità indotte da VEGF sono totalmente dipendenti da SRC (Abu-Ghazaleh et al. 2001), abbiamo ipotizzato che Dgk-α fosse richiesta per la

trasduzione del segnale angiogenico di VEGF-A. Per lo studio del ruolo di Dgk-α nell'angiogenesi in vitro abbiamo usando come modelli cellulari sia cellule PAE esprimenti VEGFR-2 (PAE-KDR) sia cellule endoteliali da cordone ombelicale umano (HUVEC).

VEGF-A_{165} attiva Dgk-α

Per verificare se fattori pro angiogenici stimolano Dgk-α abbiamo misurato in vitro in presenza di substrati esogeni (DAG ed ATP marcato) l'attività Dgk di omogenati di cellule PAE-KDR ed HUVEC stimolate o meno con VEGF-A_{165}. L'attività degli omogenati ottenuti da cellule PAE-KDR ed Huvec stimolate con VEGF-A_{165} è risultata doppia di quella ottenuta dai rispettivi controlli (Fig. 4a); l'attivazione è sostenuta nel tempo per almeno un'ora (Fig. 4b) e dipendente dalla concentrazione di VEGF-A_{165} (dati non mostrati). Come atteso l'attività Dgk di cellule PAE, che non esprimono VEGFR-2, non risulta influenzata da VEGF-A_{165} (Fig. 4a).

Questi esperimenti dimostrano che la stimolazione con VEGF-A_{165} promuove l'attività Dgk di cellule endoteliali, anche se non permettono di stabilire quali delle numerose isoforme note siano attivate da VEGF-A_{165}; Dgk-α è espressa sia in cellule PAE-KDR sia in cellule HUVEC (anche se a livelli minori, dati non mostrati). Per verificare se l'isoforma α sia regolata da VEGF-A_{165}, abbiamo misurato in vitro l'attività Dgk in immunoprecipitati da cellule PAE-KDR, controllo o stimolate con VEGF-A_{165}, ottenuti con anticorpi specifici per l'isoforma α. Come ipotizzato l'attività Dgk-α risulta maggiore in cellule stimolate con VEGF-A_{165} mentre la quantità di proteina immunoprecipitata rimane costante (Fig. 5a). L'attivazione in cellule stimolate con VEGF-A_{165} è mantenuta per almeno un'ora (Fig. 5b) ed è dipendente dalla concentrazione di VEGF-A_{165} utilizzata (Figure 5c). Immunoprecipitati di controllo ottenuti in assenza di anticorpi anti Dgk-α non contenevano né la proteina Dgk-α né attività Dgk.

Per meglio caratterizzare le attività Dgk attivate da VEGF-A_{165} in PAE-KDR e HUVEC abbiamo utilizzato R59949 un inibitore di Dgk relativamente specifico per la classe I (Jiang et al. 2000) ed i cui effetti inibitori sul signalling di HGF in cellule PAE sono annullati dalla sovraespressione di Dgk-α (Cutrupi et al. 2000). In cellule endoteliali R59949 non influenza né l'attivazione di VEGFR-2 né la vitalità cellulare misurata come esclusione di trypan blue (dati non mostrati). In vitro, 1 μM R59949 inibisce completamente l'attività Dgk sia basale sia indotta da VEGF-A_{165} in omogenati di cellule PAE-KDR (Fig. 6); contrariamente R59949, anche a 10 μM, inibisce solo parzialmente l'attività

Dgk in omogenati di cellule HUVEC (Fig. 6) suggerendo che in queste cellule vi siano altre isoforme diverse dalla α e meno sensibili a R59949.

L'espressione di un mutante dominante negativo di Dgk-α blocca l'angiogenesi indotta da VEGF-A_{165}.

Per approfondire il ruolo di Dgk-α nel signalling angiogenico di VEGF abbiamo fatto uso di un mutante cataliticamente inattivo in grado di agire da dominante negativo in cellule PAE (Dgk-α-K-, Cutrupi et al. 2000). Cellule PAE-KDR sono state infettate con i retrovirus PINCOS vuoto o trasducente Dgk-α-wt o Dgk-α-K- (Cutrupi et al. 2000).

Le linee cellulari così ottenute sono state sottoposte ad un saggio di chemiotassi su collagene I; l'espressione del mutante dominante negativo inibisce significativamente la migrazione di cellule PAE-KDR in risposta a VEGF-A_{165}, mentre l'espressione dell'enzima wt aumentava debolmente la risposta migratoria (Fig. 7a).

Le stesse linee cellulari sono state anche saggiate per la capacità proliferativa in risposta a VEGF-A_{165} misurata come incorporazione di 5-Br-desossiuridina (BrdU). L'espressione del mutante dominante negativo riduce la sintesi di DNA in cellule PAE-KDR, mentre l'espressione dell'enzima wt non ha effetto (Figure 7b).

Per esplorare ulteriormente il coinvolgimento di Dgk-α nell'angiogenesi abbiamo testato l'effetto del mutante dominante negativo o dell'inibitore R59949 in un test molto usato per saggi in vitro di fattori pro- ed anti- angiogenici. Il saggio si basa sulla capacità, tipica delle cellule endoteliali, di migrare ed organizzarsi in strutture tabulari disposte a rete quando piastrate su matrigel, una membrana basale ricostituita ricca di laminina e fattori di crescitan (Benelli and Albini, 1999). Come atteso le cellule PAE-KDR piastrate su matrigel in presenza di VEGF-A_{165} si allungano e connettono formando cordoni disposti a rete; sorprendentemente l'espressione del mutante cataliticamente negativo pur non alterando la vitalità cellulare rende le cellule incapaci di migrare ed organizzarsi in maniera appropriata (Fig. 7c).

R59949, inibitore farmacologico di Dgk, blocca l'angiogenesi in vitro indotta da VEGF-A_{165}

Dgk-α è inibita da concentrazioni micromolari di R59949, abbiamo quindi verificato se questo inibitore fosse in grado di bloccare le risposte biologiche indotte da VEGF-A_{165} in cellule HUVEC e PAE-KDR. La nostra precedente osservazione che l'effetto inibitorio di R59949 sulla chemiotasi

indotta da HGF è completamente annullato dalla sovraespressione di Dgk-α fornisce una forte indicazione della specificità di R59949 in cellule PAE (Cutrupi et al. 2000).

Come ipotizzato R599491 μM riduce drasticamente la migrazione indotta da VEGF-A_{165} in cellule PAE-KDR e HUVEC, senza influenzare significativamente la motilità spontanea (Figura 8a e 8b). Abbiamo anche studiato gli effetti di R59949 sulla sintesi di DNA, misurata come incorporazione di BrdU, dopo stimolazione con VEGF-A_{165}. In cellule PAE-KDR, mantenute in assenza di siero, VEGF-A_{165} induce un raddoppio dell'incorporazione di BrdU, effetto completamente bloccato da R59949 1 μM (Fig. 8c). Mentre la sintesi di DNA indotta da VEGF-A_{165} in HUVEC coltivate in 2% FCS risulta inibita solo da dosi di R59949 uguali superiori a 5 μM (Figura 8d). Le alte concentrazioni di R59949 richieste per bloccare la sintesi di DNA in Huvec possono dipendere o dall'effetto sequestrante del siero su R59949 (De Chaffoy de Courcelles et al. 1989) o dal coinvolgimento di altre isoforme di Dgk meno sensibili a questo inibitore (Jiang et al. 2000).

Abbiamo anche verificato che R59949 fosse in grado di inibire la formazione di strutture tubulari su matrigel, un noto saggio di angiogenesi in vitro. Come ipotizzato in presenza di R59949 1 μM sia cellule HUVEC sia cellule PAE-KDR, pur se vitali, non sono in grado di connettersi a formare la tipica struttura a rete (Fig. 7 e 9). R59949 è quindi in grado di bloccare l'angiogenesi in vitro senza influenzare né la vitalità cellulare né la fosforilazione di VEGF-R1 in risposta a VEGF-A_{165} (Fig. 9 e dati non mostrati).

siRNA specifici per Dgk-a bloccano l'angiogenesi in vitro di cellule endoteliali primarie HUVEC

Infine abbiamo voluto verificare specificamente il ruolo di Dgk-α nell'angiogenesi in vitro in cellule endoteliali primarie HUVEC. A tal fine ho realizzato tre siRNA disegnati con l'algoritmo Cenix per promuovere la degradazione specificamente del trascritto di Dgk-α, come controllo è stato utilizzato un siRNA non complementare con alcuna sequenza umana. Quando trasfettati in cellule HUVEC, tutti e tre i siRNA specifici per Dgk-α sono risultati in grado di diminuirne l'espressione misurata tramite western blot in lisati totali (Fig. 10). Inoltre i tre siRNA inibiscono l'angiogenesi in vitro su matrigel in maniera simile a R59949, mentre RNA controllo non ha effetti significativi. In questo sistema sperimentale la diminuzione delle quantità di Dgk-a non influenza significativamente la vitalità cellulare (Figura 10). Questi dati sono in ottimo accordo con quelli ottenuti precedentemente con il mutante dominante negativo e con l'inibitore R59949 supportando il coinvolgimento dell'attività Dgk-αnella trasduzione del segnale angiogenico di VEGF-A_{165}.

Ruolo di Src nell'attivazione di Dgk-α indotta da VEGF-A_{165}

Per approfondire il meccanismo di attivazione di Dgk-α in cellule stimolate con VEGF siamo partiti dall'osservazione che l'attivazione indotta da un altro fattore di crescita, HGF, era mediata dall'attività della tirosina cinasi citoplasmatica Src (Cutrupi et al., 2000). E' interessante come la letteratura riporti che l'attività di Src è necessaria per la motilità cellulare e la proliferazione indotte da VEGF-A_{165} (Abu-Ghazaleh et al., 2001; Eliceiri et al., 2002). Queste osservazioni e i dati in letteratura indicanti che un'attività Dgk associ con v-Src (Sugimoto et al. 1984) ci hanno portato ad ipotizzare che Dgk-α fosse un effettore a valle di Src nel signalling di VEGF-A.

Per verificare questa ipotesi l'attività di Src è stata inibita pretrattando con gli inibitori PP1 e PP2 rispettivamente cellule PAE-KDR o HUVEC, alla concentrazione di 5 μM questi inibitori non bloccano l'attivazione di VEGFR-2 (Figura 11a). L'inibizione dell'attività tirosina cinasica di Src blocca completamente l'attivazione di Dgk indotta da VEGF-A_{165}, dimostrando che Src è richiesto per tale attivazione (Figura 11b).

Inoltre abbiamo verificato che anche la stimolazione con VEGF-A_{165} promuovesse la formazione di un complesso tra Dgk-α e Src, analogamente a quanto già osservato in cellule stimolate con HGF (Cutrupi et al. 2000). Infatti un'attività Dgk co-immonoprecipita con Src in cellule PAE-KDR e attività è aumentata in seguito a stimolazione con VEGF-A_{165} (Figura 12a). Non siamo riusciti a rilevare tramite western blotting Dgk-α in immunoprecipitati anti Src, ma siamo riusciti ad evidenziare myc-Dgk-α co-immunoprecipitata con Src in cellule PAE-KDR infettate con PINCOS-myc-Dgk-α; inoltre la quantità di myc-Dgk-α associata a Src aumenta in cellule stimolate con VEGF-A_{165} (Figura 12b). Inoltre abbiamo osservato la formazione del complesso con proteine endogene rilevando la presenza di Src in immunoprecipitati con anticorpi anti-Dgk-α, anche in questo caso il complesso risulta incrementato in seguito a stimolazione con VEGF-A_{165} (Figura 12c).

Nell'insieme questi dati indicano che Src media l'attivazione di Dgk-α indotta da VEGF e che l'attivazione è accompagnata dalla formazione di un complesso tra Src e Dgk-α. Non è stato possibile evidenziare la presenza di Dgk-α con VEGF-R1, consistentemente a quanto osservato con HGFR, indicando che Dgk-α non forma un complesso stabile con i recettori tirosina cinasi attivati (Cutrupi et al. 2000).

Conclusioni

Concludendo siamo partiti dall'osservazione che Dgk-α viene attivata da HGF tramite la tirosina cinasi Src ed è richiesta per il movimento cellulare indotto da HGF in una linea cellulare di endotelio aortico (Cutrupi et al 2000). Questo ci ha suggerito un possibile ruolo di Dgk-α nel signalling angiogenetico; per verificare questa ipotesi abbiamo verificato il ruolo di Dgk-α nell'angiogenesi in vitro indotta da VEGF-A_{165} scelto come prototipo dei fattori di crescita pro-angiogenetici.

I dati mostrati indicano che la stimolazione con VEGF-A_{165} promuove l'attivazione, Src mediata, di Dgk-α e la formazione di un complesso Src/Dgk-α. Inoltre gli esperimenti con inibitori farmacologici, mutanti dominanti negativi e siRNA indicano che l'attività enzimatica di Dgk-α è necessaria per la proliferazione, la migrazione e l'angiogenesi in vitro sia nella linea di endotelio PAE-KDR, sia in cellule endoteliali primarie di cordone ombelicale HUVEC.

Sorprendentemente, come già osservato in cellule stimolate con HGF, anche in questo caso non è stato possibile evidenziare una consistente fosforilazione in tirosina di Dgk-α in cellule stimolate con VEGF-A_{165} (Cutrupi et al. 2000). Questo potrebbe dipendere da una fosforilazione transiente o con bassa stechiometria; alternativamente Src potrebbe fosforilare un attivatore di Dgk-α quale PI3K come suggerito da Cipres et al. 2003. L'esistenza di una fosforilazione di Dgk-α con significato attivatorio è suggerita dall'osservazione che sia la co-espressione con Src sia il trattamento con pervanadato inducono la fosforilazione in tirosina e l'attivazione di Dgk-α (Cutrupi et al. 2000 e osservazioni non pubblicate).

Nell'insieme questi dati confermano l'ipotesi iniziale che l'attivazione Src mediata di Dgk-α sia un evento necessario per l'angiogenesi in vitro, anche se non escludono la possibilità che altre isoforme partecipino a questo signalling. Inoltre questi dati suggeriscono che Dgk-α sia un nuovo possibile bersaglio di terapie farmacologiche mirate al controllo dell'angiogenesi.

L'attivazione di Dgk-α è essenziale per la mitogenesi indotta dalla cinasi dei linfomi anaplastici

Il gene ALK (anaplastic lymphoma kinase) codifica per un recettore tirosina cinasi di 200-kDa espresso esclusivamente nel sistema nervoso, alcuni studi suggeriscano che il ligando nei vertebrati siano pleiotrophin e midkine (Stoica et al. 2001, Stoika et al. 2002). Il coinvolgimento di ALK nella patogenesi di tumori ematopoietici deriva dall'osservazione che i riarrangiamenti oncogenici di ALK sono centrali nella patogenesi dei linfomi anaplastici a grandi cellule (ALCLs) un sottogruppo di linfomi non-Hodgkin con immuno-tipo T o nullo. Le forme oncogeniche di ALK derivano da riarrangiamenti cromosomici che fondono il dominio citoplasmatico tirosina cinasi di ALK con geni in genere in grado di dimerizzare, il più comune da origine alla proteina di fusione nucleophosmin/ALK (NPM/ALK) una proteina ibrida di 80-kDa prodotta dal riarrangiamento t(2;5)(p23;q35). NPM/ALK è un oncogene in grado di trasformare vari tipi cellulari in vitro ed in vivo (Kuefer et al. 1997, Chiarle et al. 2003; Lange et al. 2003; Meithing et al. 2003). Le vie di segnalazione che mediano l'attività trasformante di NPM/ALK sono tuttora poco caratterizzate ma comprendono Grb2, Shc, IRS-1, PLC-γ, p60*src*, PI3-K, Stat3 e Stat5 (Pulford et al. 2004; Nieborowska-Skorska et al. 2001).

Nel tentativo di identificare nuove vie di trasduzione del segnale attive nei tumori ALK positivi abbiamo valutato il coinvolgimento di Dgk-α basandoci sul suo ruolo nella transizione G1-S indotta da IL-2 in linfociti T (Flores et al. 1996 e 1999) ed il suo coinvolgimento nella trasduzione del segnale di fattori di crescita quali HGF e VEGF (Cutrupi et al. 2000, Baldanzi et al. 2004).

ALK attiva Dgk-α

Per valutare lo stato di attivazione di Dgk ne abbiamo misurato l'attività in vitro con substrati esogeni in estratti cellulari totali in diversi sistemi sperimentali di cellule ALK trasformate.

Il primo modello cellulare analizzato sono state cellule Karpas 299 una linea di ALCL NPM/ALK positiva; queste cellule presentano un'elevata attività Dgk basale R59949 sensibile (Fig. 13a). Come secondo modello cellulare abbiamo utilizzato cellule 32D una linea ematopoietica indifferenziata IL-3 dipendente e 32D-NPM/ALK infettate con un vettore vuoto con un retrovirus esprimente GFP-NPM/ALK. In Fig. 14a si osserva che mentre le cellule controllo 32D hanno una bassa attività Dgk le cellule 32D-NPM/ALK hanno un'attività sostanzialmente maggiore, suggerendo che la trasformazione attivi una Dgk che anche in questo caso è sensibile a R59949 suggerendo che si tratti dell'isoforma α (Fig. 13a).

Per verificare il coinvolgimento dell'isoforma a abbiamo misurato l'attività di DGK-α in immunoprecipitati ottenuti con anticorpi isoforma specifici da cellule Karpas 299 o CEM, linfoblasti T umani NPM/ALK-negativi. Entrambe le linee cellulari esprimono DGK-α a livelli comparabili ma l'attività risulta notevolmente maggiore in cellule Karpas 299 (Fig. 13B). Risultati simili sono stati ottenuti confrontando cellule CEM con linfociti TS un'altra linea NPM/ALK positiva (Fig. 13B).

Questi dati suggeriscono che NPM/ALK attivi Dgk-α; per rinforzare questa osservazione abbiamo utilizzato un recettore chimerico con la parte extracellulare di EGFR e la porzione intracellulare di ALK (Piccinini et al. 2002). Quando espresso in cellule NIH-3T3 questo recettore chimerico è attivato dal legame di EGF consentendo di studiare la trasduzione del segnale ligando indotta di ALK. In questo sistema la stimolazione con EGF induce l'aumento dell'attività Dgk misurata in omogenati totali con un massimo a 10 minuti ed un ritorno a livelli basali in 30 minuti (Fig. 14a). Gli anticorpi utilizzati precedentemente non consentono di immunoprecipitare l'enzima murino; abbiamo quindi trasfettato le stesse cellule con myc-Dgk-α e misurato l'attività in immunoprecipitati anti myc. I dati in figura 14b confermano che EGF attiva myc-Dgk-α in cellule NIH-EGFR/ALK.

L'attivazione di Dgk-α indotta da ALK è mediata da Src

Dato il coinvolgimento di tirosine cinasi della famiglia di Src nell'attivazione di Dgk-α in cellule stimolate con fattori di crescita ed interleuchine (Cutrupi et al. 2000, Baldanzi et al. 2004, Cipres et al. 2003) abbiamo ipotizzato un coinvolgimento di Src nell'attivazione di Dgk-α in cellule trasformate con NPM/ALK. Questa ipotesi è sostenuta dall'osservazione che in linfomi NPM/ALK positivi (sia Karpas 299 sia TS) l'attività Dgk-α era sostanzialmente ridotta dopo un'ora di pre-trattamento con l'inibitore di Src PP2 (Fig. 13b).

A sostegno di questa ipotesi abbiamo analizzato l'attivazione di myc-Dgk-α in cellule NIH-EGFR/ALK; in queste cellule la stimolazione con EGF induce la fosforilazione di tirosine del recettore in grado di legare ed attivare di Src (dati non mostrati). Queste cellule presentano un'attività Dgk associata a Src che è notevolmente aumentata in seguito a stimolazione con EGF mentre non si ha stimolazione in cellule NIH-EGFR/ALKY_{979}F esprimenti un recettore mutato che non recluta Src (Fig.15a). In questo sistema cellulare myc-Dgk-α co-immunoprecipitata costitutivamente con Src anche in assenza di stimolazione (Fig. 15b), confermando l'esistenza di un complesso Dgk-α/Src e suggerendo che tale complesso sia preformato in assenza di attivazione di Src almeno nelle condizioni di sovraespressione utilizzate.

Ruolo di Dgk-α nella trasduzione del segnale mitogenico di ALK

Per studiare il ruolo di Dgk-α nella proliferazione indotta da ALK abbiamo inizialmente utilizzato l'inibitore farmacologico R59949. Il trattamento con 10 μM R59949 inibisce notevolmente gli effetti proliferativi di EGF in cellule NIH-EGFR/ALK senza influenzare la proliferazione indotta da 1% FBS (Fig. 16). Simili effetti inibitori sono stati osservati in cellule 32D-NPM/ALK e Karpas utilizzando il saggio MTT (Figure 16). Non abbiamo osservato effetti tossici di 10 μM R59949 utilizzando il saggio di esclusione del trypan blue (dati non mostrati).

Per ottenere evidenze dirette del coinvolgimento dell'isoforma α nella proliferazione indotta da NPM/ALK abbiamo bloccato l'espressione della proteina in cellule Karpas 299 utilizzando siRNA specifici (Fig. 17a). A 72 ore dall'elettroporazione con i siRNA le cellule con siRNA specifico per Dgk-α mostrano una riduzione drammatica dell'incorporazione di BrdU rispetto a cellule trattate con siRNA controllo (Fig. 17b).

Concludendo questi esperimenti dimostrano che sia l'inibizione di Dgk con R59949 sia il blocco dell'espressione di Dgk-α con siRNA specifici riducono la proliferazione in cellule trasformate con NPM/ALK, suggerendo che una sua inibizione in vivo potrebbe interferire con la patogenesi degli ALCL.

Conclusioni

Nel tentativo di identificare nuove vie di trasduzione del segnale responsabili della trasformazione cellulare negli ALCL abbiamo verificato il coinvolgimento di Dgk-α dimostrando che: 1) Dgk-α è costitutivamente attiva in linee cellulari NPM/ALK positive derivate da ALCL ed in cellule 32D infettate con NPM/ALK, 2) l'attivazione della chimera EGFR/ALK in cellule NIH-3T3 induce una contemporanea attivazione di Src e di Dgk-α, 3) l'attivazione di Dgk-α è mediata da Src con cui Dgk-α forma un complesso costitutivo, 4) l'inibizione di Dgk-α con R59949 o la riduzione con siRNA specifici diminuisce drasticamente la proliferazione sostenuta con NPM/ALK.

La sorprendente mancanza di regolazione nella formazione del complesso Dgk-α/Src può dipendere dalla parziale attivazione di Src promossa dal signalling integrinico o può essere dovuta alla sovraespressione sperimentale di myc-Dgk-α nei fibroblasti che normalmente esprimono bassi livelli di Dgk-α. Nonostante questi risultati suggeriscano che Dgk-α sia un substrato di Src non è stato possibile rilevare alcuna fosforilazione di Dgk-α nei sistemi cellulari utilizzati, analogamente a quanto osservato con HGFR e VEGFR-2 (Cutrupi et al. 2000 e Baldanzi et al. 2004).

Infine queste osservazioni indicano Dgk-α come un possibile bersaglio farmacologico per il controllo della proliferazione dei linfomi anaplastici a grandi cellule.

La fosforilazione di Dgk-α sulla tirosina 335 ad opera di Src media l'attivazione, la traslocazione in membrana e la motilità cellulare indotta da HGF.

Nei lavori precedenti avevamo evidenziato come Dgk-α sia regolata da numerosi fattori di crescita quali VEGF ed HGF in cellule epiteliali ed endoteliali (Cutrupi et al., 2000; Baldanzi et al., 2004); IL-2 in linfociti T (Flores et al., 1999; Cipres et al., 2003). L'inibizione di Dgk-α con inibitori farmacologici, siRNA o mutanti dominanti negativi blocca il movimento cellulare e la proliferazione in svariati modelli cellulari (Cutrupi et al., 2000; Baldanzi et al., 2004; Bacchiocchi et al., 2005, Flores et al., 1999).

L'attivazione di Dgk-α ad opera dei recettori per fattori di crescita o citochine richiede l'attività di tirosine cinasi della famiglia di Src e l'associazione di Dgk-α con Src o Lck (Cutrupi et al., 2000; Cipres et al., 2003; Baldanzi et al.,2004; Bacchiocchi et al., 2005). Inoltre inibendo le fosfatasi cellulari con pervanadato o trasfettando tirosine cinsi della famiglia di Src si osserva una costitutiva fosforilazione ed attivazione Dgk-α (Cutrupi et al., 2000; Cipres et al., 2003). Nonostante questi dati suggeriscano che Dgk-α sia regolata tramite fosforilazione in tirosina non è stata osservata fosforilazione in tirosina dopo stimolazione con fattori quali HGF, VEGF, IL-2 od in seguito ad attivazione del recettore ALK, facendo pensare ad una fosforilazione transitoria od a bassa stechiometria oppure ad una attivazione medita da effettori di Src quali PI3K (Cutrupi et al., 2000; Cipres et al., 2003; Baldanzi et al., 2004; Bacchiocchi et al., 2005). L'attivazione di Dgk-α è strettamente coordinata con il suo reclutamento in membrana, dove trova il substrato; i due fenomeni sembrano essere sottoposti a più livelli di regolazione. Cipres e colleghi dimostrano che l'attivazione e presumibilmente il reclutamento in membrana di Dgk-α in linfociti T stimolati con IL-2 è mediato dal legame al PIP3 e richiede i domini C1; suggerendo inoltre che questi domini sono mascherati in Dgk-α e che altri eventi quali il legame del calcio ai due domini EF-hand sono richiesti per esporli (Sanjuan et al., 2001; Cipres et al., 2003).

La tirosina 335 e la coda ricca in proline di Dgk-α sono richieste per la fosforilazione Src mediata interagendo rispettivamente con i domini SH2 e SH3 di Src

Per verificare se Dgk-α sia un substrato diretto di Src abbiamo incubato una Dgk-α parzialmente purificata (come proteina di fusione con GST) con Src ricombinante in presenza di ATP e Mg^{++}. In queste condizioni Src promuove una forte fosforilazione in tirosina di GST-Dgk-α come

evidenziato con western blot con anticorpi anti-fosfotirosina (Fig. 18a). Osservando la sequenza primaria di Dgk-α abbiamo notato due tirosine con la caratteristica isoleucina in posizione -1: FLKIY$_{60}$LEVDN e PPSSIY$_{335}$PSVLA che conferisce specificità di substrato per Src (Songyang e Cantley, 1995; Schmitz et al., 1996). Per verificare se queste due tirosine sono substrato per l'attività tirosina cinasi di Src in cellule intatte abbiamo co-espresso Src con i rispettivi mutanti a fenil-alanina. Myc-Dgk-α-wt è fosforilata in tirosina quando co-espressa in cellule COS con Src come precedentemente riportato (Cutrupi et al. 2000); nello stesso saggio anche Myc-Dgk-α-Y$_{60}$F è fosforilata in tirosina mentre myc-Dgk-α-Y$_{335}$F non presenta una fosforilazione in tirosina rilevabile (Fig. 18b). Questi esperimenti indicano che Dgk-α può essere fosforilata direttamente da Src e che Y$_{335}$ è il principale sito di fosforilazione.

Dato che i siti ottimali per la fosforilazione ad opera di Src sono anche siti per il legame del dominio SH2 di Src stesso (Songyang et al., 1993; Songyang and Cantley, 1995), abbiamo deciso di investigare se Y$_{335}$ mediasse anche l'interazione tra Dgk-α ed il dominio SH2 di Src. Il saggio di interazione è stato condotto utilizzando una proteina ricombinante purificata GST-Src-SH2 immobilizzata su resina ed un lisato di cellule COS trasfettate con Myc-Dgk-α wt o mutanti. Myc-Dgk-α wt associa GST-SrcSH2 e non il solo GST, indicando un'interazione specifica con il dominio SH2 di Src (Fig 19a). Come controlli di specificità abbiamo utilizzato il mutante GST-Src-SH2 R$_{175}$L che non riconosce la tirosina fosforilata dei ligandi (Yeo et al., 2006) e come atteso non associa con Myc-Dgk-α (Tabella 3). Inoltre il mutante Myc-Dgk-α-Y$_{335}$F, che non è fosforilato da Src, non associa a GST-Src-SH2 mentre il mutante Myc-Dgk-α-Y$_{60}$F associa come il wt. Concludendo questa serie di esperimenti dimostra che la tirosina 335 fosforilata di Dgk-α interagisce selettivamente con il dominio SH2 di Src. In vitro questa interazione non è limitata a Src, ma è stata osservata anche con Btk, C-PLCγ, Grb2 e Lck ma non con i domini SH2 di Abl, n-PLCγ e n-p85 (Tabella 3).

Dato che numerosi substrati di Src, come p130Cas, vengono fosforilati dopo un'interazione tra il dominio SH3 di Src ed una sequenza ricca in proline (Pellicena e Miller, 2001), abbiamo verificato una possibile interazione tra Dgk-α ed il dominio SH3 di Src nel saggio precedentemente illustrato. Myc-Dgk-α-wt and Myc-Dgk-α-Y$_{335}$F associano specificamente a GST-Src-SH3 ma non al solo GST (Fig. 15b), confermando l'interazione tra Dgk-α ed il dominio SH3 di Src. A conferma della specificità di tale interazione non abbiamo osservato associazione tra Dgk-α e il mutante GST-Src-SH3-D$_{99}$N, che non lega sequenze di poli-proline (Weng et al., 1995). Nonostante Dgk-α non contenga una sequenza consenso per l'interazione con il dominio SH3 canonica (PxxP), presenta una seguenza C terminale

ricca in proline molto conservata (PMLMGPPPR). Abbiamo quindi generato due mutanti di delezione mancanti della metà C terminale di Dgk-α (Myc-Dgk-α-STOP) o degli ultimi 13 aa (Myc-Dgk-α-ΔP). Entrambi i mutanti mancanti del C-terminale non associano a GST-Src-SH3 (Fig. 19b); indicando che la coda ricca in proline è richiesta per l'interazione tra Dgk-α e il dominio SH3 di Src. L'interazione non è limitata al dominio SH3 di Src; almeno in vitro anche quelli di Lck ed Abl interagiscono ugualmente bene (tabella 3).

Basandoci sul modello di fosforilazione processiva di p130Cas (Pellicena e Miller, 2001), abbiamo ipotizzato che la coda ricca in proline di Dgk-α sia richiesta per la fosforilazione in tirosina ad opera di Src. Per verificare questa ipotesi abbiamo coespresso in cellule 293 T Myc-Dgk-α wt, ΔP o $Y_{335}F$ con Src-$Y_{527}F$, una forma di Src costitutivamente attiva. La figura 20 mostra che sia Myc-Dgk-α-ΔP sia Myc-Dgk-α-$Y_{335}F$ non sono fosforilate in seguito a coespressione con Src-$Y_{527}F$; mentre Myc-Dgk-α-wt è fosforilata in tirosina come atteso. Concludendo questi dati dimostrano che la coda ricca in proline di Dgk-α è richiesta per l'interazione con il dominio SH3 di Src e suggeriscono che tale interazione preceda la fosforilazione della tirosina 335.

La tirosina 335 e la coda riccha in proline sono richieste per l'attivazione di Dgk-a da HGF e vSrc

Gli esperimenti presentati dimostrano che la Y_{335} e la coda ricca in prolina di Dgk-α sono i principali determinanti molecolari per la fosforilazione ad opera di Src. Inoltre i rispettivi mutanti possono essere utilizzati per verificare se la fosforilazione della Y_{335} è necessaria per l'attivazione di Dgk-α. Infatti, anche se numerose evidenze indicano un ruolo dell'attività tirosina cinasica di Src, l'esistenza di una fosforilazione attivatoria di Dgk-α indotta da fattori di crescita rimane dibattuta (Cutrupi et al., 2000; Cipres et al., 2003; Baldanzi et al., 2004; Bacchiocchi et al., 2005).

Inizialmente abbiamo esaminato l'attività enzimatica di omogenati di cellule Cos esprimenti Myc-Dgk-α wt, $Y_{335}F$ o ΔP, dopo co-incubazione con omogenati di cellule esprimenti Src. Come precedentemente osservato l'attività enzimatica di Myc-Dgk-α wt è significativamente aumentata dopo co-incubazione con Src (Cutrupi et al., 2000; Fig. 21). Al contrario l'attività enzimatica di Myc-Dgk-α–$Y_{335}F$ e di Myc-Dgk-α–ΔP non è significativamente stimolata dalla co-incubazione con src in vitro (Fig. 21); dimostrando che sia Y_{335} sia la coda ricca in proline sono richieste per l'attivazione in vitro.

Abbiamo quindi investigato se queste sequenze fossero parimenti richieste per l'attivazione da HGF in cellule intatte, misurando l'attività enzimatica in cellule COS trasfettate con Myc-Dgk-α-wt, $Y_{335}F$ o ΔP. La figura 22 indica che, mentre l'attività enzimatica di Myc-Dgk-α wt è stimolata da HGF,

l'attività di Myc-Dgk-α–Y_{335}F o Myc-Dgk-α–ΔP non è stimolata da HGF (Fig. 21a). Consistentemente l'attività del doppio mutante Myc-Dgk-α–Y_{335}F–ΔP, pur se basalmente molto ridotta, non è ulteriormente attivata da HGF (Fig. 22b). Questi risultati suggeriscono che l'attivazione di Dgk-α in seguito a stimolazione con HGF sia mediata dalla fosforilazione della Y_{335} ad opera di Src e che l'interazione tra la coda ricca in proline di Dgk-α con il dominio SH3 di Src preceda l'attivazione.

Inoltre abbiamo verificato se Dgk-α sia un effettore del prototipo dell'oncogene tirosina cinasi v-Src, utilizzando come modello cellule MDCK stabilmente esprimenti un mutante di Src temperatura sensibile. Alla temperatura non permissiva di 40°C ts-v-Src è inattivo, mentre viene attivato portando le cellule a 35°C (Behrens et al., 1993). In questo sistema cellulare Myc-Dgk-α è espressa a basso livello e non influenza significativamente l'attività misurata in omogenati totali (Fig. 23b). Spostando le cellule MDCK-ts-v-Src alla temperatura permissiva si ha sia la fosforilazione in tirosina (Fig. 23a) e attivazione (Fig. 23b) di Myc-Dgk-α wt. Al contrario l'attivazione di ts-v-Src non induce ne la fosforilazione in tirosina nè l'attivazione di Myc-Dgk-α-Y_{335}F e Myc-Dgk-α–ΔP (Fig. 23). Questi esperimenti indicano che Dgk-α è un substrato di v-Src, che fosforila la tirosina 335 di Dgk-α, suggerendo inoltre che l'interazione tra la coda ricca in proline di Dgk-α con il dominio SH3 di Src preceda la fosforilazione in tirosina e l'attivazione.

Il reclutamento in membrana di Dgk-α indotto da HGF richiede la Y_{335} e la coda ricca di proline

Dgk-α è un enzima citoplasmatico che associa alla membrana plasmatica in seguito a stimolazione con fattori di crescita (Flores et al., 1996; Sanjuan et al., 2003); abbiamo quindi verificato se la fosforilazione di Dgk-α sulla Y_{335} regoli il reclutamento alla membrana in cellule stimolate con HGF. La localizzazione è stata studiata in cellule MDCK trasfettate con Dgk-α wt o mutante fusa all'N-terminale con GFP.

Nella maggioranza delle cellule controllo GFP-Dgk-α wt è localizzata esclusivamente nel citosol e che in seguito a stimolazione con HGF trasloca alla membrana plasmatica della maggioranza delle cellule trasfettate (70%; Fig. 24a e 25c). Un comportamento simile ha GFP-Dgk-α-k^-: diffusa nel citoplasma delle cellule controllo ed associata alla membrana plasmatica in cellule stimolate con HGF (Fig. 24b). Il reclutamento alla membrana in cellule stimolate con HGF è dipendente dall'attività di Src, dato che viene ridotto del 50% dall'inibitore di Src PP2 (Fig. 24a).

Per verificare se la fosforilazione in tirosina di Dgk-α sia richiesta per il reclutamento alla membrana indotto da HGF abbiamo verificato la localizzazione subcellulare dei mutanti Y_{335}F e ΔP.

Sorprendentemente, nella maggioranza delle cellule, entrambi i mutanti sono accumulati in strutture intracellulari (presumibilmente vescicole) e non traslocano alla membrana cellulare in seguito a stimolazione con HGF (Fig. 25). Queste osservazioni dimostrano che la tirosina 335 e la sequenza ricca in proline sono richieste per la corretta localizzazione di Dgk-α, suggerendo che la fosforilazione in tirosina sia un evento chiave nel controllo del reclutamento in membrana. Inoltre la localizzazione in strutture vescicolari intracellulari dei mutanti GFP-Dgk-α-Y_{335}F e GFP-Dgk-α-ΔP suggerisce che il reclutamento coinvolga un traffico vescicolare verso la membrana plasmatica. Se questa ipotesi è corretta l'inibizione del traffico vescicolare tra Golgi e membrana plasmatica con Brefeldina A (Lippincott-Schwartz et al., 1989) dovrebbe bloccare GFP-Dgk-α-wt sulle stesse vescicole. Effettivamente il trattamento per 15 minuti con 10 μM Brefeldina A induce l'accumulo su vescicole di GFP-Dgk-α wt in cellule controllo e impedisce la traslocazione in membrana in seguito a stimolazione con HGF (Fig. 26).

Queste osservazioni indicano che il reclutamento alla membrana di Dgk-α in cellule stimolate da HGF dipenda da un traffico vescicolare e richieda la fosforilazione della Y_{335} ad opera di Src ma non l'attività diacilglicerolo cinasica.

Il reclutamento alla membrana e l'attivazione di Dgk-α sono eventi necessari e sufficienti per trasdurre il segnale chemiotattico di HGF e sufficienti ad indurre motilità cellulare.

Dato che l'attivazione di Dgk-α è un evento necessario per la migrazione cellulare indotta da HGF e VEGF (Cutrupi et al., 2000; Baldanzi et al., 2004), abbiamo verificato se la Y_{335} contribuisce alla trasduzione del segnale chemiotattico di HGF. Come modello cellulare abbiamo utilizzato cellule COS-7 che non migrano sensibilmente in risposta ad HGF in un sistema di chemiotassi quantitativa in transwell ma diventano responsive se trasfettate transientemente con Myc-Dgk-α-wt (Fig. 27a). Nello stesso saggio il mutante Myc-Dgk-α-Y_{335}F non è in grado di trasdurre il segnale promigratorio di HGF (Fig. 27a), suggerendo che l'attivazione ed il reclutamento in membrana di Dgk-α mediati dalla fosforilazione della Y_{335} siano richiesti per il segnale chemiotattico di HGF.

Inoltre abbiamo utilizzato una Dgk-α miristoilata, che Sanjuan et al. dimostrano essere costitutivamente attiva e reclutata in membrana, per verificare se una Dgk-α costitutivamente attiva sia sufficiente a stimolare la motilità cellulare. L'espressione transiente di myr-Dgk-α in cellule COS triplica la motilità cellulare in cellule COS-7 non stimolate misurata sia in transwell sia in un saggio di

wound healing (Fig. 27b e c). Quindi Dgk-α attiva in membrana produce un segnale intracellulare necessario e sufficiente per la motilità cellulare.

Conclusioni

Riassumendo abbiamo identificato la tirosina 335 e la coda ricca in proline come determinanti molecolari di Dgk-α necessari per: (i) la sua fosforilazione in tirosina ed attivazione in seguito a stimolazione da HGF o v-Src, (ii) il reclutamento alla membrana plasmatica, (iii) la capacità di trasdurre il segnale chemiotattico di HGF.

L'osservazione che la coda ricca in proline di Dgk-α è necessaria per la fosforilazione della tirosina 335 e l'attivazione ci porta a proporre un modello di interazione tra Src e Dgk-α in cui: Src recluta Dgk-α tramite l'iterazione tra il dominio SH3 e la coda ricca in proline, seguita dalla fosforilazione della tirosina 335 che diviene un sito di legame per il dominio SH2 portando alla stabilizzazione del complesso ed eventualmente alla fosforilazione di altri siti quali la tirosina 60. Questi dati sono altamente consistenti con il modello di fosforilazione processiva proposti per substrati di Src quali p130Cas (Kanemitsu et al., 1997; Scott e Miller, 2000). Alternativamente la tirosina 335 di Dgk-α potrebbe essere un sito di interazione per altri domini SH2 portando alla formazione di un complesso.

La fosforilazione della tirosina 335 non è di per se un evento attivatorio (dati in vitro non pubblicati) ma risulta necessaria per l'attivazione da Src o da fattori di crescita; questo fa supporre che questa fosforilazione sia un pre-requisito per un'altro evento quale l'attivazione ed il reclutamento in membrana da Ca^{++}, PIP_3 od interattori ancora non identificati. Un modello simile è stato proposto successivamente sulla base di osservazioni simili alle nostre condotte in linfociti T (Fig. 28). Merino et al. indicanti che: 1) i due domini C1 atipici sono necessari per il reclutamento alla membrana interagendo con lipidi/proteine ancora non identificati; 2) la sequenza C terminale è essenziale per il reclutamento alla membrana in modo PA dipendente; 3) in assenza dei domini EF leganti il calcio la traslocazione alla membrana richiede la tirosina 335. Inoltre alcuni dati in letteratura indicano che la fosforilazione della Y_{334} della Dgk-α murina ad opera di Src media la traslocazione alla membrana plasmatica e l'attivazione indotte da vitamina E (Fukunaga-Takenaka et al., 2005).

La sorprendente osservazione che i mutanti GFP-Dgk-α-Y335F e GFP-Dgk-α-ΔP sono costitutivamente associati a vescicole intracellulari e che GFP-Dgk-α-wt viene bloccata da Brefeldina A in vescicole citoplasmatiche suggerisce che la traslocazione in membrana sia mediata da un traffico

vescicolare originato da Golgi. In questo modello l'interazione con Src e la fosforilazione della Y_{335} sono necessarie per accoppiare Dgk-α a questo traffico vescicolare ed loro assenza l'enzima rimane bloccato durante il trasporto possibilmente interferendo con il traffico vescicolare; questo potrebbe giustificare l'osservazione che la sovraespressione dei due mutanti interferisce con la motilità cellulare (osservazioni non pubblicate). Consistentemente ci sono indicazioni che Dgk-α si localizza: all'apparato di Golgi in CHO stimolate con acido arachidonico (Shirai et al., 2000), al trans Golgi network e late endosomal compartment regolando la secrezione FAS-L in cellule T (Alonso et al., 2005). La caratterizzazione delle vescicole in cui sono localizzati GFP-Dgk-α-Y_{335}F e GFP-Dgk-α–ΔP è in corso; abbiamo osservato una parziale co-localizzazione di GFP-Dgk-α-Y_{335}F con marker dei compartimenti acidi e di GFP-Dgk-α–ΔP con il reticolo endoplasmatico (E. Rainero osservazioni non pubblicate).

L'importanza biologica dell'attivazione e reclutamento in membrana di Dgk-α nella trasduzione del segnale è ribadita dall'osservazione che in cellule COS la motilità indotta da HGF richiede l'espressione di DGK-α e la tirosina 335 e che myr-Dgk-α costitutivamente attiva in membrana è sufficiente ad indurre una motilità cellulare spontanea. Questi esperimenti, pur se realizzati in un sistema di sovraespressione sperimentale, suggeriscono che il livello di attività Dgk-α in membrana sia limitante per la motilità cellulare. Dgk-α è stata anche individuata in una recente analisi proteomica degli invadopodi, strutture subcellulari deputate all'invasione della matrice, facendo supporre un suo ruolo anche nell'invasione della matrice e nella metastatizzazione (Jia et al., 2005). In linea con questi risultati il nostro gruppo di ricerca ha recentemente dimostrato l'importanza di Dgk-α nell'organizzazione del citoscheletro e nell'invasione della matrice (Chianale et al. 2007).

CONCLUSIONI: Effettori di Dgk-α nella trasduzione del segnale chemiotattico e proliferativo?

Concludendo nei lavori qui presentati viene presentato in dettaglio il ruolo di Dgk-α nella trasduzione del segnale chemiotattico e proliferativo di HGF e VEGF; esplorano inoltre in dettaglio il meccanismo di attivazione Src mediato di Dgk-α in cellule epiteliali ed endoteliali stimolate con fattori di crescita. I risultati ottenuti sono coerenti con quelli ottenuti da altri gruppi sia per quanto riguarda il ruolo nella trasduzione del segnale dei fattori di crescita quali PDGF, EGF o IL-2 (Xiangnan et al. 2001; Wollina et al., 2003; Flores et al. 1996) sia per quanto riguarda il meccanismo di reclutamento alla membrana ad opera di Src in linfociti T (Merino et al. 2007).

Sorprendentemente, mentre nei linfociti T è stato ben dimostrato un ruolo di Dgk-α nella regolazione di RAS-GRP1 e PKCθ (Sanjuan et al. 2001 e 2003, Carasco e Merida 2004), non è noto quali effettori giustificano il blocco della proliferazione e movimento osservato in cellule epiteliali ed endoteliali. Nei sistemi sperimentali discussi l'inibizione di Dgk-α ha come conseguenze prevedibili l'accumulo di DAG ed una mancata produzione di PA alla membrana plasmatica ed al Golgi (le membrane ove Dgk-α localizza in seguito a stimolazione con fattori di crescita) e potrebbe alterare la funzione di numerosi effettori (Fig. 1). Un accumulo di DAG non è da solo in grado di spiegare i fenotipi osservati risultando pro-migratorio e pro-proliferativo in numerosi tipi cellulari (Wellner et al., 1999; Matsumoto e Claesson-Welsh, 2001; Wang et al., 2002). Anche se non è stato caratterizzato un dominio definito di legame al PA questo è in grado di legare ed attivare numerose proteine coinvolte nella trsduzione del segnale dei fattori di crescita tra cui: PI(4)P 5-kinase, mTor, PKC-e, Raf ed il complesso NADPH ossidasi (Topham and Prescott, 1999). Alternativamente il rapporto PA/LPA controlla la curvatura delle membrane durante la secrezione e l'endocitosi (Ohashi et al.,1995; Kooijman et al., 2003), in linea con il possibile ruolo di Dgk-α nel traffico vescicolare (Sakane et al. 2007).

In cellule trattate con inibitori di Dgk non sono state osservate modificazioni rilevanti nell'attivazione di classici effettori dei fattori di crescita quali AKT, ERK1/2 e Src (dati non pubblicati e Yanagisawa et al. 2007). Il nostro gruppo di ricerca ha recentemente pubblicato che Dgk-α è richiesta per l'attivazione di Rac in cellule epiteliali stimolate con HGF e questo potrebbe giustificare il blocco della riorganizzazione citoscheletrica e del movimento cellulare (Chianale et al 2007); parallelamente Dgk-α regola l'attivazione di NF-κB in linee cellulari derivate da melanomi sopprimendo l'apoptosi indotta da TNF-α (Yanagisawa et al 2007). Queste osservazioni non definiscono quali proteine siano regolate da Dgk-α poiché né Rac né NF-κB sono direttamente regolati da DAG o PA. Inoltre non è

immediato ricollegare i fenotipi osservati con un difetto nell'attivazione di Rac ed NF-κB, suggerendo l'esistenza di ulteriori vie di trasduzione del segnale Dgk-α dipendenti.

ABBREVIAZIONI

DAG diacylglycerol; PA phosphatidic acid; DGK, Diacylglycerol kinase; HGF hepatocyte growth factor; VEGF vascular endothelial growth factor; GnRH Gonadotropin-releasing hormone; EGF, epidermal growth factor; TCR, T cell receptor; PDGF platelet-derived growth factor; TNF tumor necrosis factor; PMA phorbol 12-myristate 13-acetate; siRNA smal interfering RNA; BrdU 5-Br-desossiuridina; $PI_{4,5}P2$ fosfatidilinositolo 4,5 bifosfato; PIP3 fosfatidilinositolo (3,4,5)-trisfosfato; PI3-K phosphatidylinositol 3-kinase; PIP5K phosphatidylinositol-4-phosphate 5-kinase; SphK sphingosine kinase; Grb2 growth factor receptor–bound protein 2; Shc Src homology and collagen, IRS-1 insulin receptor substrate-1, PLC-γ phospholipase Cγ; Btk Bruton's tyrosine kinase; GST glutathione-S transferase; PLD phospholipase D; cPKC conventional protein kinase C; nPKC, novel PKC; aPKC atypical PKC; RasGRP Ras guanyl nucleotide-releasing protein; TRPC transient receptor potential channel; RasGAP Ras GTPase activating protein; PAK p21-activated kinase; PP1c protein phosphatase-1 catalytic subunit; mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase; pRB retinoblastoma protein; NF-κB, nuclear factor κB; CD catalytic domain; MARCKS myristoylated alanine-rich C-kinase substrate; PSD phosphorylation-site domain; PDZ PSD-95 DLG ZO-1 domain; SAM sterile α motif; RVH, recoverin homology; PH pleckstrin homology; SAM, sterile α motif; PDZ, PSD-95, DLG, ZO-1; RA, Ras-associating.

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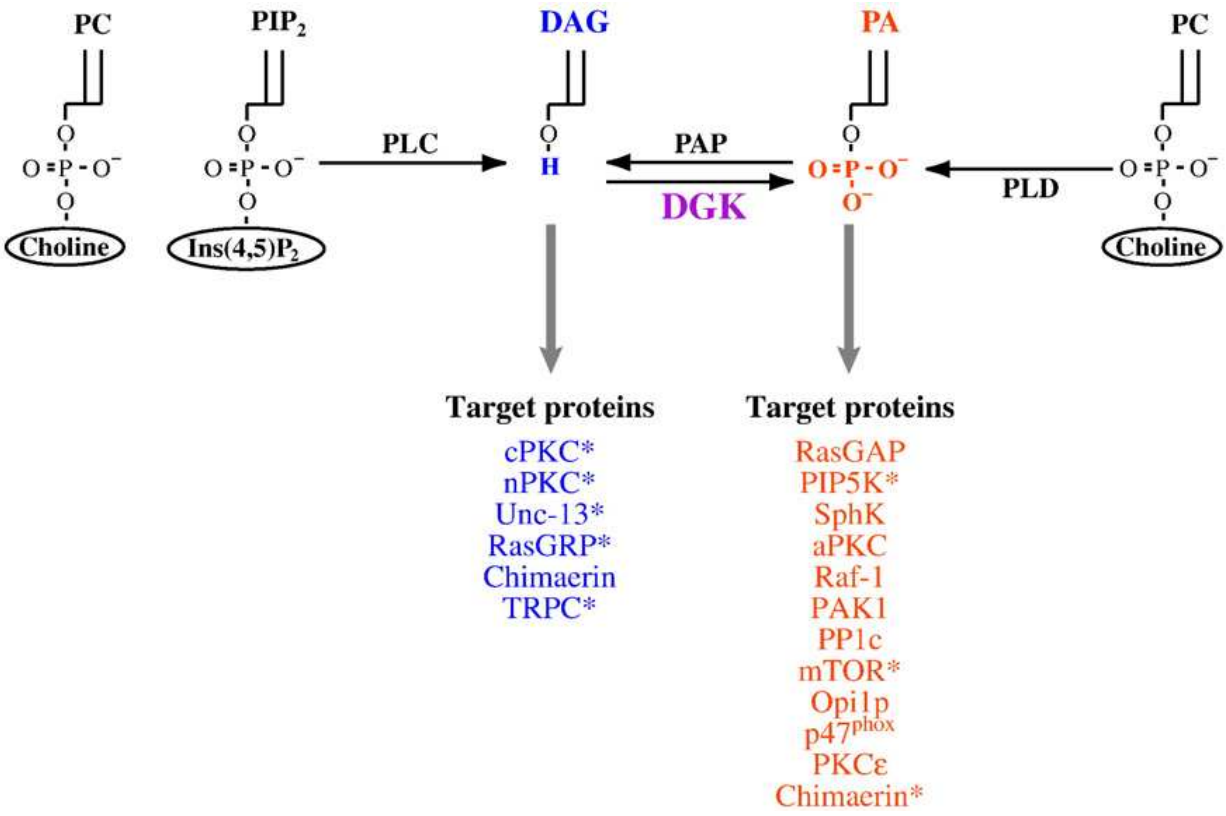

Fig. 1. **Proteine regolate da DAG o da PA**
Gli asterischi indicano proteine note per essere regolate da DGK tramite metabolismo di DAG o produzione di PA.
Adattato da Sakane et al. 2007

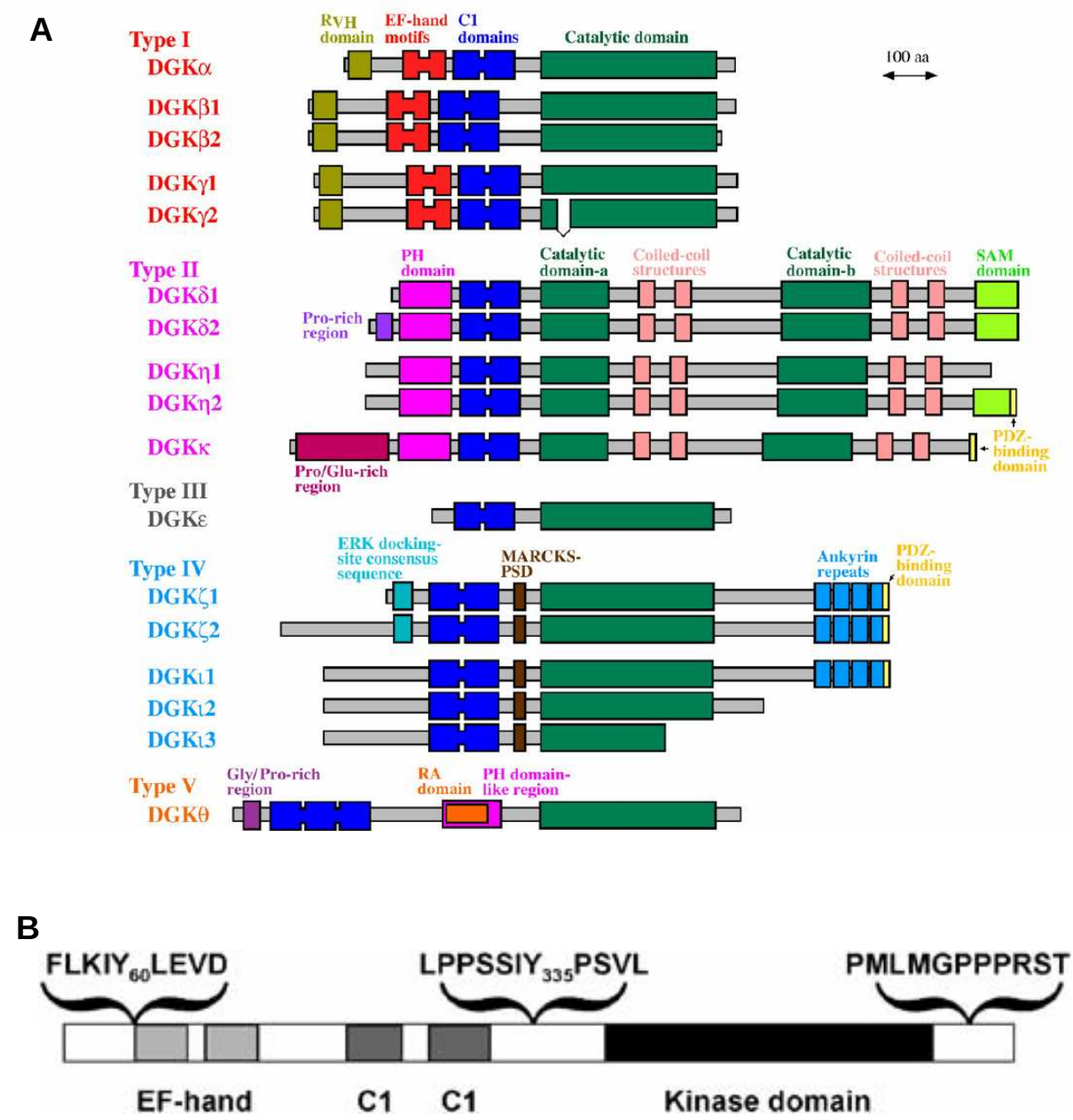

Fig. 2 **Struttura delle isoforme di Dgk**

A) Classi di Dgk. RVH, recoverin homology; PH, pleckstrin homology; SAM, sterile α motif; PDZ, PSD-95, DLG, ZO-1; MARCKS, myristoylated alanine-rich C kinase substrate; PSD, phosphorylation-site domain; RA, Ras-associating. Adattato da Sakane et al. 2007.

B) Struttura Dgk-α. Dgk-α contiene due domini EF-hand, due domini C1 atipici ed il dominio catalitico. La posizione delle tirosine 60 e 335 e della coda ricca in proline è indicata.

Table 1
Upstream regulators of DGKs

Upstream regulator	DGK isoform	Interaction region in DGK	Effect on DGK	Cell stimulation	Ref.
Tyr kinase					
Src*	DGKα	?	Tyr phosphorylation, Activation	HGF, VEGF	[54,55]
Src	DGKα	–	Phosphorylation at Tyr-334, Activation	α-Tocopherol	[120]
Src*	DGKζ	?	Activation	GnRH	[106]
SFK (Src)	DGKκ	–	Phosphorylation at Tyr-78	H_2O_2	[40]
Ser/Thr kinase					
cPKC (PKCα)	DGKδ1	–	Phosphorylation at Ser-22/Ser-26 (within the PH domain), Inhibition of translocation to the plasma membrane	PMA, EGF	[94]
PKCα*	DGKζ	aa 467–605 within C-terminal half of CD	Phosphorylation at MARCKS-PSD (Ser-258/Ser-265/Ser-270/Ser-271), Inactivation, Inhibition of the nuclear localization	PMA	[80,82,83]
			Enhancement of translocation to the plasma membrane	?	[108]
			Inhibition of interaction with Rac1	?	[76]
PKCγ*	DGKγ	C-terminal half of CD	Phosphorylation at Ser-776/Ser-779, Activation	PMA	[81]
ERK	DGKζ	ERK docking-site consensus sequence?	Phosphorylation at Ser-768/Ser-773/Ser-776, Inhibition of translocation to the actin cytoskeleton	?	[108]
Second messenger					
Ca^{2+}*	DGKα	EF-hands (K_d=0.3 μM)	Activation	?	[48,121]
Ca^{2+}*	DGKβ	EF-hands (K_d=$\sim10^{-8}$ M)	Activation	?	[49]
Ca^{2+}*	DGKγ	EF-hands (K_d=$<10^{-8}$ M)	?	?	[49]
PI3K products ($PI(3,4)P_2$ $PI(3,4,5)P_3$)	DGKα	?	Activation, Translocation to the plasma membrane	IL-2	[53]
PIP5K product ($PI(4,5)P_2$)	DGKβ	?	Activation	?	[53]
Phorbol ester* (DAG?)	DGKβ	First C1 domain (C1A domain)	?	?	[122]
Phorbol ester* (DAG?)	DGKγ	First C1 domain (C1A domain)	Translocation to the plasma membrane?	?	[122,123]
Receptor					
Leptin receptor* (long form)	DGKζ	Ankyrin repeats	?	Leptin	[124]
Cytoskeleton-related protein including small GTPase					
Syntrophin* (α1 and γ1)	DGKζ	PDZ-binding domain	Recruitment to the actin cytoskeleton	?	[107,108]
RhoA* (active)	DGKθ	?	Inactivation	?	[115]
Inhibitor of cell-cycle progression					
pRB*	DGKζ	?	Activation	?	[100]
Adaptor					
RACK1*	DGKδ	aa 1015–1097 (DGKδ1)	?	?	Unpublished work
DGK					
DGKs δ1*, δ2* and η2*	DGKδ1	SAM domain	Oligomer formation, Inhibition of translocation to the plasma membrane	? (Inhibited by PMA)	[42,43,91]

*: interaction is demonstrated.

Table 2
Downstream effectors of DGKs

Downstream effector	DGK isoform	Interaction region in DGK	Mediator	Effect on effector	Cell stimulation	Ref.
Ser/Thr kinase						
PKCα*	DGKζ	aa 467–605 within C-terminal half of CD	DAG	Inhibition	PMA	[82,83]
cPKC (α*) and nPKC (δ*, ε*, η*)	DGKδ	?	DAG	Inhibition	EGF	[84]
PKCβI	DGKβ	–	DAG	Inhibition	Ag-IgG-FcγR	[69]
PKCγ*	DGKγ	C-terminal half of CD	DAG	Inhibition	PMA	[81]
mTOR	DGKζ	–	PA	Activation	Serum	[112]
Regulatory protein of small GTPase						
RasGRP1*-Ras	DGKζ	aa 467–605 within C-terminal half of CD	DAG	Inhibition	Ag-TCR	[102]
RasGRP1-Ras	DGKα	–	DAG	Inhibition	Ag-TCR	[61,62]
RasGRP3*-Rap1	DGKι	?	DAG	Inhibition	?	[87]
β2-Chimaerin* (RacGAP)	DGKγ	?	PA?	Activation	EGF	[72]
Small GTPase						
Rac1*	DGKγ	?	–	Inactivation (via β2-chimaerin?)	PDGF	[71]
Rac1*	DGKζ	C1 domains	–	Activation? (via PIP5K Iα?)	Serum-depletion	[76]
Lipid kinase						
PIP5K Iα*	DGKζ	?	PA	Activation	Serum	[77]
Transcription factor						
NF-κB	DGKα	?	DAG/PA?	Activation (indirectly?)	TNF-α	[59]
Channel						
TRPC2	Type I DGK (α, β, γ)?	?	DAG	Inhibition	Pheromone	[7]

*: interaction is demonstrated.

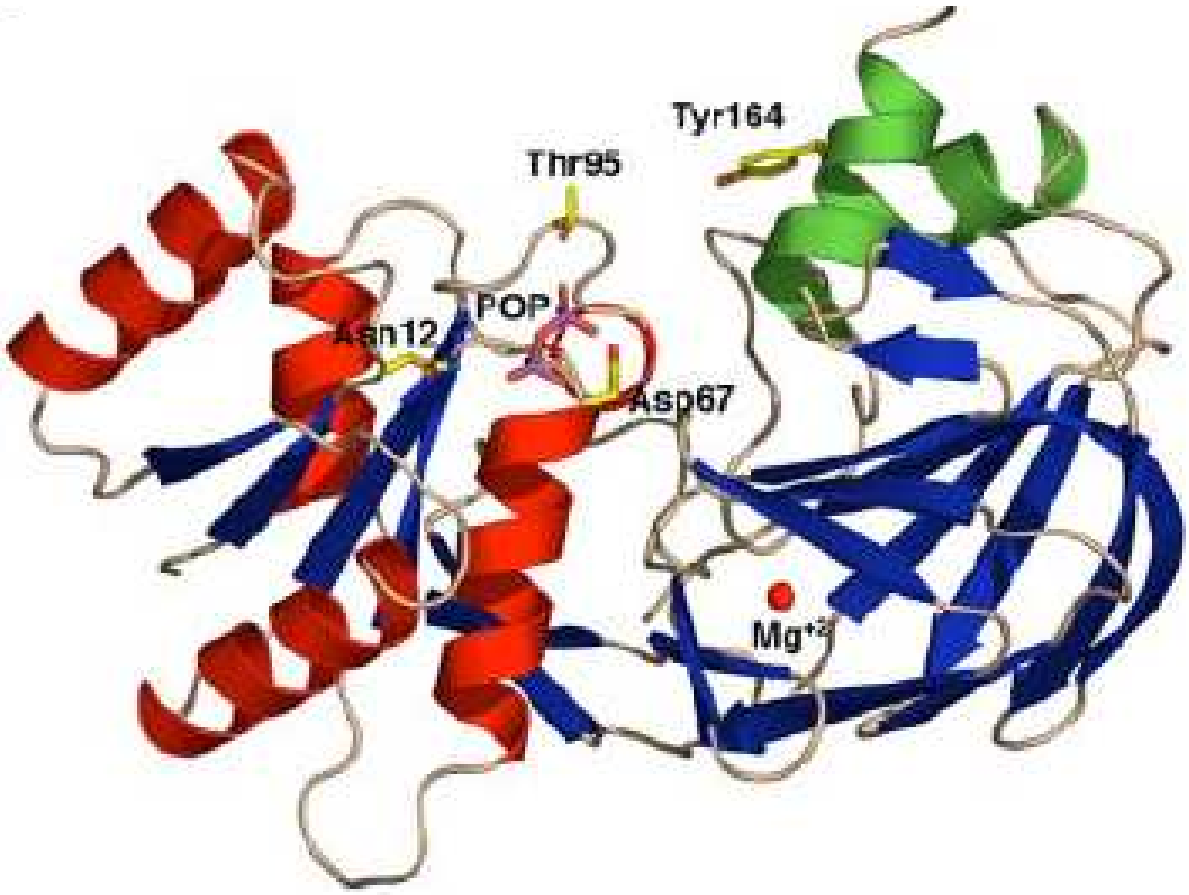

Fig. 3 **Struttura di YegS**.
Sono evidenziati i fosfati dell'ADP (*POP*) ed i residui chiave tipici delle lipide cinasi. Mg^{+2} individua il novo sito di legame per metalli. Il dominio N-terminale è il sito di legame dei nucleotidi mentre le eliche in verde rappresentano il putativo sito di legame del substrato. Adattato da Bakali et al. 2007

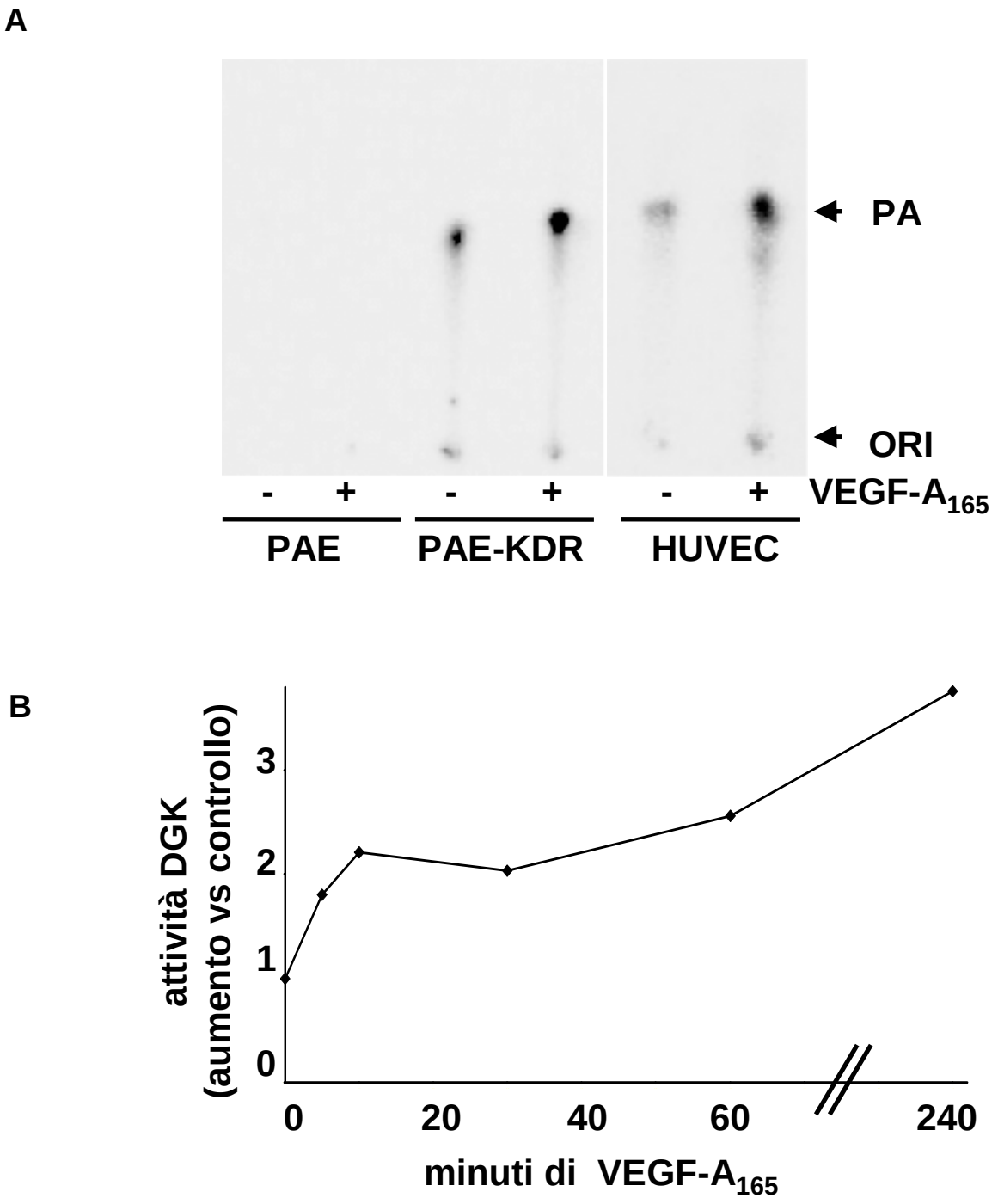

Fig. 4 **VEGF-A_{165} attiva DGK in cellule PAE-KDR ed HUVEC**
A) Attività Dgk misurata in omogenati totali di cellule PAE, PAE-KDR e HUVEC controllo o stimolate con VEGF-A_{165} (10 ng/ml, 10 minuti).
C) Cinetica di attivazione di Dgk in cellule PAE-KDR stimolate con VEGF-A_{165} 50 ng/ml.

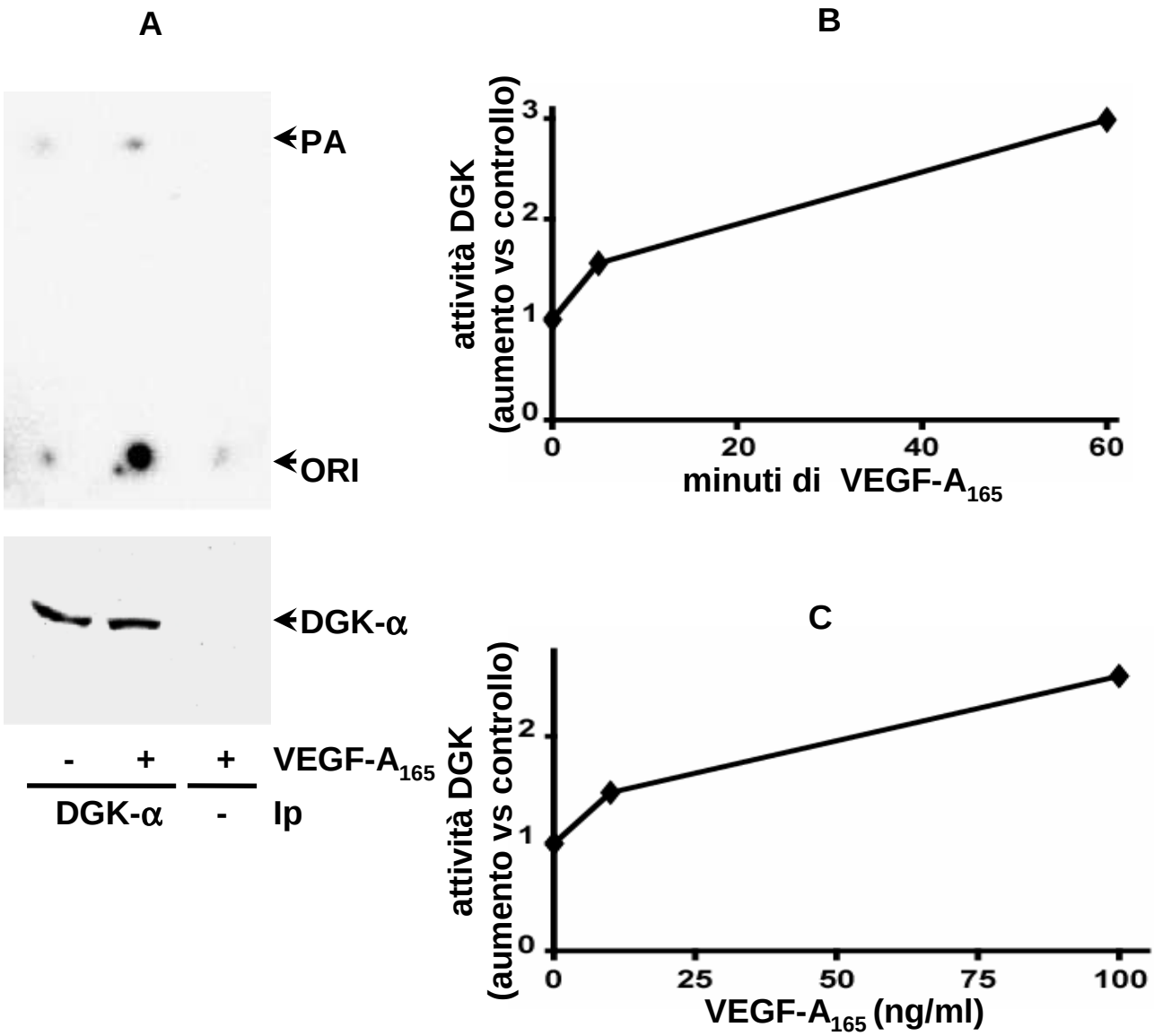

Fig. 5 **VEGF-A_{165} attiva DGK-α**

A) Attività Dgk-α (pannello superiore) e proteina (pannello inferiore) misurate in immunoprecipitati anti-Dgk-α da lisati di cellule PAE-KDR controllo o stimolate (VEGF-A_{165} (10 ng/ml per 10 minuti.

B) Cinetica di attivazione di Dgk-a misurata in immunoprecipitati anti-Dgk-α da cellule PAE-KDR stimolate con 50 ng/ml VEGF-$A_{165.}$

C) Dose risposta di attivazione di Dgk-α misurata in immunoprecipitati anti-Dgk-α da cellule PAE-KDR stimolate per 15 minuti.

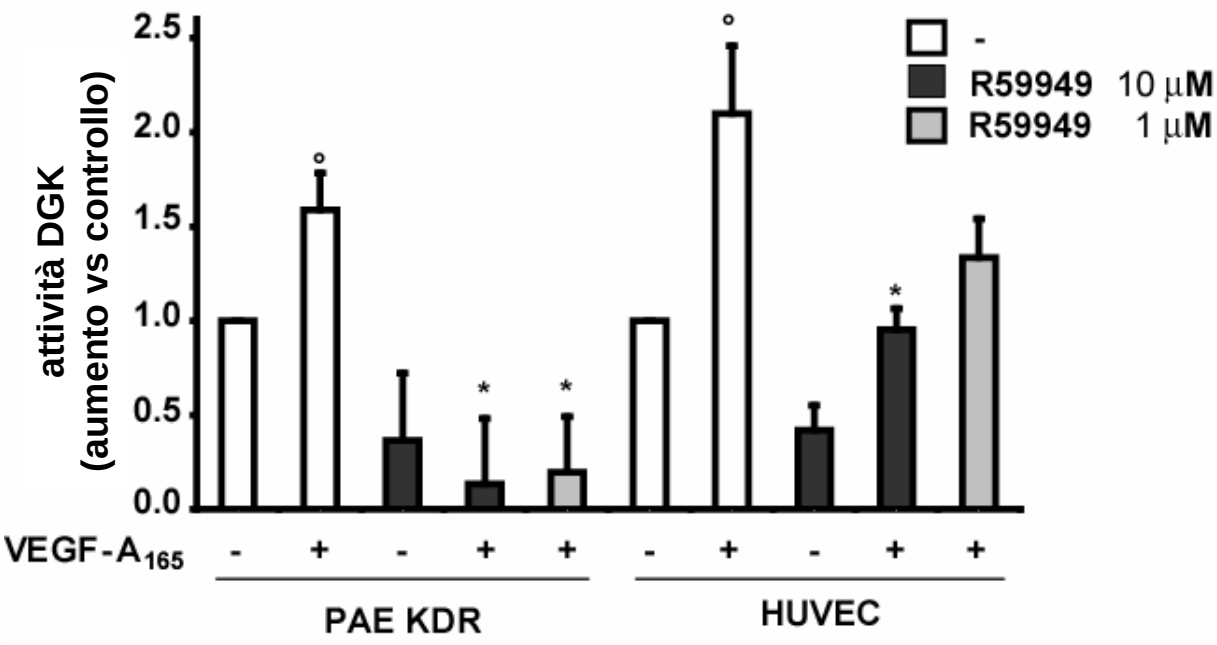

Fig. 6 **L'attività Dgk stimolata da VEGF-A_{165} è sensibile a R59949**
Attività Dgk totale misurata in omogenati totali di cellule PAE-KDR o HUVEC controllo o stimolate con VEGF-A_{165} (10 ng/ml, 10 minuti) in presenza delle concentrazioni indicate di R59949. I dati sono la media ± errore standard di 5 esperimenti indipendenti normalizzati per il controllo; ℗t-test vs control, *t-test vs VEGF-A_{165}, $P < 0,05$)

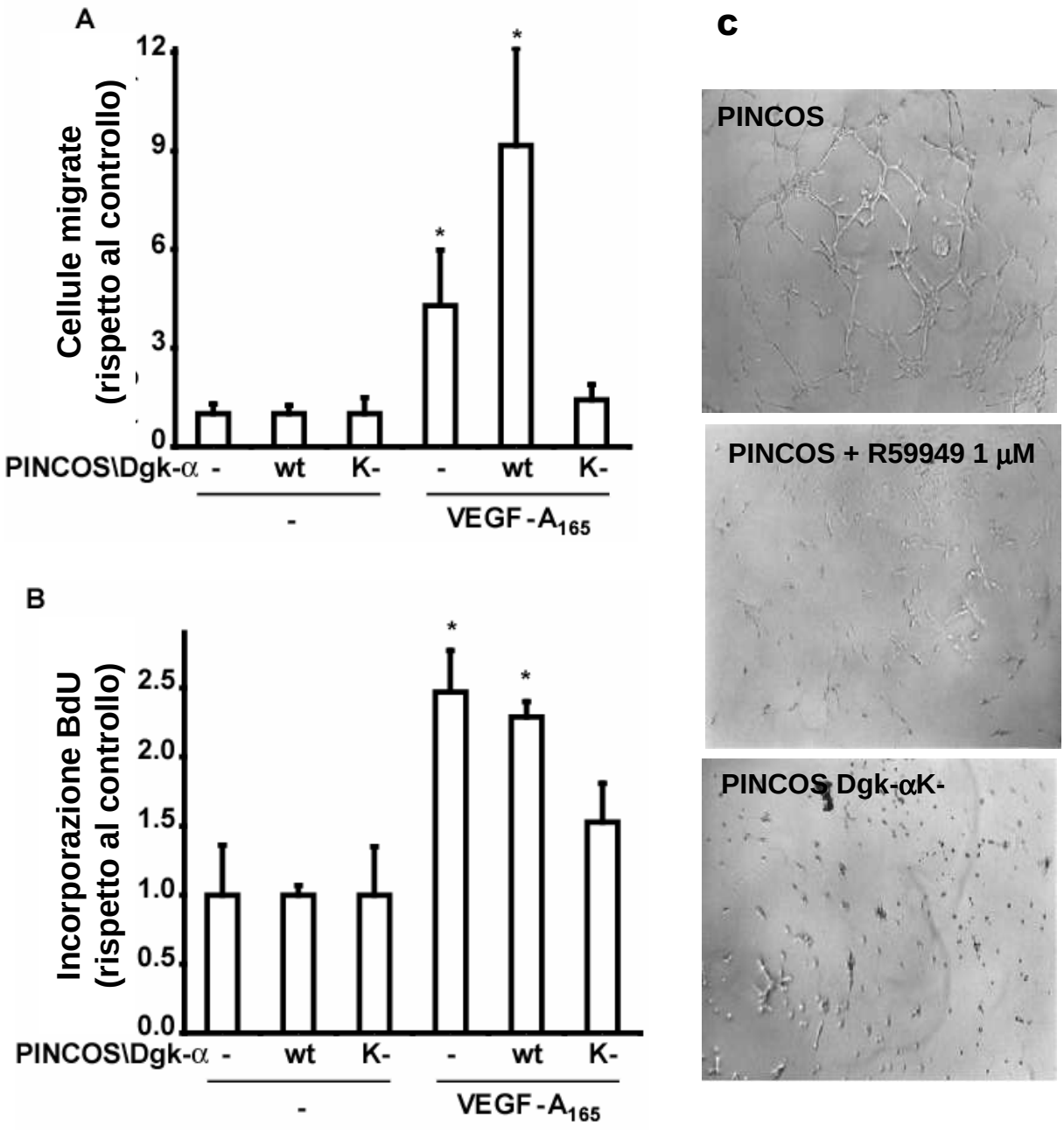

Fig. 7 **Un mutante dominante negativo di Dgk-α inibisce il signalling angiogenico di VEGF-A_{165}**

Cellule PAE-KDR infettate con un vettore vuoto o esprimente Myc-Dgk-α-wt or Myc-Dgk-α-K- sono state confrontate in saggi di:

A) migrazione in camera di Boyden su filtro ricoperto di collagene I in presenza di 20 ng/ml VEGF-A_{165} per 6 ore. I dati sono la media ± errore standard di 6 punti indipendenti normalizzati per il controllo; *t-test $P < 0,05$);

B) incorporazione di BrdU in piastre da 96 pozzetti in presenza od assenza di VEGF-A_{165} (10 ng/ml, 24 ore). I dati sono la media ± errore standard di 8 punti indipendenti normalizzati per il controllo; *t-test $P < 0,01$);

C) organizzazione su matrigel in presenza di VEGF-A_{165} (25 ng/ml) per 24 ore, in presenza od assenza di 1 μM R59949. Le cellule sono state fotografate in contrasto di fase.

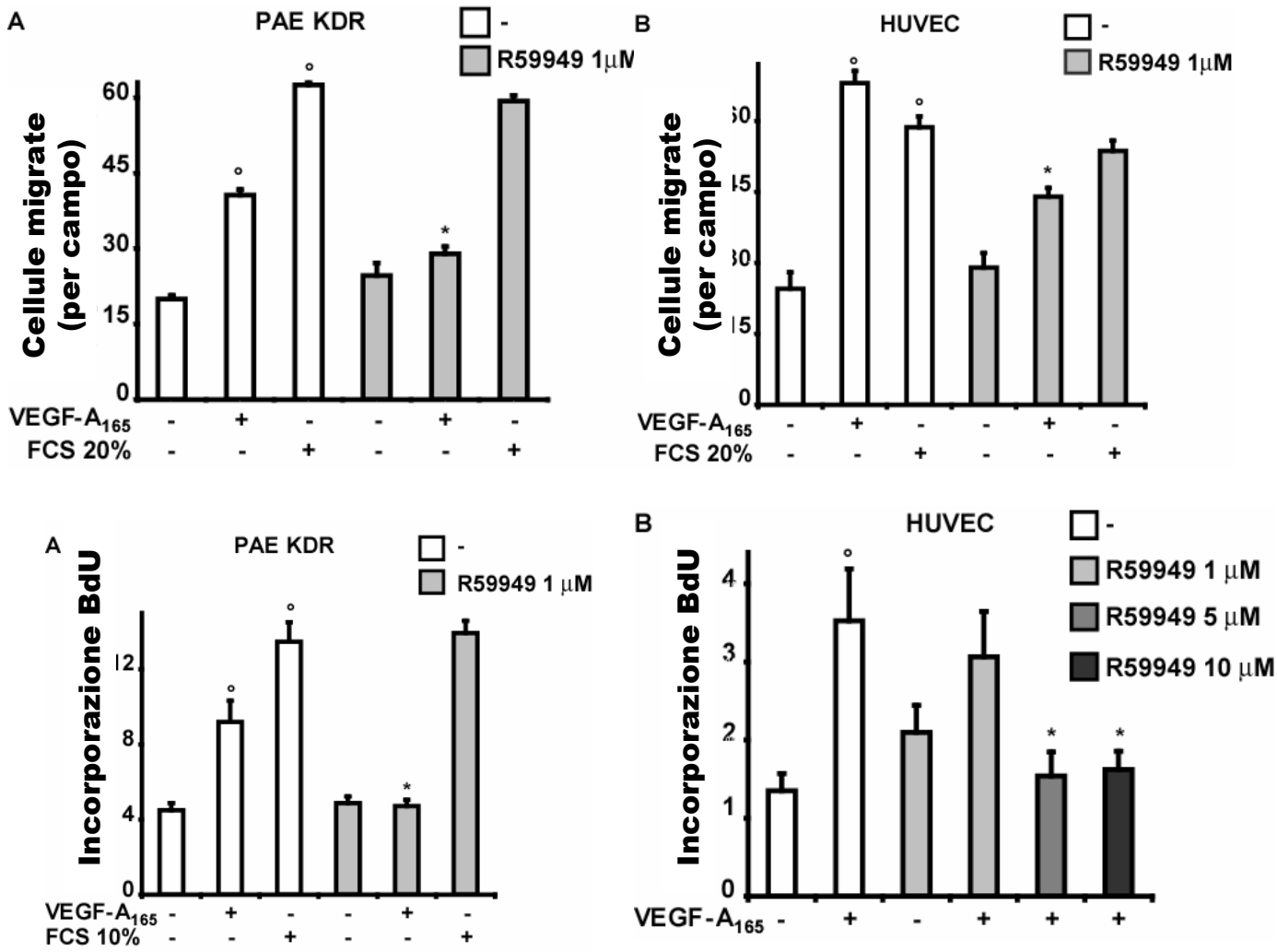

Fig. 8 **R59949 inibisce la migrazione e la proliferazione indotte da VEGF-A_{165} in cellule PAE-KDR e HUVEC.**

Cellule PAEKDR (A) e HUVEC (B) sono state saggiate per migrazione in una camera di Boyden con filtro ricoperto di gelatina in presenza di 10 ng/ml (A) o 20 ng/ml (B) di VEGF-A_{165} o 20% FCS per 6 ore, in presenza od assenza di 1 μM R59949. I dati sono la media ± errore standard di triplicati (°t-test vs controllo, *t-te st vs VEGF-A_{165}, P<0,01). Cellule PAE-KDR (C) o HUVEC (D) sono state saggiate per incorporazione di BrdU in piastre da 96 pozzetti per 24 (C) o 48 ore (D) dopo trattamento con VEGF-A_{165} 25 ng/ml in assenza di siero (C), VEGF-A_{165} 10 ng/ml, 2% FCS (D) o 10% FCS in presenza od assenza di R59949 alle concentrazioni indicate. I dati sono media ± errore standard di otto punti indipendenti (°t-test vs cont rol, *t-test vs VEGF-A_{165}, P<0,01)

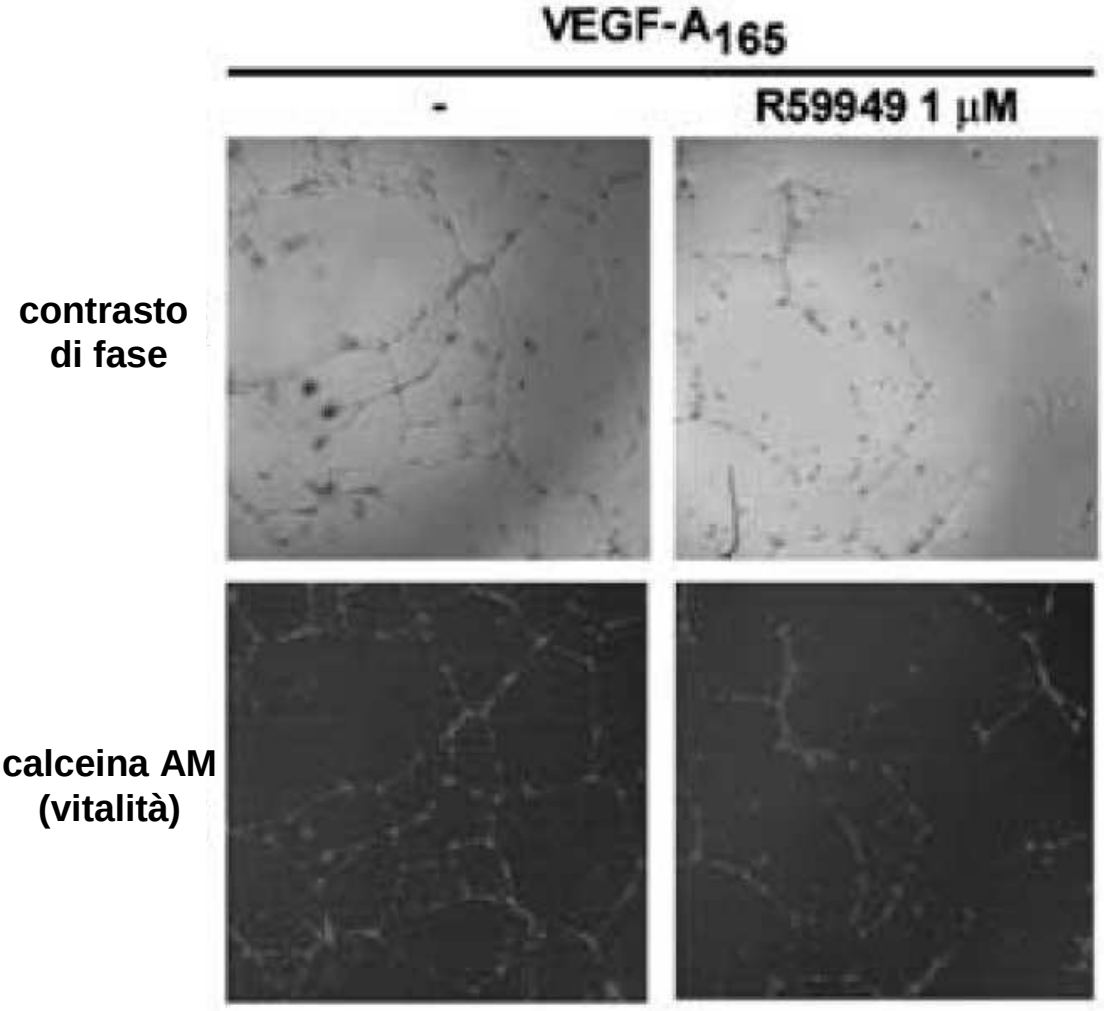

Fig. 9 **R59949 inibisce l'angiogenesi indotta da VEGF-A_{165} in HUVEC**

HUVEC piastarate su matrigel in presenza di VEGF-A_{165} (25 ng/ml) per 24 ore in presenza od assenza di 1 μM R59949. Le cellule sono state caricate con la sonda fluorescente per cellule vive Calcein-AM e fotografate in contrasto di fase (pannello superiore) o in fluorescenza (pannello inferiore).

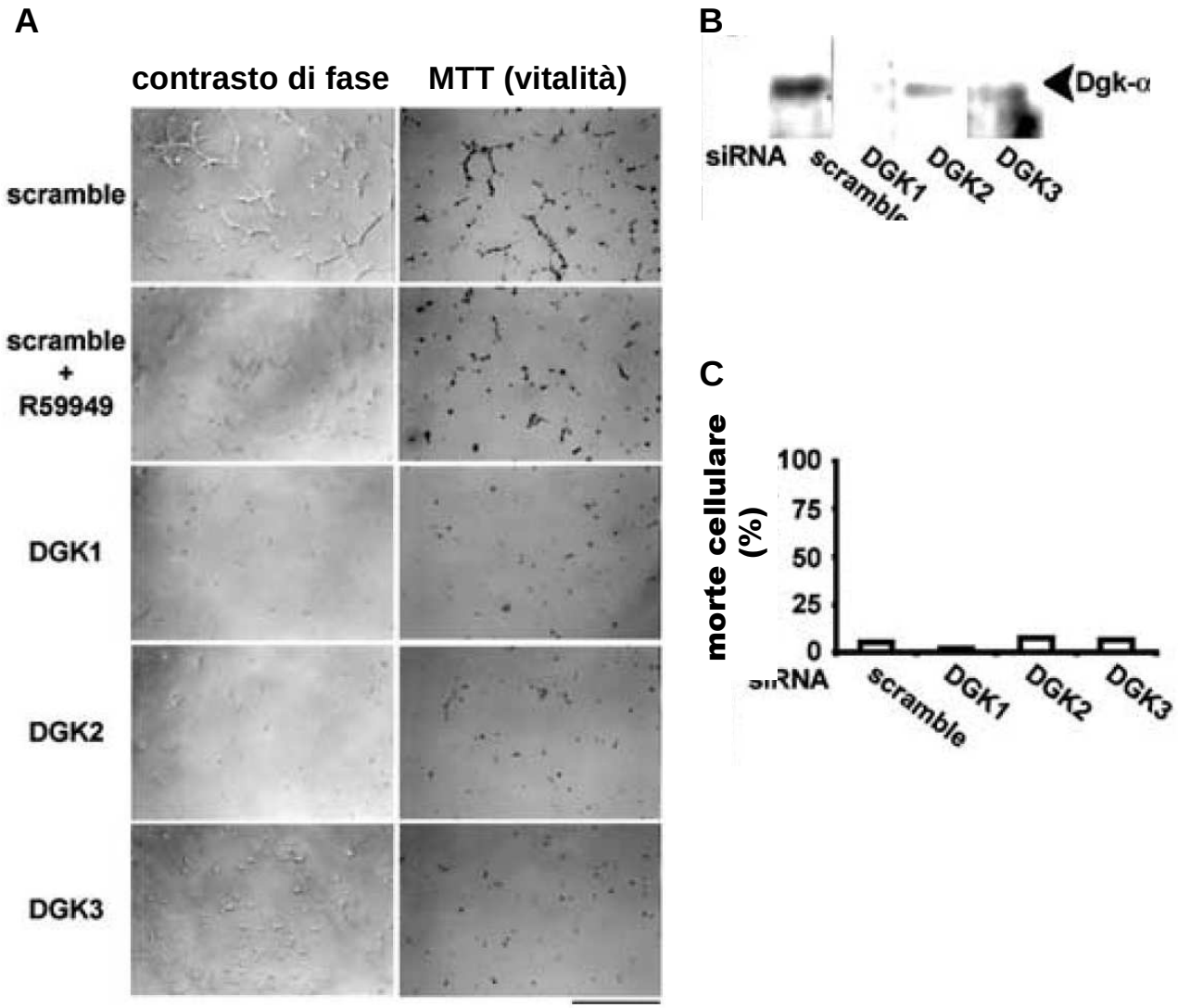

Fig. 10 **L'inibizione dell'espressione di Dgk-α con siRNA inibisce l'angiogenesi in vitro in HUVEC**

Cellule HUVEC sono state trasfettate con i siRNA indicati e dopo 48 ore:

A) piastrate su matrigel in presenza od assenza di 1 μM R59949 per ulteriori 24 ore. Le cellule sono state prima fotografate in contrasto di fase e successivamente le cellule vitali colorate con MTT e fotografate in campo chiaro;

B) lisate ed analizzate per il contenuto di Dgk-α per western blot con anticorpi anti-Dgk-α;

C) coltivate per ulteriori 24 ore e testate per vitalità cellulare con il saggio trypan blue.

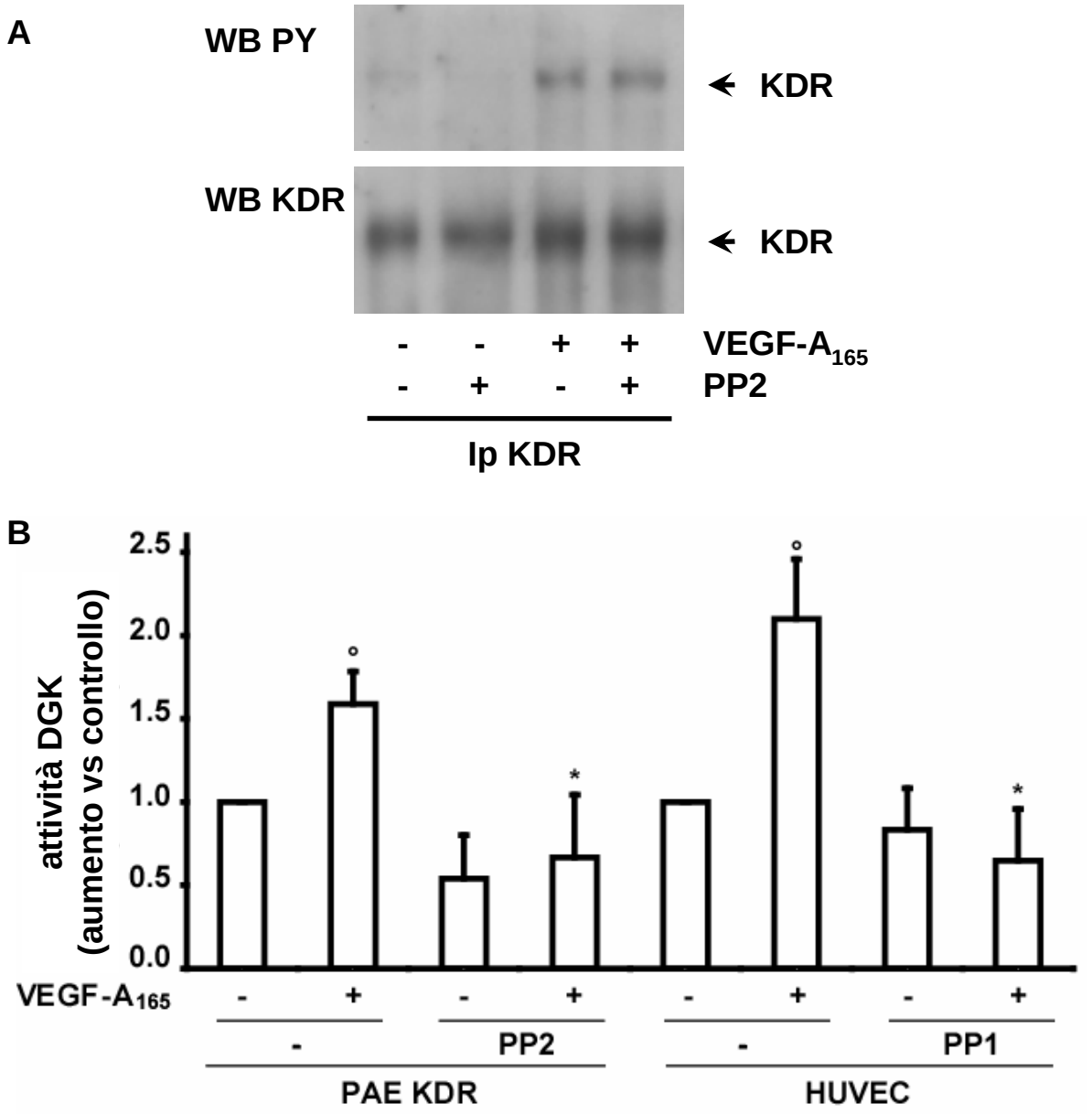

Fig. 11 **L'attività tirosina cinasica di Src è richiesta per l'attivazione di Dgk in cellule stimolate con VEGF-A_{165}**

A) Western blot anti-fosfotirosina (pannelo superiore) e anti-VEGFR-2 (pannello inferiore) di immunoprecipitati anti-VEGFR-2 da elisati di cellule HUVEC pretrattate con 5 μM PP2 per 15 minuti e stimolate con VEGF-A_{165} (10 ng/ml per 10 minuti).

B) Attività Dgk in omogenati totali di cellule PAE-KDR o HUVEC pretrattate per 15 minuti con 5 μM PP2 o PP1 e stimolate con VEGF-A_{165} (10 ng/ml per 10 minuti). I dati sono la media ± errore standard di cinque esperimenti indipendenti normalizzati per il controllo (ϒ test vs controllo, *t-test vs VEGF-A_{165}, $P<0.05$).

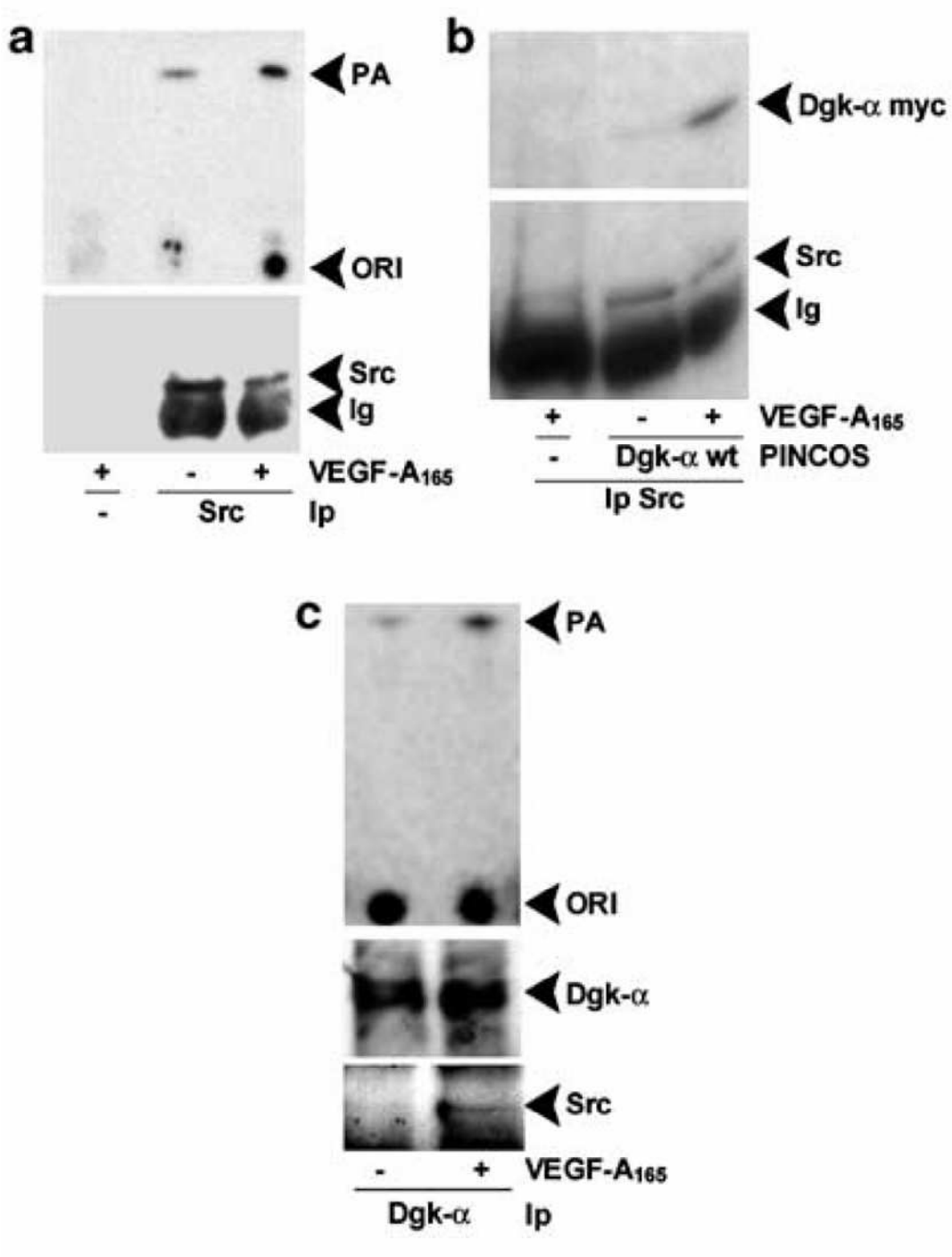

Fig. 12 **Associazione tra Dgk-α e Src in cellule stimolate con VEGF-A_{165}**
a) Attività Dgk (pannello superiore) e Western blot anti Src (pannello inferiore) di immunoprecipitati anti Src di cellule PAE-KDR controllo o stimolate con VEGF-A_{165} (50 ng/ml per 10 minuti).
b) Cellule PAE-KDR infettate con il vettore PINCOS o PINCOS-Myc-Dgk-α e trattate con VEGF-A_{165} (50 ng/ml per 10 minuti) sono state immunoprecipitate con anticorpi anti Src. La presenza di Myc-Dgk-α (pannello superiore) e Src (pannello inferiore) e stata rilevata tramite western blot.
c) Attività enzimatica Dgk (pannello superiore), proteina Dgk-α in Western blot (pannello centrale) e Src in western blot (pannello inferiore) in immunoprecipitati anti-Src da lisati di cellule PAE-KDR controllo o stimolate con VEGF-A_{165} (50 ng/ml per 10 minuti)

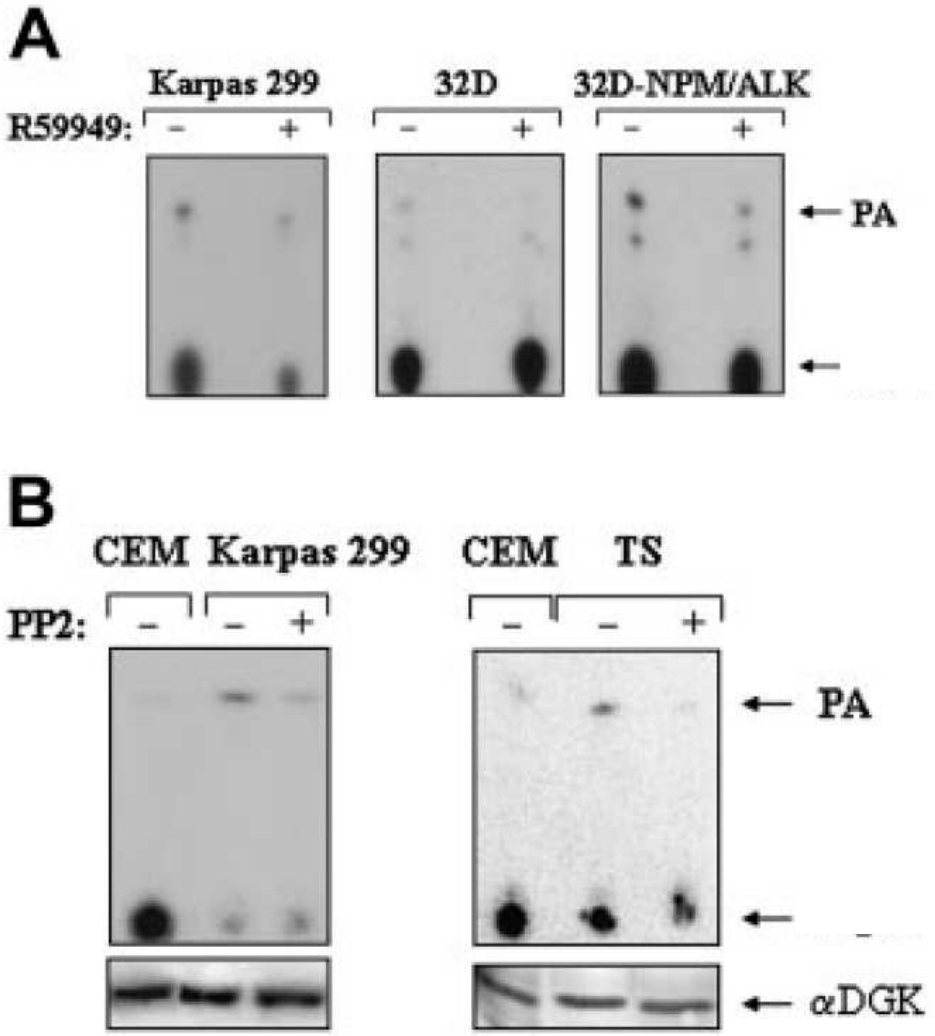

Fig. 13**. Attività DGK-α in cellule NPM/ALK-positive**
Le linee cellulari indicate sono state pretrattate per 30' con 10 μM R59949 o 1 μM PP2. L'attività Dgk di omogenati totali (A) o immunoprecipitati anti-DGK-α (B, pannello superiore) è stata analizzata in vitro. In B una aliquota di ciascun immunoprecipitato è stata analizzata in werstern blot con anticorpi anti-Dgk-α (B, pannello inferiore).

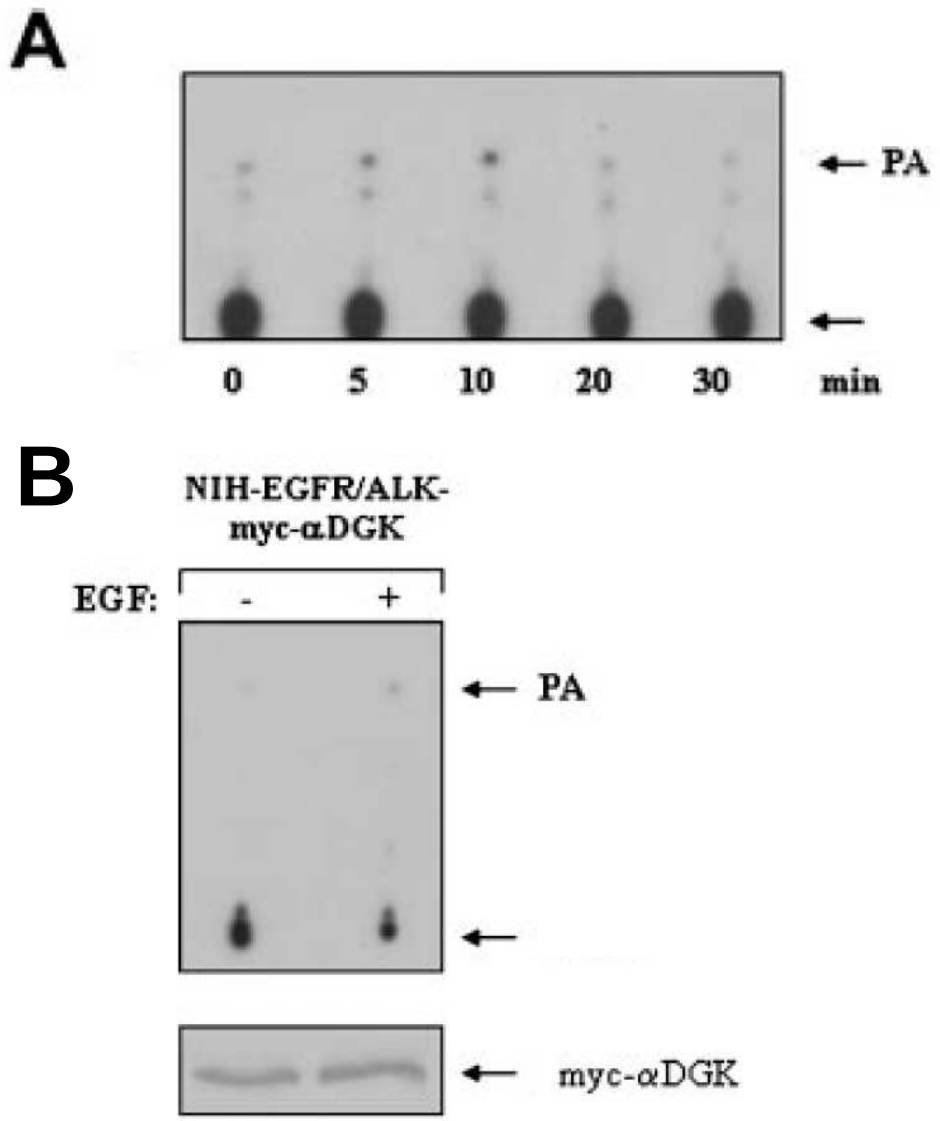

Fig. 14. In cellule NIH-EGFR/ALK la stimolazione con EGF attiva Dgk-α

A) Cellule NIH-EGFR/ALK starvate per 18 ore e trattate con 100 ng/mL EGF per i tempi indicati sono state omogenate ed analizzate per l'attività Dgk.

B) cellule NIH-EGFR/ALK trasfettate transientemente con myc-DGK-α Sono state starvate per 18 ore e stimolate con EGF (10 minuti, 100 ng/mL). Le cellule sono state lisate ed immunoprecipitate con anticorpi anti-myc; gli immunoprecipitati sono stati analizzati per attività Dgk (pannello superiore) e western blot con anticorpi anti-myc.

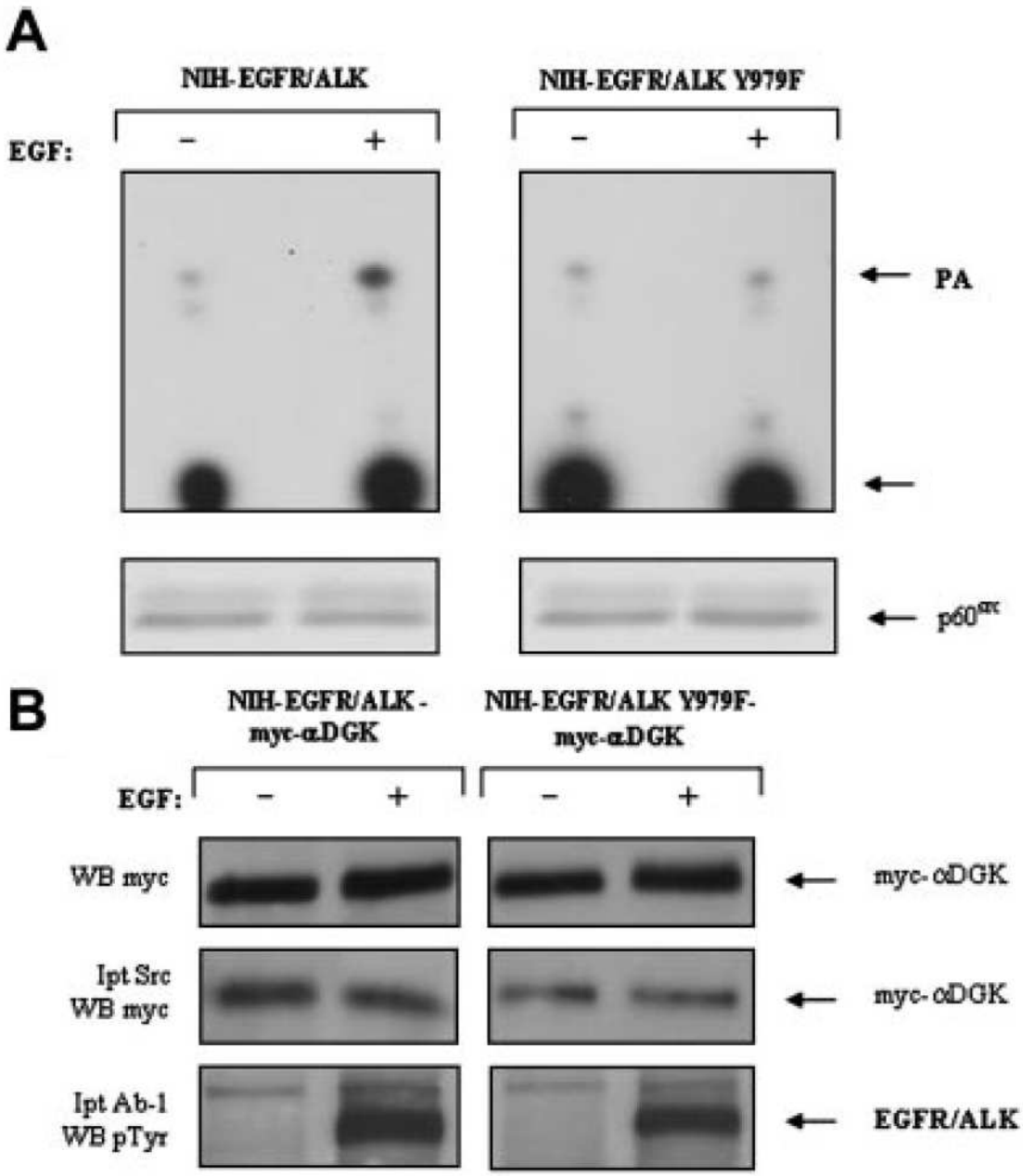

Fig. 15 **Associazione DGK-α / p60src**

A) Cellule NIH-EGFR/ALK e NIH-EGFR/ALKY_{979}F starvate per 18 ore e trattate con EGF (5 minuti, 100 ng/mL) sono state lisate ed immunoprecipitate con anticorpi anti-p60src. Gli immunoprecipitati sono stati analizzati per attività Dgk in vitro (pannello superiore) e western blot con anticorpi anti-Src (pannello inferiore).

B) Cellule NIH-EGFR/ALK e NIH-EGFR/ALK-Y979F trasfettate transientemente con myc-DGK-α starvate per 18 ore e trattate con EGF (5 minuti, 100 ng/mL) e lisate. Il pannello superiore mostra i lisati cellulari totali saggiati in western blot anti myc. Il pannello centrale mostra immunoprecipitati anti Src saggiati in western blot anti myc. Il pannello inferiore mostra immunoprecipitati con anticorpi contro il dominio extracellulare di EGFR saggiati per western blot con anticorpi anti-fosfotirosina.

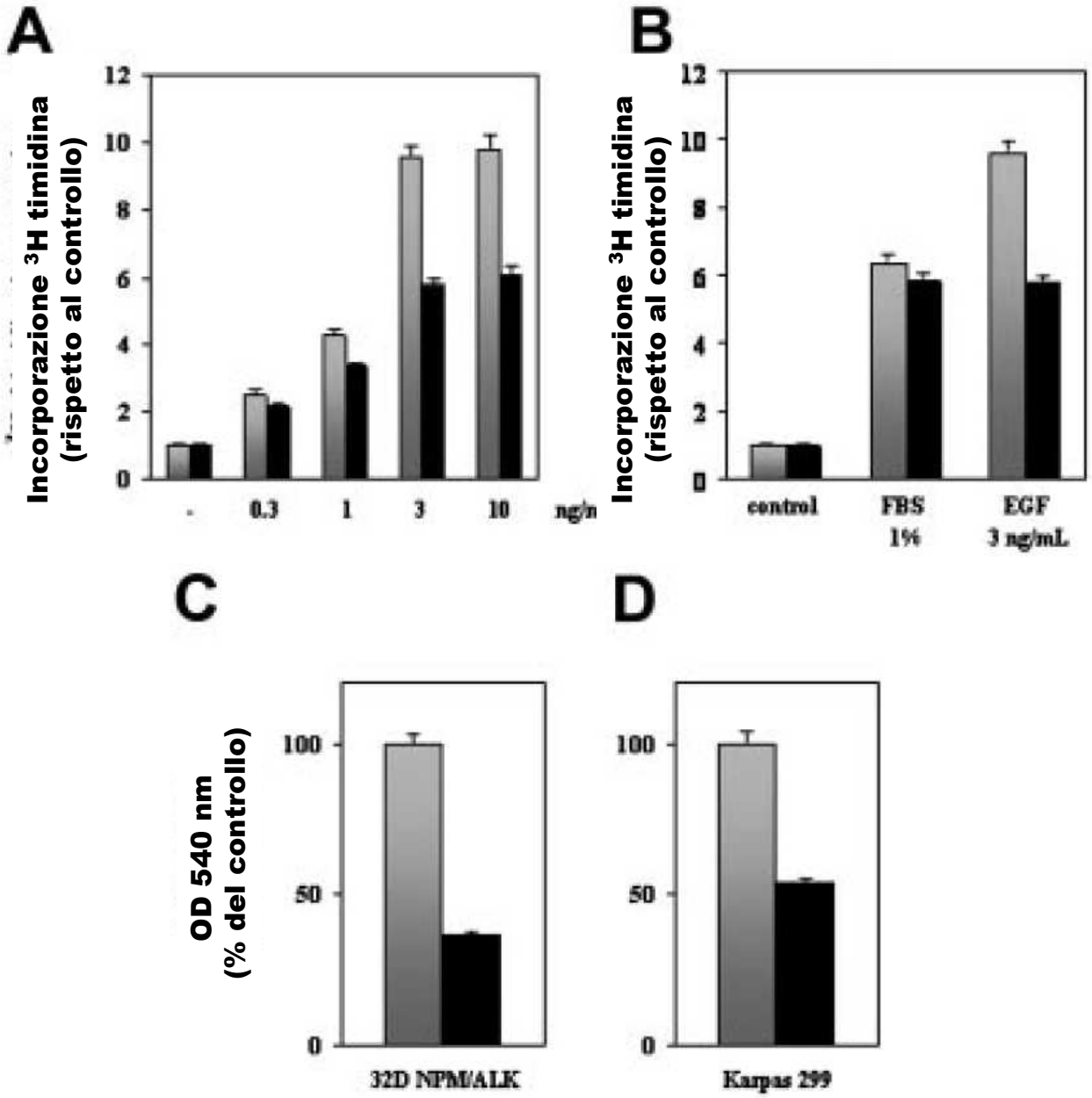

Fig. 16 **Effetto di R59949 sulla proliferazione di NIH-EGFR/ALK, 32D-NPM/ALK e Karpas 299**

A-B) Cellule NIH-EGFR/ALK starvate per 72 ore e pretrattate per 30 minuti con 1 μM R59949 (■) o DMSO (▭) e successivamente stimolate con le dosi indicate di EGF o 1%FBS. La proliferazione è stata misurata come incorporazione di [*methyl*-^{3}H] thymidine in 22 ore; i risultati sono espressi come aumento rispetto al controllo (media ± deviazione standard di triplicati).

C-D) cellule 32D NPM/ALK e Karpas 299 coltivate per 48 ore in FBS 0,1% e BSA 0,2 mg/mL in presenza di 10 μM R59949 (■) o DMSO 0,02% (▭). La proliferzione è stata misurata con il saggio MTT, i dati sono espressi come percentuale del controllo (media ± deviazione standard di triplicati).

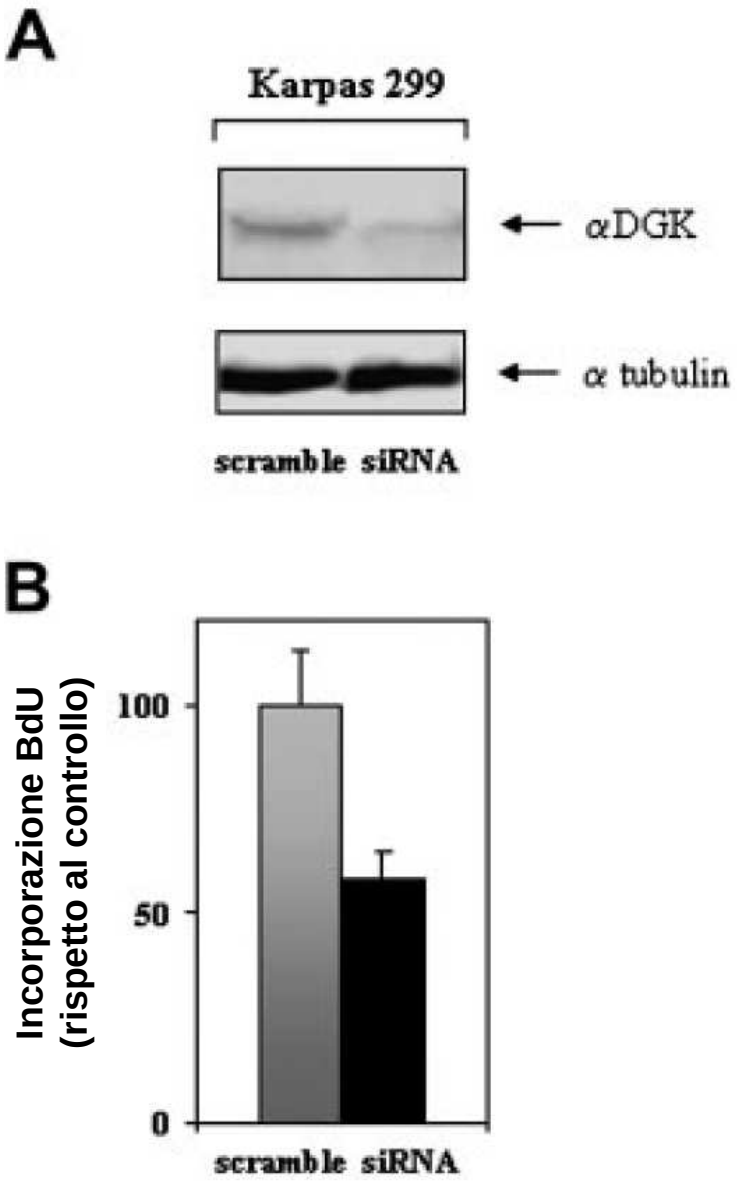

Fig 17 **Il blocco dell'espressione di DGK-α con siRNA specifici blocca la proliferazione di cellule Karpas 299**
A) Cellule Karpas 299 trasfettate con i siRNA indicati sono state lisate a 72 ore dalla trasfezione ed analizzate in Western blot con anticorpi anti-DGK-α (pannello superiore) e anti–-tubulina (pannello inferiore).
B) Proliferazione misurata come incorporazione di BrdU di cellule Karpas 299 a 72 ore dalla trasfezione con i siRNA indicati. I risultati sono espressi come percentuale del controllo (media ± deviazione standard di triplicati).

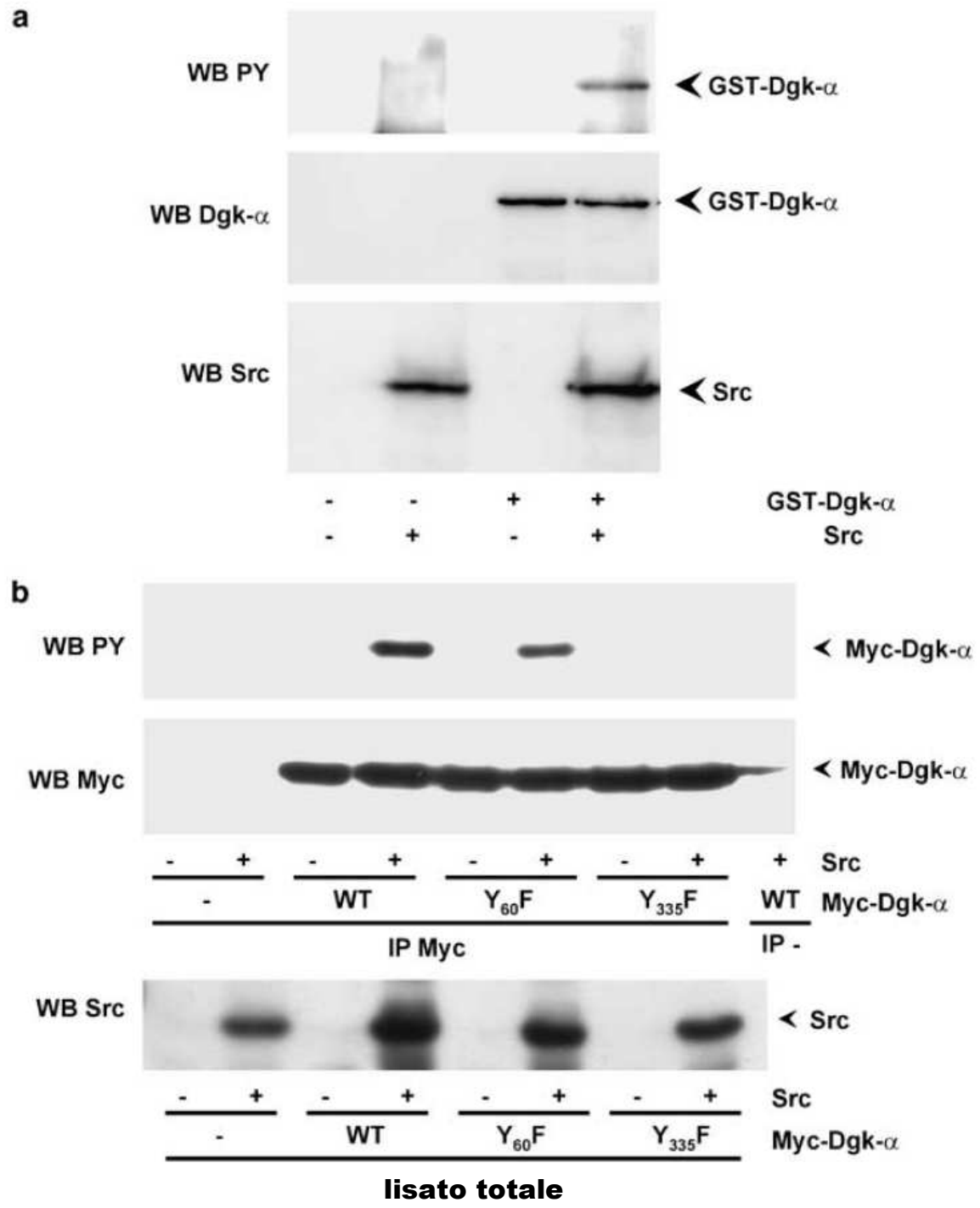

Fig. 18 **Src fosforila Dgk-α sulla tirosina 335**

a) GST-Dgk-α o GST sono stati incubati con o senza Src ricombinante per 10 minuti at 30℃. I campioni sono stati analizzati per western blot con anticorpi anti-fosfotirosina (pannello superiore), anti-Dgk-α (pannello centrale) o anti-Src (pannello inferiore).

b) Cellule COS-7 co-trasfettate con i costrutti indicati sono state lisate ed immunoprecipitate con anticorpi anti-myc. Gli immunoprecipitati sono stati analizzati per western blot con anticorpi anti-fosfotirosina (pannello superiore) e anti-myc (pannello centrale). Il pannello centrale mostra un western blot dei lisati totali corrispondenti con anticorpi anti-Src.

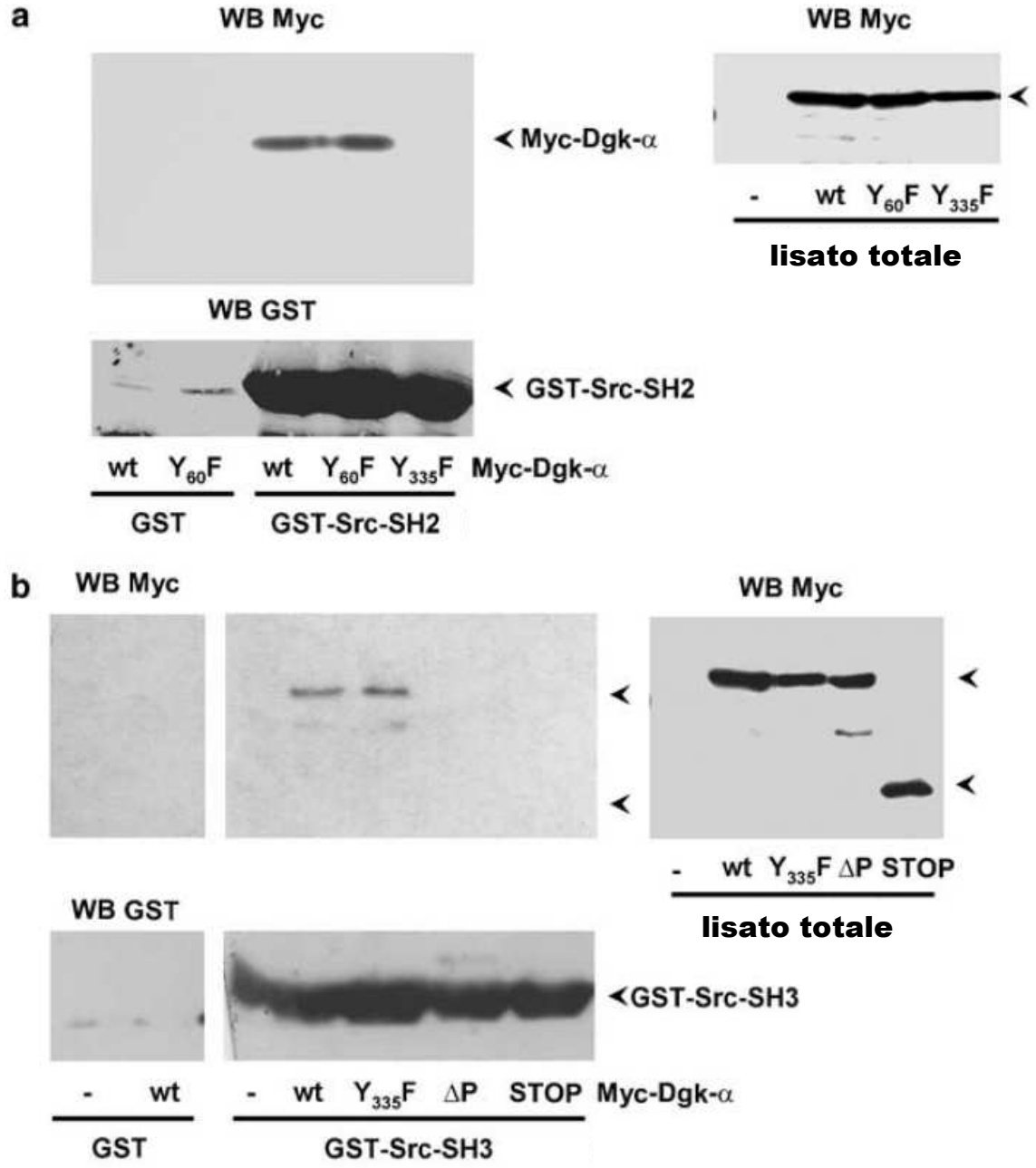

Fig. 19 **Dgk-α interagisce con i domini SH2 e SH3 di Src.**
a) Cellule COS-7 trasfettate con i costrutti Myc-Dgk-α indicati sono state lisate e sottoposte ad un saggio di associazione a GST o GST-Src-SH2 immobilizzate su agarosio. Myc-Dgk-α associata alla matrice (pannello di sinistra) e Myc-Dgk-α nei lisati totali (pannello di destra) sono state rlevate per western blot con anticorpi anti-myc. GST-Src-SH2 immobilizzato alla matrice è stato rilevato per western blot con anticorpi anti-GST (pannello inferiore).
b) Cellule COS-7 trasfettate con i costrutti Myc-Dgk-α indicati sono state lisate e sottoposte ad un saggio di associazione a GST o GST-Src-SH3 immobilizzate su agarosio. Myc-Dgk-α associata alla matrice (pannello di sinistra) e Myc-Dgk-α nei lisati totali (pannello di destra) sono state rlevate per western blot con anticorpi anti-myc. GST-Src-SH3 immobilizzato alla matrice è stato rilevato per western blot con anticorpi anti-GST (pannello inferiore).

Proteina immobilizzata	***Legame (++ forte, + debole, -nessuno)***
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	+

Tabella 3 **Associazione di myc-Dgk-α a domini GST-SH2 e GST-SH3**

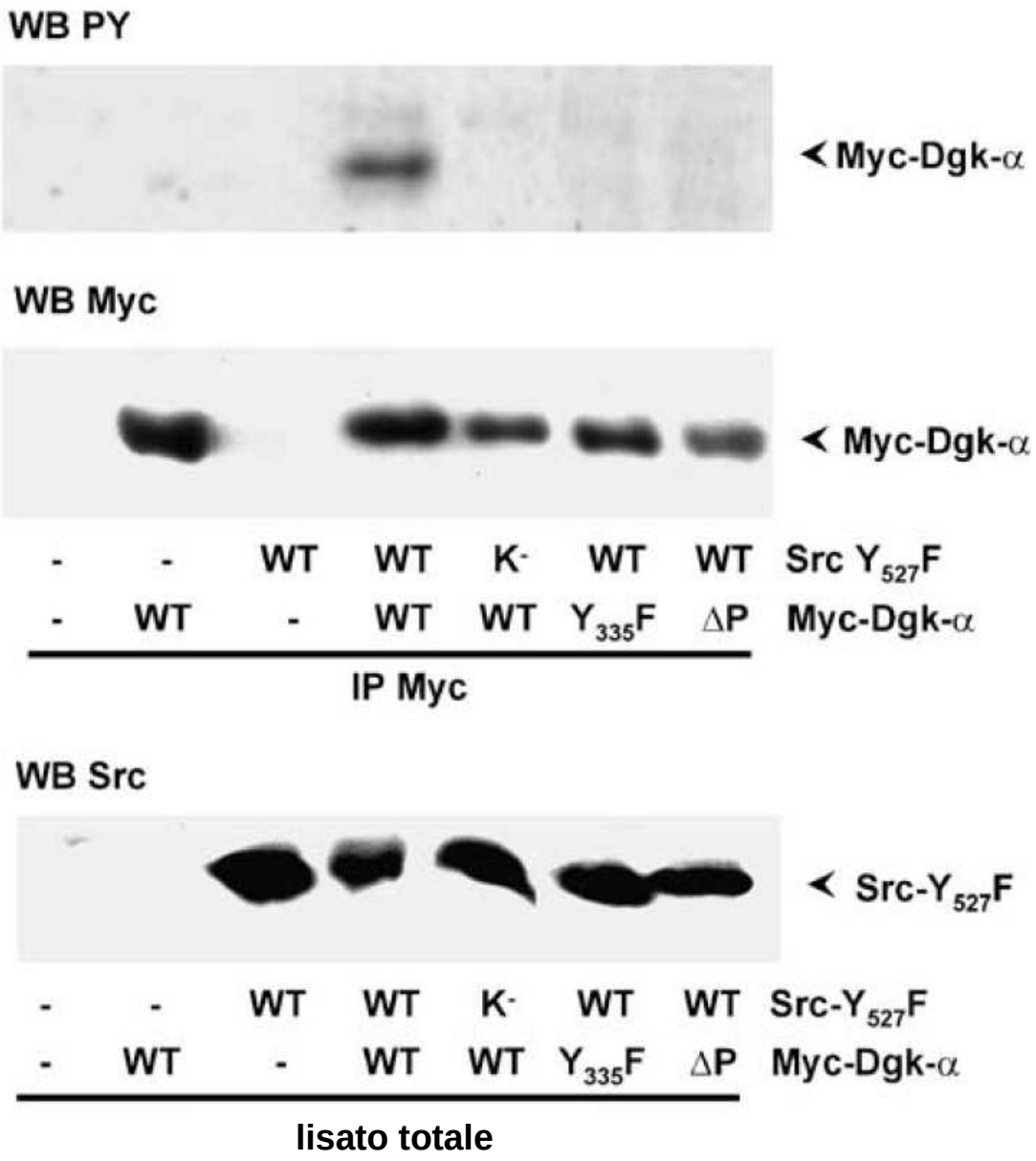

Fig. 20 **La fosforilazione di Dgk-α mediata da Src richiede Y_{335} ed il C-terminale ricco in proline**

HEK 293T co-trasfettate con i costrutti di Myc-Dgk-α e Src-Y_{527}F sono state lisate ed immunoprecipitate con anticorpi anti-myc. Gli immunoprecipitati anti-myc sono stati analizzati con anticorpi anti-fosfotirosina (pannello superiore) e anti-myc (pannello centrale). Il pannello inferiore mostra i lisati cellulari totali immunoprecipitati con anticorpi anti-Src (pannello inferiore).

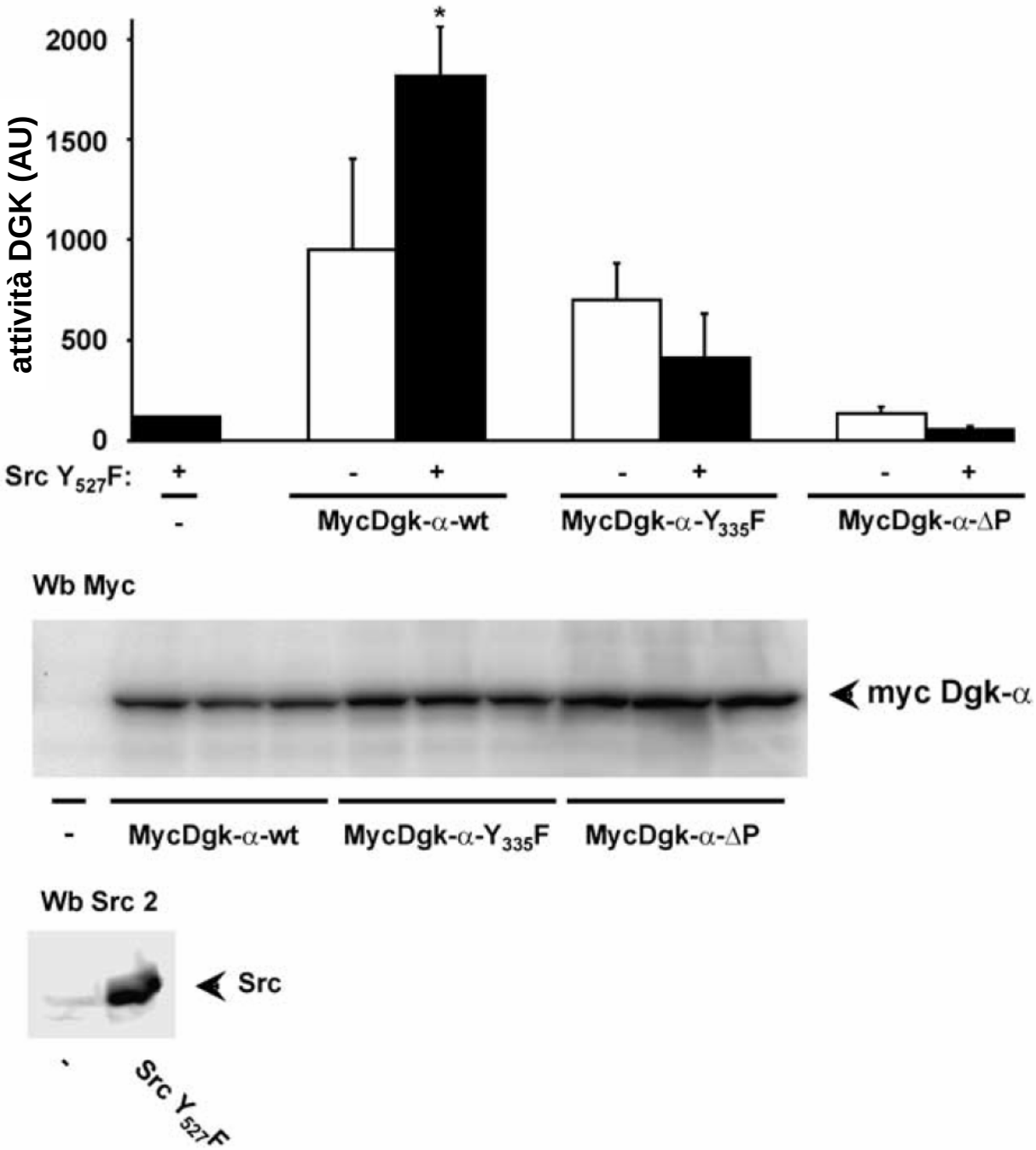

Fig. 21 **L'attivazione di Dgk-α da c-Src richiede Y_{335} ed il C-terminale ricco in proline**

Cellule COS-7 trasfettate con Myc-Dgk-α wt, Myc-Dgk-α-Y_{335}F, Myc-Dgk-α-ΔP o Src sono state omogenate. Gli omogenati sono stati mescolati come indicato ed incubati in presenza di 1 mM ATP per 15 minuti e successivamente analizzati per attività Dgk (pannello superiore) i dati sono la media ± di triplicati; *t-test, $P<0,05$. L'espressione di Myc-Dgk-α e Src è stata verificata per western blot con anticorpi anti-myc e anti-src.

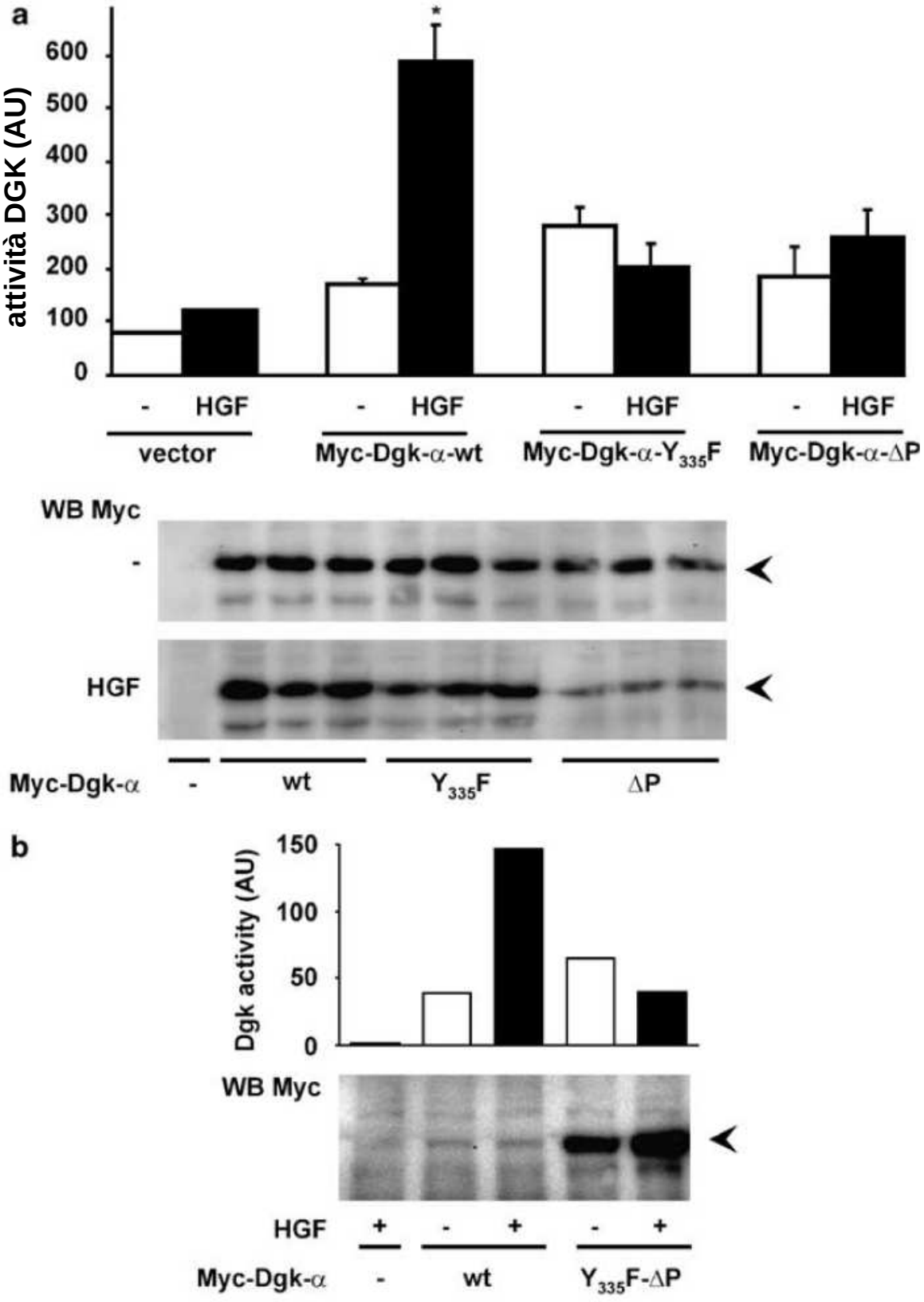

Fig. 22 **L'attivazione di Dgk-α da HGF in vivo richiede Y335 ed il C-terminale ricco in proline**

Cellule COS-7 transfettate con i costrutti indicati sono state stimolate con HGF (A 100 ng/ml, B 200 ng/ml, 15 minuti) ed omogenate. Gli omogenati sono stati saggiati per western blot con anticorpi anti-myc (pannello inferiore) e per attività Dgk (pannello superiore). I dati sono mostrati come media±errore standard di triplicati (*t-test P<0,05).

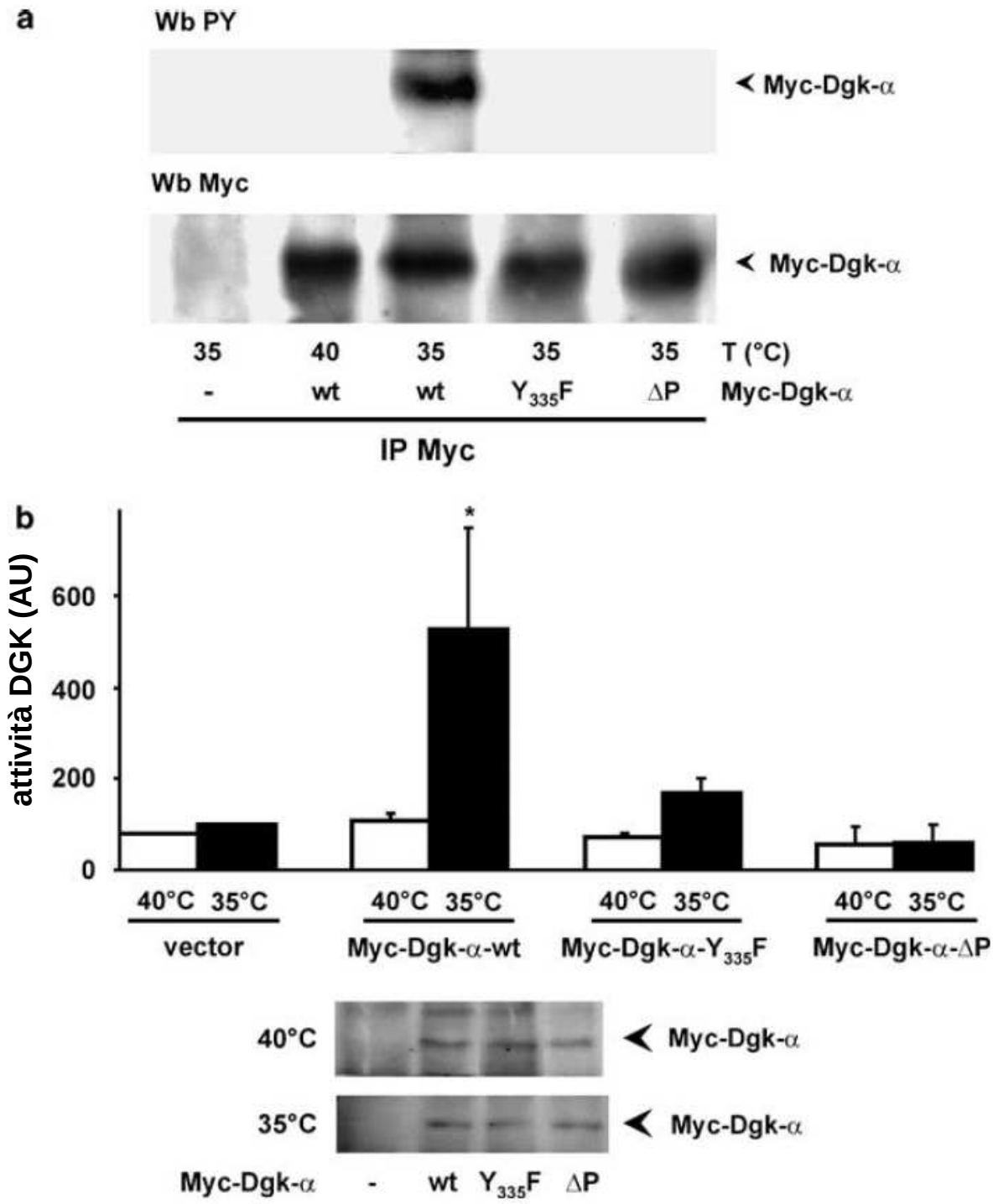

Fig. 23 **Fosforilazione e attivazione di Dgk-α indotte da v-Src richiedono la Y_{335} e il C terminale ricco in proline**

Cellul Ts-v-Src/MDCK trasfettate con i construtti indicati e coltivate alla temperatura non permissiva (40℃) sono state sposta te alla temperatura permissiva (35℃) per 1 ora.

a) Le cellule sono state lisate e immunoprecipitate con anticorpi anti myc ed analizzate per western blot con aticorpi anti-fosfotirosina (pannello superiore) e anti-myc (pannello inferiore).

b) Le cellule sono state omogenizzate e saggiate per western blot con anticorpi anti myc (pannello inferiore) o per attività Dgk (pannello superiore). I dati sono mostrati come media±errore standard di triplicati (*t-test P<0,05).

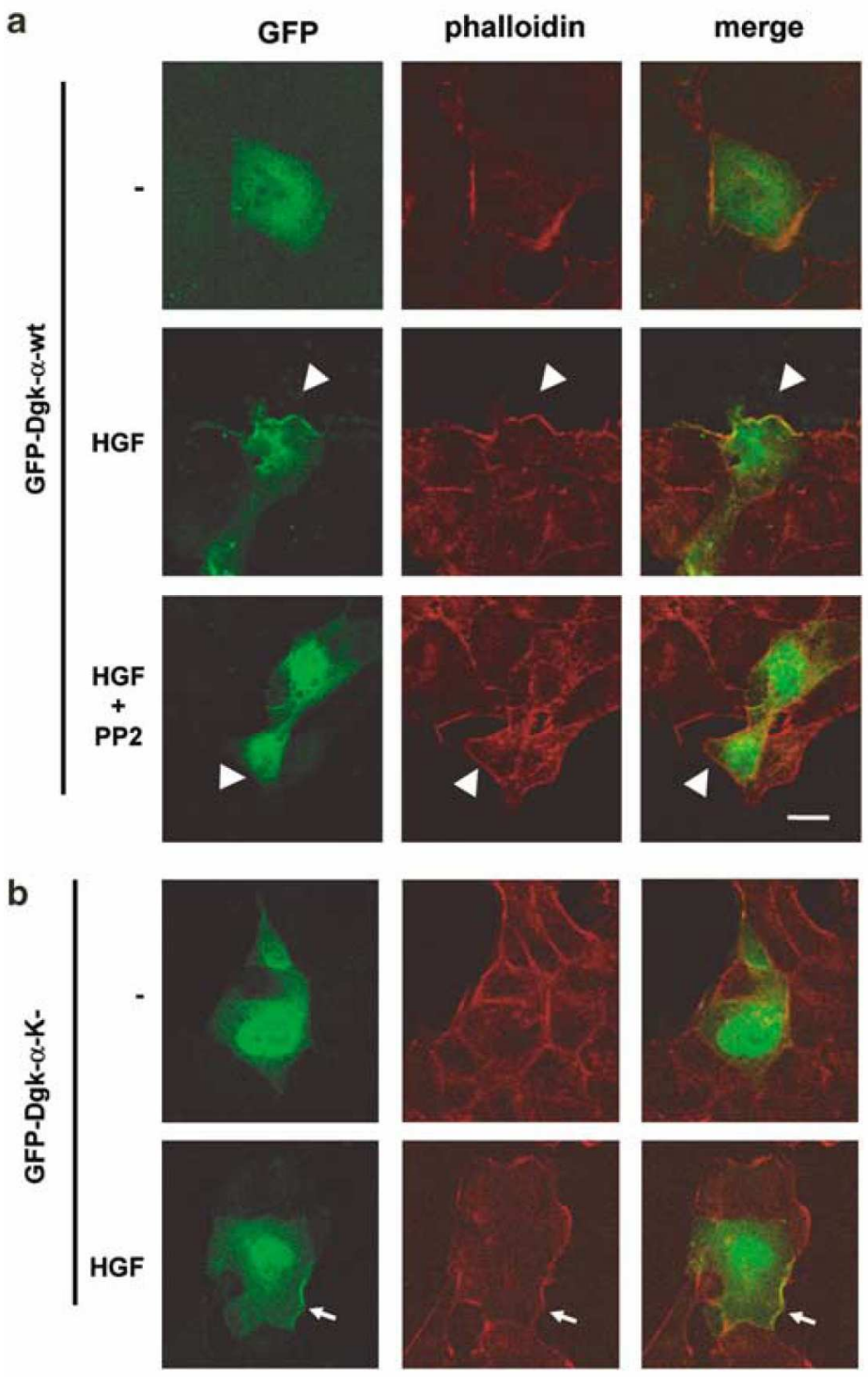

Fig. 24 **Dgk-α è reclutata alla membrana in seguito a trattamento con HGF**

Cellule MDCK trasfettate con GFP-Dgk-α wt or GFP-Dgk-α-K- e trattate con HGF (50 ng/ml, 15minuti). Ove indicato le cellule sono state pretrattate con PP2 (10 μM PP2 per 15 minuti). Le cellule sono state decorate con phalloidin-TRITC e le immagini acquisite al microscopio cofocale (barra 16 μm).

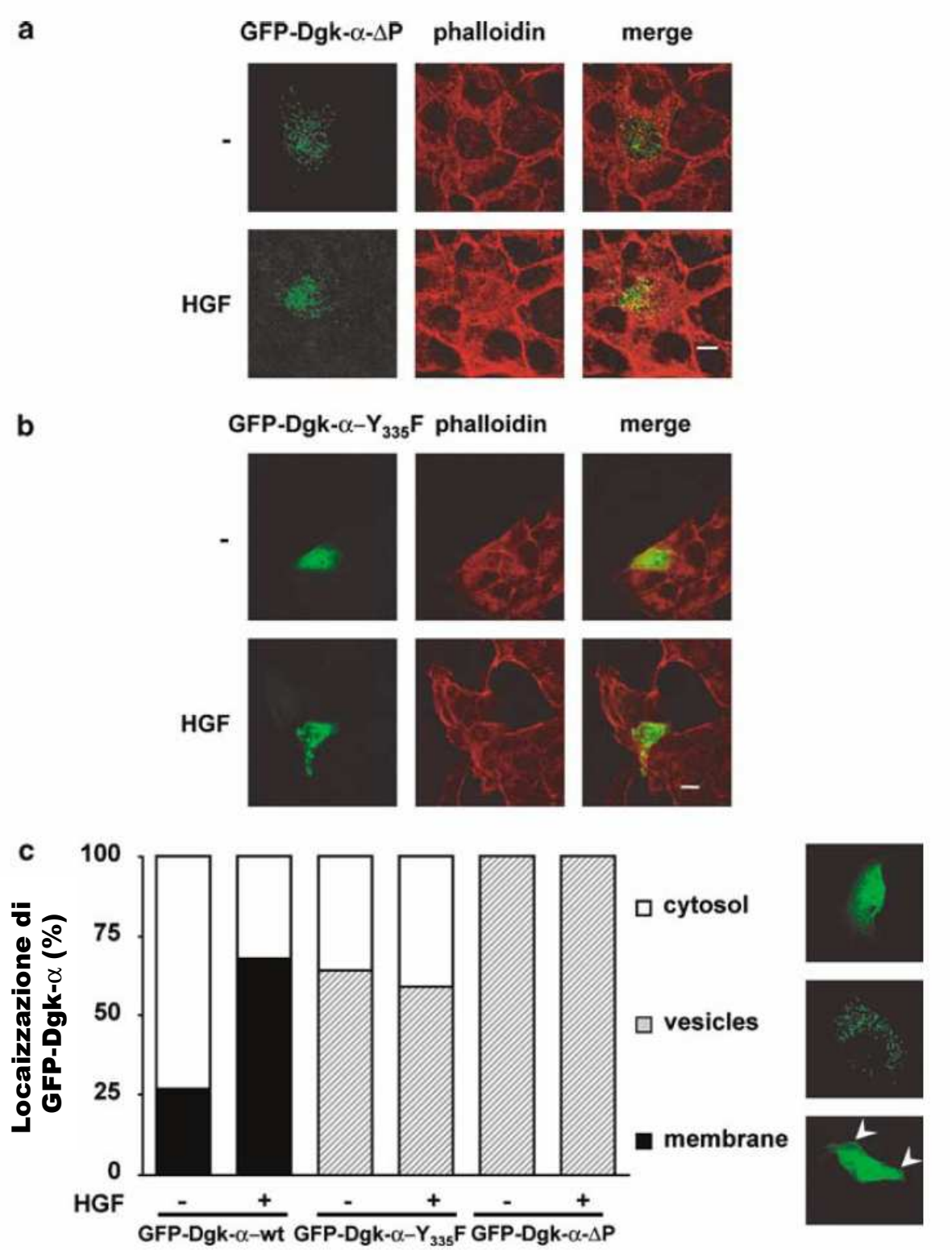

Fig. 25 **Il reclutamento di Dgk-α alla membrana cellulare richiede Y335 ed il C terminale ricco in proline**

Cellule MDCK trasfettate con GFP-Dgk-α-wt, GFP-Dgk-α-DP (a) o GFP-Dgk-α-Y_{335}F (b) sono state stimolate con HGF (50 ng/ml, 15 minuti). Le cellule sono state decorate con phalloidin-TRITC e le immagini acquisite al microscopio cofocale (barra 16 mm).

c) Per ogni punto sperimentale 100 o più cellule sono state valutate per la localizzazione: membrana (barre piene), citoplasma (barre vuote), vescicole (barre tratteggiate), esempi di ciascuna categoria sono mostrati a destra.

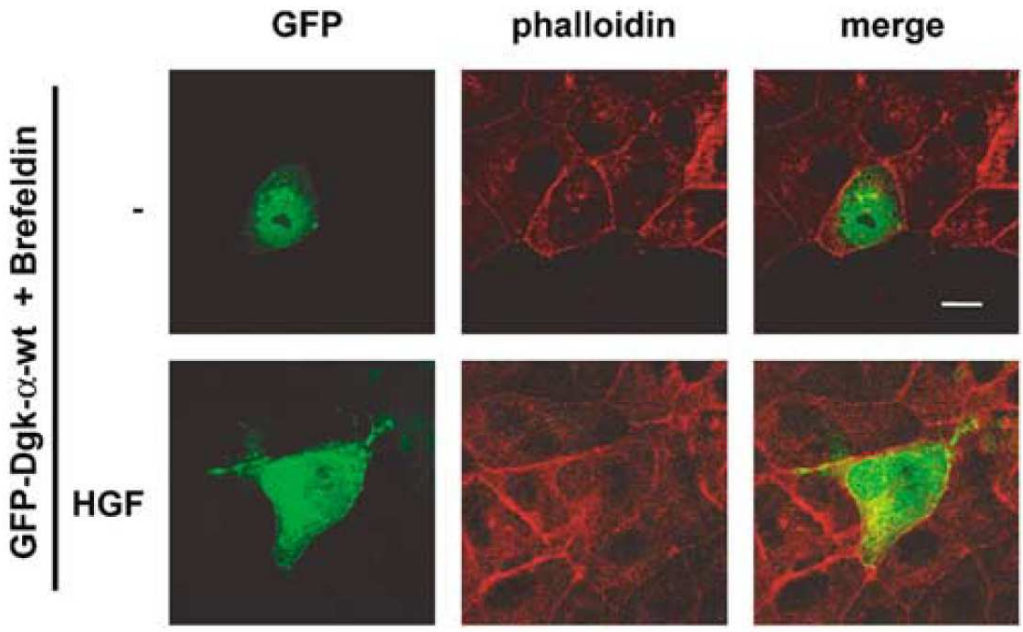

Fig. 26 **Brefeldina promuove l'accumulo di Dgk-α su vescicole citoplasmatiche**
Cellule MDCK trasfettate con GFP-Dgk-α-wt sono state pretrattate con BFA (10 μM BFA per 15 minuti) e stimolate con HGF (50 ng/ml, 15 minuti). Le cellule sono state decorate con phalloidin-TRITC e le immagini acquisite al microscopio cofocale (barra 16 mm).

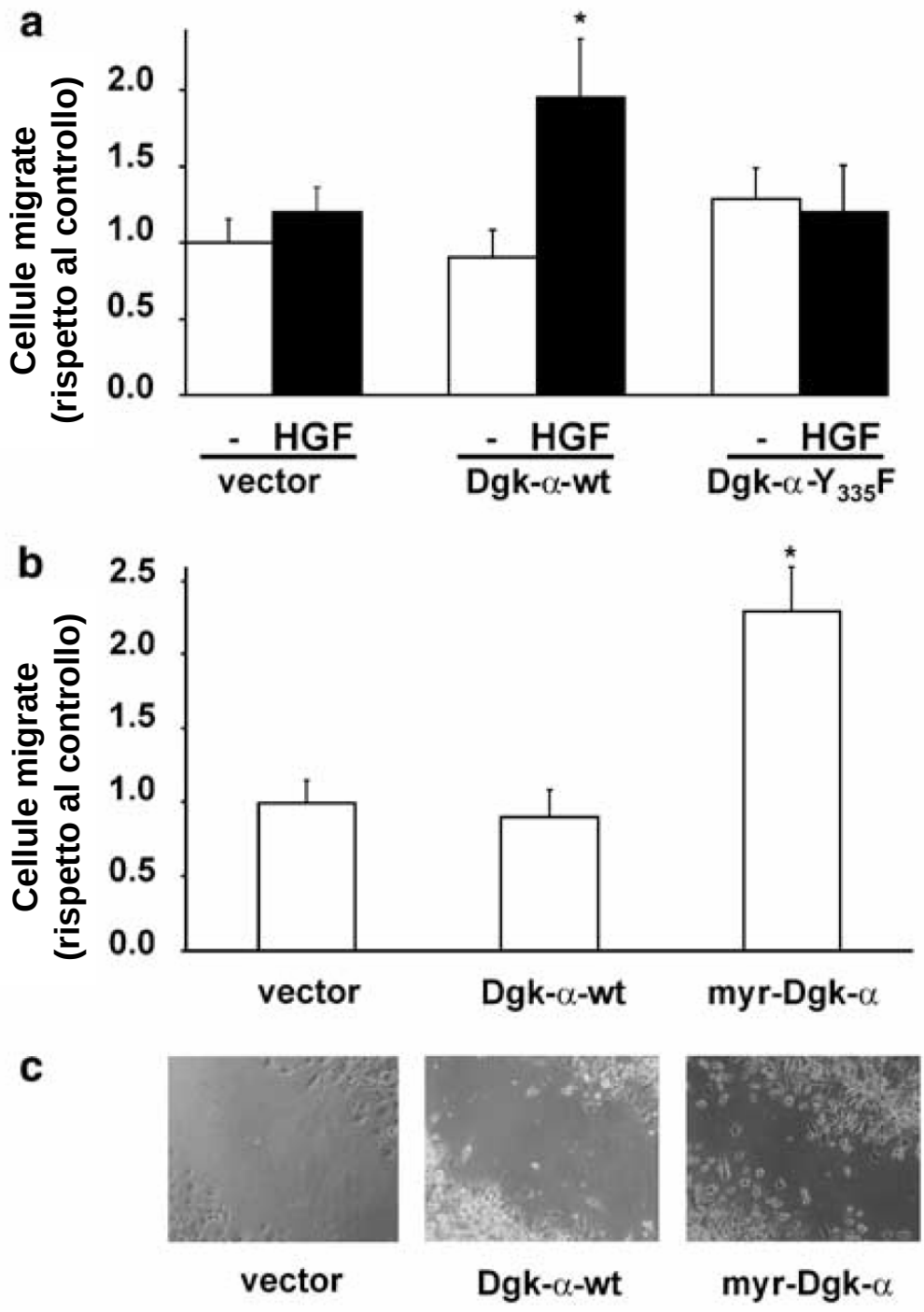

Fig. 27 **Il reclutamento alla membrana di Dgk-α è necessario e sufficiente per la motilità cellulare.**

a) Cellule COS-7 trasfettate come indicato sono state indotte a migrare in un saggio di migrazione in transwell stimolando con HGF (100 ng/ml). I dati sono espressi come aumento rispetto al controllo (media±deviazione standard di 4 esperimenti indipendenti; * t-test accoppiato, $P<0.07$).

b) migrazione spontanea in un saggio di migrazione in transwell di cellule COS-7 trasfettate come indicato. I dati sono espressi come aumento rispetto al controllo (media±deviazione standard di 4 esperimenti indipendenti; *t-test accoppiato, $P<0.06$).

c) migrazione spontanea in un saggio di wound healing di cellule COS-7 trasfettate come indicato. Un campo rappresentativo è stato fotografato in contrasto di fase.

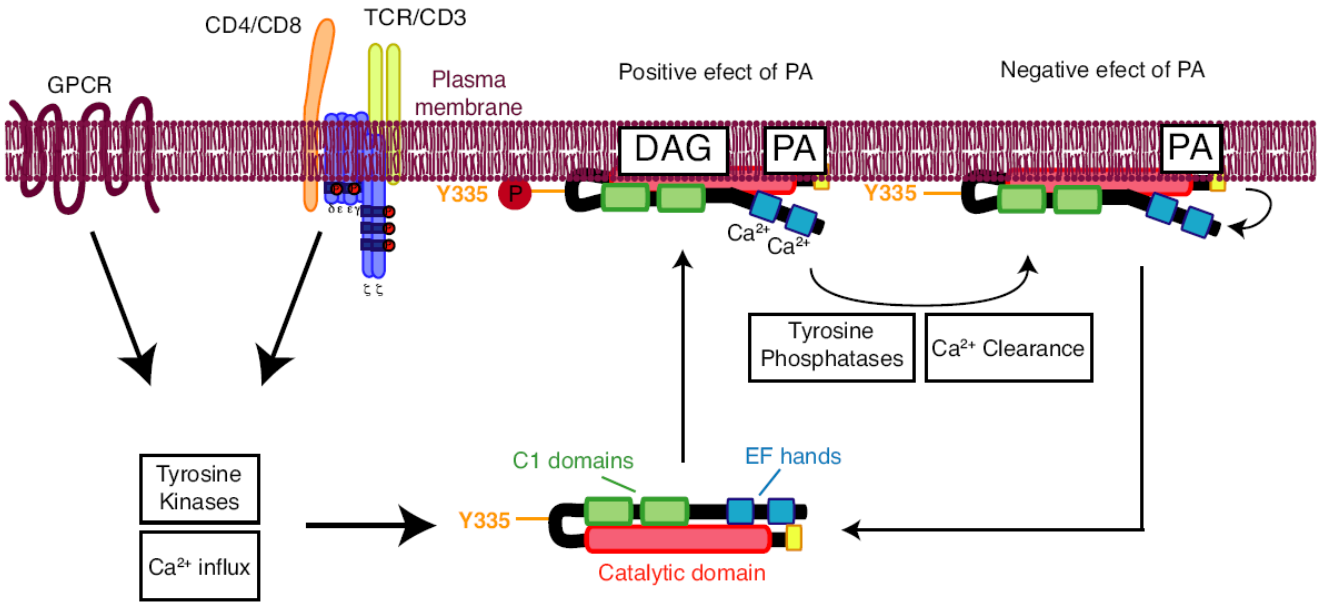

Fig. 28. **Regolazione dell'attività e traslocazione in membrana di Dgk-α**

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ORIGINAL PAPER

Activation of diacylglycerol kinase α is required for VEGF-induced angiogenic signaling *in vitro*

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Vascular endothelial growth factor-A (VEGF-A) promotes angiogenesis by stimulating migration, proliferation and organization of endothelium, through the activation of signaling pathways involving Src tyrosine kinase. As we had previously shown that Src-mediated activation of diacylglycerol kinase-α (Dgk-α) is required for hepatocytes growth factor-stimulated cell migration, we asked whether Dgk-α is involved in the transduction of angiogenic signaling. In PAE-KDR cells, an endothelial-derived cell line expressing VEGFR-2, VEGF-A_{165}, stimulates the enzymatic activity of Dgk-α: activation is inhibited by R59949, an isoform-specific Dgk inhibitor, and is dependent on Src tyrosine kinase, with which Dgk-α forms a complex. Conversely in HUVEC, VEGF-A_{165}-induced activation of Dgk is only partially sensitive to R59949, suggesting that also other isoforms may be activated, albeit still dependent on Src tyrosine kinase. Specific inhibition of Dgk-α, obtained in both cells by R59949 and in PAE-KDR by expression of Dgk-α dominant-negative mutant, impairs VEGF-A_{165}-dependent chemotaxis, proliferation and *in vitro* angiogenesis. In addition, in HUVEC, specific downregulation of Dgk-α by siRNA impairs *in vitro* angiogenesis on matrigel, further suggesting the requirement for Dgk-α in angiogenic signaling in HUVEC. Thus, we propose that activation of Dgk-α generates a signal essential for both proliferative and migratory response to VEGF-A_{165}, suggesting that it may constitute a novel pharmacological target for angiogenesis control.
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Keywords: diacylglycerol kinase; VEGF; phosphatidic acid; Src; angiogenesis

Introduction

Tumor growth and metastasis to distant organs, as well as revascularization of ischemic tissues and female cycle are dependent on angiogenesis, the formation of new blood vessels by branching from pre-existing ones (Folkman, 1972; Carmeliet and Jain, 2000). Angiogenesis is promoted by angiogenic factors secreted by hypoxic tissues, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocytes growth factor (HGF) (Bussolino *et al*., 1992; Matsumoto and Claesson-Welsh, 2001). In particular, VEGF-A, whose receptors are mostly expressed on endothelial cells, plays a crucial role in the angiogenesis *in vivo* (Folkman, 1972; Carmeliet and Jain, 2000; Matsumoto and Claesson-Welsh, 2001). Because antiangiogenic therapy is currently believed to be a promising approach to cancer treatment (Folkman, 1972; Carmeliet and Jain, 2000; Matsumoto and Claesson-Welsh, 2001), the identification of new proteins involved in the biochemical mechanisms transducing angiogenic extracellular signals would provide novel biochemical targets for pharmacological intervention. VEGF-A stimulates angiogenesis by activating several signaling pathways in a spatially and timely coordinate manner, leading to endothelial cells migration, proliferation and organization in tubular structures (reviewed in Matsumoto and Claesson-Welsh, 2001). Among the numerous pathways activated, the generation of phosphatidylinositol(4,5)bis-phosphate ($PI(4,5)P_2$)-derived second messengers plays a crucial role in VEGF-A angiogenic signaling. Indeed, VEGF-A-induced angiogenic signaling requires both phosphorylation and hydrolysis of $PI(4,5)P_2$, mediated, respectively, by phosphatidylinositol 3-kinase (PI 3-kinase) and phospholipase C-γ (PLC-γ to generate phosphatidylinositol(3,4,5)tris-phosphate ($PI(3,4,5)P_3$) and diacylglycerol (DG). While $PI(3,4,5)P_3$ is required for activation of Akt, which mediates VEGF-A-induced survival signaling (Gerber *et al*., 1998), DG is required for activation of PKC-α, which mediates VEGF-A-induced proliferative and chemotactic signaling (Wellner *et al*., 1999; Matsumoto and Claesson-Welsh, 2001). Diacylglycerol kinase (Dgk) enzymes phosphorylate

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diacylglycerol to generate phosphatidic acid (PA), regulating in a reciprocal manner the level of two lipid second messengers (Topham and Prescott, 1999). Thus Dgk enzymes may act both as terminators of DG-mediated signaling and as activators of PA-mediated signals. Indeed, activation of Dgk enzymes has been reported to downregulate DG-regulated enzymes such as PKCs and Ras GTP releasing proteins (RasGRP), respectively, in platelets and T cells (De Chaffoy de Courcelles *et al*., 1989; Jones *et al*., 2002; Zhong *et al*., 2002). On the other hand, activation of Dgk-α has been reported to convey proliferative and motility signals, respectively, in IL-2-stimulated T lymphocytes and in HGF-stimulated endothelial cells (Flores *et al*., 1996; Cutrupi *et al*., 2000). In addition, generation of PA through phospholipase D (PLD)-mediated hydrolysis of phospholipid is required for vescicular trafficking, lamellipodia, cell migration and proliferation in different cellular systems (English, 1996; Honda *et al*., 1999; O'Luanaigh *et al*., 2002).

To date, nine distinct Dgk isoforms have been cloned in mammalians, encoding soluble proteins that reversibly associate to the membrane or to the nucleus (reviewed in Topham and Prescott, 1999). All isoforms share a highly conserved catalytic domain, preceded by at least two Zn fingers, homologous to the C1 domain of PKCs. Five different families have been defined according to the different N-terminal regulatory domains. Class I Dgk, comprising α-, β- and γ-isoforms, shares a common N-terminal autoinhibitory motif and a pair of EF-hand calcium-binding domains preceding the Zn fingers. Dgk-α is abundant in T lymphocytes, but is also expressed in endothelial and epithelial cells, fibroblasts and oligodendrocytes (Schaap *et al*. 1990; Cutrupi *et al*., 2000; data not shown). The biological functions and biochemical regulation of Dgk enzymes are currently under investigation (reviewed in van Blitterswijk and Houssa, 2000). We have previously shown that upon HGF stimulation of a porcine aortic cell line (PAE), Dgk-α is activated and associates in a complex with Src. In addition, we have also shown that Dgk-α is regulated by tyrosine phosphorylation and that its activation requires Src tyrosine kinase activity (Cutrupi *et al*., 2000). Specific inhibition of Dgk-α, either through expression of a dominant-negative mutant or by cell treatment with R59949, a pharmacological inhibitor, impairs HGF-induced chemotaxis of endothelial cells (Cutrupi *et al*., 2000) and IL-2-induced proliferation of T lymphocytes (Flores *et al*., 1996).

Thus, based on these data and on the absolute requirement for Src tyrosine kinase activity in VEGF-A-induced proliferation and motility of endothelial cells (Abu-Ghazaleh *et al*., 2001), we set to investigate the role of Dgk-α in VEGF-A signaling in PAE cells expressing VEGFR-2 (PAE-KDR) and in human umbilical vein endothelial cells (HUVEC). Herein we report that (i) VEGF-A_{165} stimulates Dgk-α activity in an Src-dependent manner and, at least in PAE-KDR, induces the formation of a Dgk-α/Src complex; (ii) both pharmacological inhibition of Dgk-α in both cells and expression of Dgk-α dominant-negative mutant in PAE-KDR impair VEGF-A_{165}-induced chemotaxis and proliferation, as well as *in vitro* formation of tubule structures in matrigel; (iii) specific downregulation of Dgk-α by siRNA in HUVEC impairs *in vitro* formation of tubule structures in matrigel. These are the first evidences indicating the involvement of Dgk-α in VEGF-A signal transduction, providing the first demonstration that Dgk-α activation is necessary for the VEGF-A-triggered angiogenic program. Furthermore, these data indicate Dgk-α as a promising novel pharmacological target for angiogenesis control.

Results

VEGF-A_{165} activates Dgk-α

Stimulation of VEGFR-2 tyrosine kinase activity mediates the activation of the angiogenetic response induced by VEGF-A in HUVEC and PAE-KDR cells (Waltenberger *et al*., 1994; Matsumoto and Claesson-Welsh, 2001). Thus, we have measured Dgk activity *in vitro* in cell homogenates obtained from either control or VEGF-A_{165}-stimulated PAE, PAE-KDR and HUVEC. Dgk activity was measured *in vitro* as phosphorylation of exogenous DG in the presence of radiolabeled ATP. The activity of homogenates obtained from PAE-KDR and HUVEC stimulated with VEGF-A_{165} was twofold higher than from untreated control cells (Figure 1a). The activation was sustained for at least 1 h (Figure 1b) and dependent on VEGF concentration (data not shown). On the other hand, Dgk activity from PAE cells, which do not express VEGFR-2 at significant

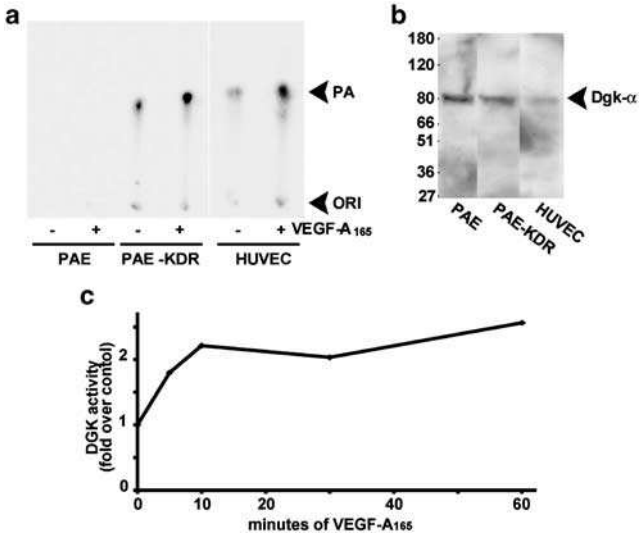

Figure 1 VEGF-A_{165} activates DGK in PAE-KDR and HUVEC. **(a)** Dgk activity assayed on whole-cell homogenates from either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent PAE, PAE-KDR and HUVEC cells. **(b)** Dgk-α protein assayed by Western blot with anti-Dgk-α antibodies in whole-cell extracts from PAE, PAE-KDR and HUVEC. **(c)** Time course of Dgk activation in VEGF-A_{165}-stimulated (VEGF-A_{165} 50 ng/ml) PAE-KDR cells

levels, was not increased upon VEGF-A_{165} stimulation (Figure 1a). Intriguingly, even in the absence of exogenous VEGF-A_{165}, basal Dgk activity from PAE-KDR was much higher than from PAE cells, suggesting that the sole expression of VEGFR-2, putatively through an autocrine loop, is sufficient to generate a signal that upregulates Dgk activity. Indeed, PAE and PAE-KDR express VEGF, as measured by RT–PCR, and both basal ERK-1/2 and Src activities are upregulated in unstimulated PAE-KDR cells compared with PAE cells (data not shown).

However, these experiments, which clearly indicate that VEGF-A_{165} stimulates a Dgk activity, do not allow to establish which of the different Dgk isoforms are activated upon VEGF-A_{165} stimulation of these cells. Dgk-α is expressed at similar levels in PAE and PAE-KDR cells, as measured by Western blot with Dgk-α antibodies, while in HUVEC its expression is significantly lower (Figure 1c). Thus in order to verify whether Dgk-α is regulated by VEGF-A_{165}, we assayed *in vitro* the Dgk activity in anti-Dgk-α immunoprecipitates from lysates of PAE-KDR cells, either control or VEGF-A_{165} stimulated. Indeed, Dgk activity in anti-Dgk-α immunoprecipitates from VEGF-A_{165}-stimulated cells was higher than from unstimulated cells, while the amount of Dgk-α protein immunoprecipitated did not change (Figure 2a). VEGF-induced activation of Dgk-α was sustained for at least 60 min (Figure 2b), and was dependent on VEGF concentration (Figure 2c). Mock immunoprecipitates carried out in the absence of anti-Dgk-α antibodies did not contain either Dgk-α protein or activity.

In order to further characterize the Dgk activity stimulated by VEGF-A_{165} in PAE-KDR cells and HUVEC cells, we have verified whether it is inhibited *in vitro* by R59949, a class I Dgk-specific inhibitor with a strong preference for the α- and β-isoform rather than the γ-isoform (Kai *et al*., 1994), whose effects on tyrosine kinase signaling in PAE cells are reverted by overexpression of Dgk-α (Cutrupi *et al*., 2000). In endothelial cells, R59949 cell treatment did not affect both VEGFR-2 tyrosine phosphorylation (Figure 3a) and cell viability as measured by Trypan blue (Figure 3b). *In vitro*, 1 μM R59949 inhibited completely both basal and VEGF-induced Dgk activity assayed in PAE-KDR cell homogenates (Figure 3c). Conversely, R59949, even at 10 μM, inhibited only part of both basal and VEGF-stimulated Dgk activity assayed in HUVEC homogenates (Figure 3c), suggesting that in these cells VEGF-A_{165} may stimulate other Dgk isoforms also that are less sensitive to R59949.

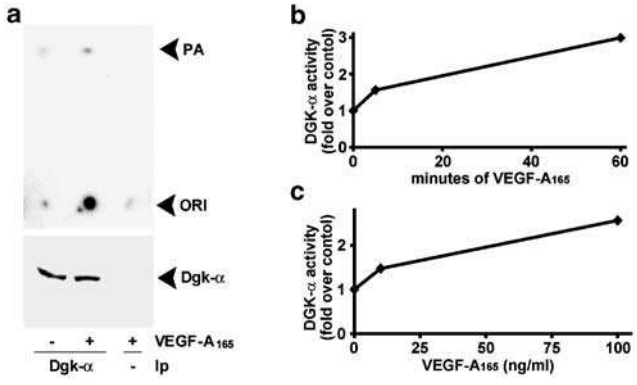

Figure 2 VEGF-A_{165} activates Dgk-α. **(a)** Dgk-α activity (upper panel) and protein (lower panel) assayed on anti-Dgk-α immunoprecipitates from lysates of either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated PAE-KDR cells. Time course **(b)** and VEGF concentration dependence **(c)** of Dgk-α activation, assayed in anti-Dgk-α immunoprecipitates from PAE-KDR stimulated, respectively, with 50 ng/ml VEGF-A_{165} and upon 15 min stimulation

Expression of a dominant-negative mutant of Dgk-α impairs VEGF-A_{165}-induced in vitro angiogenesis

To further investigate the involvement of Dgk-α VEGF signaling, we expressed a catalytic inactive mutant of Dgk-α Dgk-α-K^-), which acts as dominant negative in PAE cells (Cutrupi *et al*., 2000). PAE-KDR cells were infected with PINCOS retrovirus containing myc-tagged Dgk-α-K^-, obtained as described previously (Cutrupi *et al*., 2000). The efficiency of infection, measured as green fluorescent protein (GFP) expression by FACS analysis, was about 70% (data not shown), while the expression of myc-tagged Dgk-α, either wt or K^-, was about two to threefold the expression of endogenous Dgk-α (data not shown). Thus the PAE-KDR-PINCOS cell lines, containing either empty vector, Dgk-α-wt or

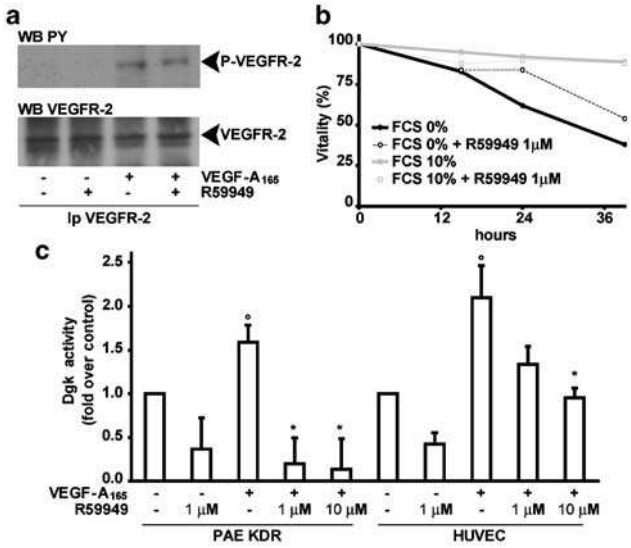

Figure 3 VEGF-A_{165}-stimulated Dgk is sensitive to R59949. (**a**) Antiphosphotyrosine (upper panel) and anti-VEGFR-2 (lower panel) Western blot of anti-VEGFR-2 immunoprecipitates from either unstimulated or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated HUVEC, pretreated for 15 min with either R59949 1 μM or vehicle. **(b)** Cell viability, quantified by Trypan blue exclusion, of PAE-KDR cells maintained in the presence (10%) or absence of FCS and R59949 (1 μM) for the time indicated. **(c)** Dgk activity assayed in the presence of the indicated concentrations of R59949 on whole-cell homogenates from either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent PAE-KDR and HUVEC cells (data are mean ±s.e.m. of five independent experiments normalized for control, °*t*-test *vs* control, **t*-test *vs* VEGF-A_{165}, $P<0.05$)

dominant-negative mutant, were assayed in a chemotaxis assay on a collagen I matrix. The expression of Dgk-α dominant-negative mutant in PAE-KDR significantly inhibited VEGF-A_{165}-induced cell migration (Figure 4a), while it did not affect cell migration induced by fetal calf serum (FCS) (data not shown). Conversely, the expression of Dgk-α-wt enhanced the migratory response of PAE-KDR cells to VEGF by about twofold, although the difference was not statistically significant (Figure 4a).

The same cell lines were also assayed for VEGF-A_{165}-induced DNA synthesis. The expression of Dgk-α dominant-negative mutant reduced VEGF-A_{165}-induced DNA synthesis of PAE-KDR, while the expression of wt Dgk-α did not affect DNA synthesis of these cells upon VEGF-A_{165} treatment (Figure 4b). Similar to the cell migration assay, the expression of Dgk-α dominant-negative mutant did not affect FCS-induced DNA synthesis of PAE-KDR cells (data not shown).

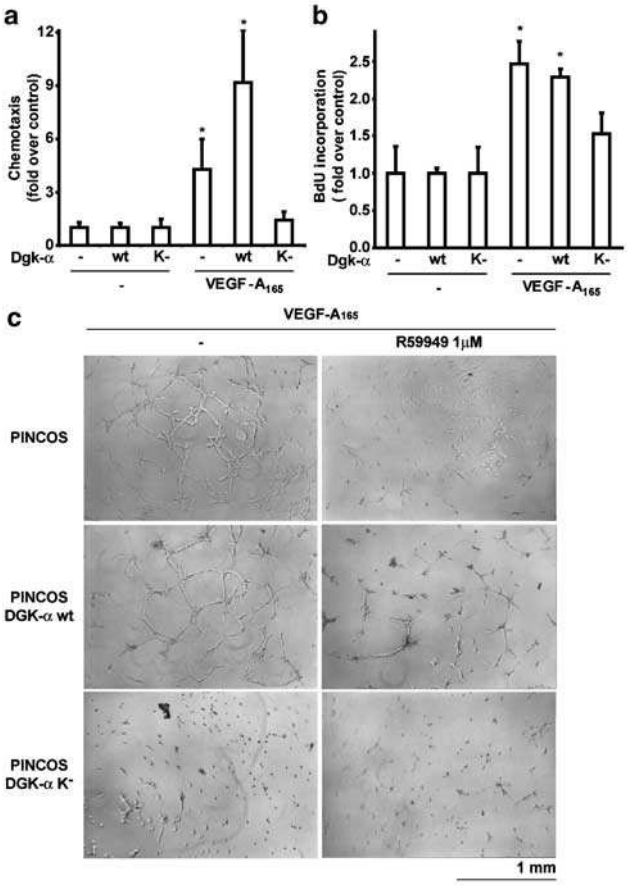

Figure 4 Expression of Dgk-α dominant negative inhibits VEGF-A_{165}-induced *in vitro* angiogenetic signaling. Quiescent PAE-KDR infected with either empty or Myc-Dgk-α-wt or Myc-Dgk-α-K^- virus were assayed for chemotaxis **(a)**, DNA synthesis **(b)** and network formation on matrigel **(c)** as follows. **(a)** Cells were plated in a modified Boyden chamber coated with collagen I and induced to migrate with 20 ng/ml of VEGF-A_{165} for 6 h. Data are shown as fold increase over control; each value is the mean ± s.e. of six points (**t*-test, $P<0.01\%$). **(b)** Cells were cultured in 96-well plates in the presence or absence of VEGF-A_{165} (10 ng/ml, absence of serum, 24 h). BrdU incorporation assay was carried out as described. Data are shown as fold increase over control; each value is the mean ± s.e. of eight points (**t*-test, $P<0.01\%$). **(c)** Cells were plated on growth factor-rich matrigel, and stimulated with VEGF-A_{165} (25 ng/ml) for 24 h, in the presence or absence of 1 μM R59949. Cells were photographed with contrast phase microscopy

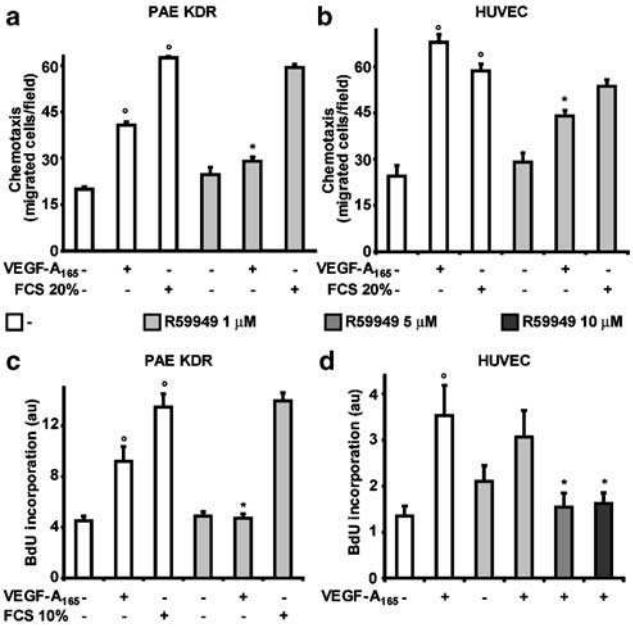

Figure 5 R59949 5 R59949 inhibits VEGF-A-induced cell motility and proliferation of PAE-KDR and HUVEC. Quiescent PAE-KDR (**a,c**) and HUVEC (**b,d**) were assayed for chemotaxis **(a,b)** and DNA synthesis **(c,d)** as follows: quiescent PAE-KDR **(a)** or HUVEC **(b)** were plated in a modified Boyden chamber coated with gelatin and induced to migrate in the presence of either 10 ng/ml **(a)** or 20 ng/ml **(b)** of VEGF-A_{165} or 20% FCS for 6 h, in the presence or absence of 1 μM R59949. Each value is the mean ± s.e. of triplicates (°*t*-test *vs* control, **t*-test *vs* VEGF-A_{165} stimulated, $P<0.01\%$). Quiescent PAE-KDR **(c)** or HUVEC **(d)** were cultured in 96-wells plates for either 24 h **(c)** or 48 h **(d)** with or without VEGF-A_{165} (**c**) 25 ng/ml in the absence of serum, (**d**) 10 ng/ml, 2% serum) or 10% FCS and in the presence or absence of R59949 at the indicated concentrations. BrdU incorporation was assayed as described. Each value is the mean ± s.e. of eight points (°*t*-test *vs* control, **t*-test *vs* VEGF-A_{165} stimulated, $P<0.01\%$)

To further investigate the involvement of Dgk-α in VEGF-A_{165} angiogenic signaling, we have verified whether the expression of Dgk dominant-negative mutant impairs the ability of endothelial cells to form tubular-like structures on matrigel, a mixture of laminin-rich basement membrane enriched in growth factors. In this assay, which is currently used to test *in vitro* pro- or antiangiogenic properties of molecules, endothelial cells are stimulated to migrate and to organize themselves in a chord structure network (Benelli and Albini, 1999). PAE-KDR cells infected with empty PINCOS vector or expressing Dgk-wt once plated on matrigel in the presence of VEGF-A_{165} (10 ng/ml) elongate and connect through chord-like structures to generate a complex network. However, PAE-KDR expressing Dgk-K^-, although still viable, fail to connect and to differentiate in chord-like structures, resulting in

a severe impairment of the formation of the characteristic network (Figure 4c).

Pharmacological inhibition of Dgk-α impairs in vitro VEGF-A_{165}-induced angiogenesis

Based on the sensitivity of Dgk-α to the inhibition by micromolar concentrations of R59949, we assayed its ability to inhibit VEGF-A_{165}-induced biological responses in both HUVEC and PAE-KDR cells. Indeed we had previously shown that R59949 inhibition of HGF-induced chemotaxis was completely reverted by overexpression of Dgk-α, providing a strong indication for the specificity of R59949-induced inhibition (Cutrupi *et al*., 2000).

In the presence of 1 μM R59949, VEGF-A_{165}-stimulated cell migration was inhibited by about 50 and 60%, respectively, in PAE-KDR and HUVEC, while basal cell motility of unstimulated cells was not significantly affected. However, in both PAE-KDR and HUVEC, R59949 did not inhibit cell migration induced by serum, suggesting that Dgk-α stands in the signaling pathway conveying signals specifically from the VEGFR-2 to the cell motility machinery (Figure 5a and b).

We then investigated the ability of R59949 to inhibit VEGF-A_{165}-induced DNA synthesis in both PAE-KDR and HUVEC. In serum-starved PAE-KDR, VEGF-A_{165} induced a twofold increase of DNA synthesis, which was completely abolished in the presence of 1 μM R59949 (Figure 5c). In HUVEC cultured in 2% FCS, VEGF-A_{165} stimulates DNA synthesis, which was inhibited by R59949 at concentrations equal or higher than 5 μM (Figure 5d). The reduced ability of R59949 to inhibit DNA synthesis in HUVEC may depend on serum sequestration of R59949 (De Chaffoy de Courcelles *et al*., 1989), although the involvement of other Dgk isoforms, less sensitive to the inhibitor (Jiang *et al*., 2000), could not be ruled out.

In addition, we have assayed the ability of R59949 to inhibit tubular-like structure formation on matrigel both by HUVEC and PAE-KDR-PINCOS. In the presence of 1 μM R59949, both PAE-KDR-PINCOS and HUVEC, although still viable, fail to connect and to differentiate in chord-like structures, resulting in a severe impairment of the formation of the characteristic complex network (Figures 4c, 6a and b). Cell viability in the presence of R59949 was verified by three distinct assays, Trypan blue, calcein-AM and MTT, reflecting three different cellular functions, respectively membrane impermeability, cytoplasmic esterase activity and mitochondrial respiratory chain activity (Poole *et al*., 1993; Baldanzi *et al*., 2002). R59949 treatment of HUVEC did not affect their viability as measured by calcein-AM and MTT (Figure 6a and b), while drug treatement of PAE-KDR cells did not affect cell viability as measured by Trypan blue and MTT (Figure3b and data not shown).

Interestingly, the specificity of R59949 in impairing angiogenic signaling through inhibition of Dgk-α is further suggested by the observation that in PAE-KDR overexpression of Dgk-wt reverts significantly the inhibition of chord-like formation on matrigel

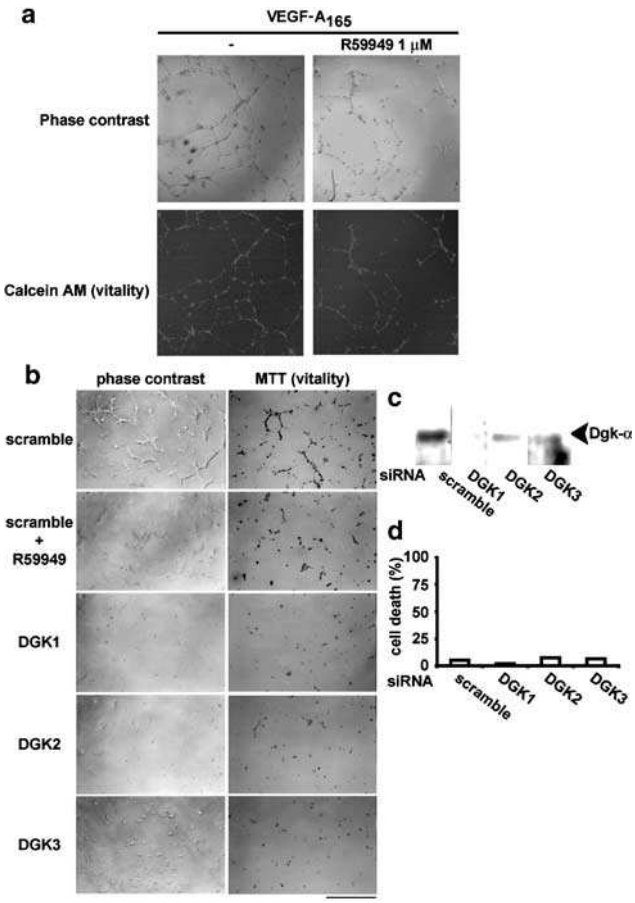

Figure 6 Downregulation of Dgk-α by RNA interference or R59949 treatment inhibits VEGF-A_{165}-induced *in vitro* angiogenesis in HUVEC. **(a)** HUVEC were plated on a growth factor rich matrigel and stimulated with VEGF-A_{165} (25 ng/ml) for 24 h in the presence or absence of 1 μM R59949. Cells were loaded with Calcein-AM fluorescent viability dye and photographed with contrast phase microscopy (upper panel) and green fluorescence (lower panel). **(b)** siRNA-transfected HUVEC were plated at 48 h following transfection on a growth factor rich matrigel for 24 h in the presence or absence of 1 μM R59949. Following image acquisition at contrast phase microscopy (left), cells were labeled with MTT vital dye and photographed with bright field microscopy (right). **(c)** siRNA-transfected HUVEC were homogenized, at 48 h from transfection, and their Dgk-α content was assayed by Western blot with anti-Dgk-α antibodies. **(d)** Cell death, quantified by Trypan blue exclusion assay, of siRNA-transfected HUVEC, 70 h following transfection

(Figure 4c). This finding is consistent with the reported ability of Dgk-α overexpression to revert the inhibition of HGF-induced chemotaxis exerted by R59949 (Cutrupi *et al*., 2000).

In addition, we have also verified whether R59949 impairs VEGF signaling through downregulation of VEGFR-2 tyrosine phosphorylation. However, VEGFR-2 tyrosine phosphorylation, as detected by antiphosphotyrosine Western blot of anti-VEGFR-2 immunoprecipitates, was not affected by cell treatment with 1 μM R59949 in VEGF-stimulated HUVEC (Figure 3a).

Downregulation of Dgk-α expression by RNA interference impairs HUVEC endothelial cell organization on matrigel

In order to provide further evidence that Dgk-α is involved in VEGF angiogenic signaling in HUVEC, we synthesized three single interfering RNAs for the Dgk-α transcript designed with Cenix algorithm, which did not overlap with any other known transcript from the human genome. Transfection of HUVEC cells with any of the three interfering RNAs lowers but does not abolish the expression of Dgk-α, as detected by Western blot on whole-cell lysates (Figure 6c). Transfection with the three interfering RNAs impairs the formation of chord-like structures on matrigel with efficacy similar to that obtained with 1 μM R59949 cell treatment. Conversely, transfection with a scrambled siRNA, which does not overlap with any known transcript, did not affect chord-like formation. In addition, downregulation of Dgk-α expression did not affect cell viability of HUVEC, as measured by Trypan blue exclusion (Figure 6d) and MTT (Figure 6b).

In summary, these data indicate that either inhibition of Dgk-α catalytic activity by both expression of dominant-negative mutant or cell treatment with a specific inhibitor, either downregulation of its expression by interfering RNA, severely impair the ability of endothelial cells to migrate, proliferate and organize as chord-like networks, providing further support to the claim that catalytic function of Dgk-α is required for the transduction of VEGF-A_{165} angiogenic signal.

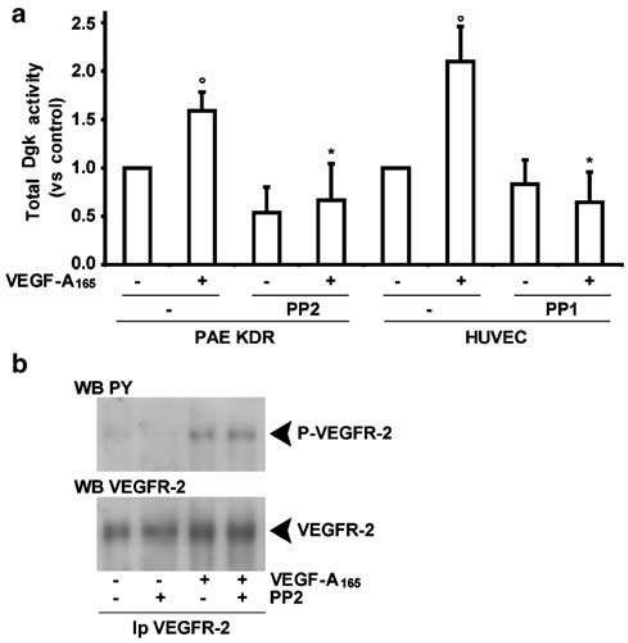

Figure 7 Src kinase activity is required for VEGF-A_{165}-induced activation of Dgk. **(a)** Dgk activity assayed in homogenates from either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent PAE-KDR or HUVEC cells, pretreated for 15 min with either vehicle or with 5 μM PP2 or PP1 respectively (data are mean ± s.e.m. of five independent experiments normalized for control, °*t*-test *vs* control, **t*-test *vs* VEGF-A_{165}, $P<0.05$). **(b)** Antiphosphotyrosine (upper panel) and anti-VEGFR-2 (lower panel) Western blot of anti-VEGFR-2 immunoprecipitates obtained from cell lysates of either control or VEGF-A_{165} (10 ng/ml for 10 min)-stimulated quiescent HUVEC, pretreated with either vehicle or 5 μM PP2

VEGF-A_{165}-induced activation of Dgk-α is mediated by Src

Overall, these data demonstrate that VEGF-induced activation Dgk-α is required for VEGF angiogenic signaling in endothelial cells. We had previously shown that activation of Dgk-α by HGF is mediated by Src (Cutrupi *et al*., 2000), whose activation is also required for VEGF-A_{165}-induced cell motility, proliferation and increase of permeability (Abu-Ghazaleh *et al*., 2001; Eliceiri *et al*., 2002). Thus, we have explored the hypothesis that Dgk-α may stand as a downstream target of Src in VEGF-A signaling. Src tyrosine kinase activity was inhibited by pretreating both PAE-KDR and HUVEC, respectively, with 5 μM PP2 and PP1. At these concentrations, either PP1 or PP2 does not affect VEGFR-2 tyrosine kinase activity (Figure7b and data not shown) (Waltenberger *et al*., 1999; Abu-Ghazaleh *et al*., 2001). Inhibition of Src tyrosine kinase activity completely abrogated the stimulation of total Dgk activity induced by VEGF-A_{165}, demonstrating that Src mediates the activation of VEGF-A_{165}-induced stimulation of Dgk in both PAE-KDR and HUVEC (Figure 7a).

Then we investigated whether VEGF-A_{165} induces the formation of a Dgk-α/Src complex, by assaying Dgk activity co-purified with Src in anti-Src immunoprecipitates from either control or VEGF-A_{165}-stimulated PAE-KDR cells. Indeed, Dgk activity in anti-Src immunocomplexes from stimulated cells was higher than from control cells (Figure 8a, upper panel), while mock immunoprecipitates from VEGF-A_{165}-stimulated cells did not contain any Src protein and Dgk activity. No Dgk-α protein could be detected by Western blot in anti-Src immunoprecipitates, suggesting that the stoichiometry of Dgk-α association to Src is very low, below the level of sensitivity of the Dgk-α antibodies (data not shown). Indeed even upon HGF stimulation of PAE, only endogenous Dgk activity, but no endogenous Dgk-α protein, could be detected in a complex with Src (Cutrupi *et al*., 2000).

Thus in order to provide a more conclusive evidence for the formation of the complex between the two proteins, we have assayed the ability of myc-tagged Dgk-α to co-purify with Src in anti-Src immunoprecipitates. Myc-tagged Dgk-α was expressed in PAE-KDR cells by infection with PINCOS retrovirus containing myc-tagged Dgk-α, obtained as described previously (Cutrupi *et al*., 2000). Indeed in infected cells more myc-Dgk-α was detected by Western blot of anti-Src immunoprecipitates obtained from VEGF-A_{165}-stimulated rather than unstimulated cells (Figure 5b, upper panel). The amount of immunoprecipitated Src protein was not affected by VEGF-A_{165} stimulation, while no

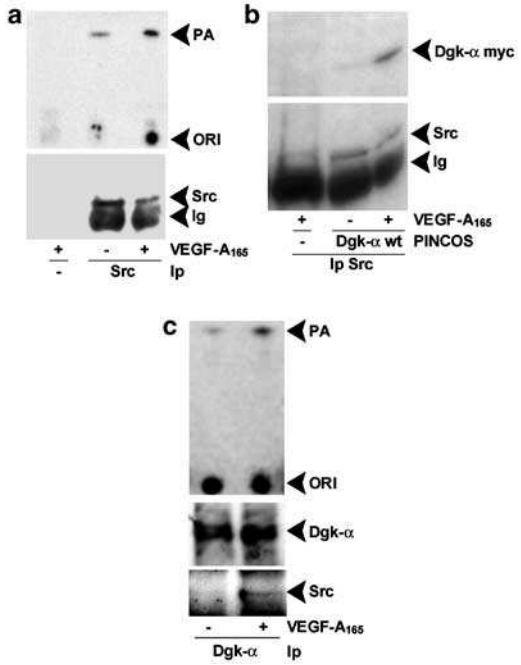

Figure 8 Dgk-α protein and activity are associated with Src in VEGF-A_{165}-stimulated cells. **(a)** Dgk enzymatic activity (upper panel) and Western blot of Src protein (lower panel) assayed in anti-Src immunoprecipitates obtained from lysates of either control or VEGF-A_{165} (50 ng/ml for 10 min)-stimulated quiescent PAE-KDR cells. (**b**) Myc-Dgk protein (upper panel) and Src protein (lower panel) assayed by Western blot, respectively, with anti-myc and anti-Src antibodies, of anti-Src immunoprecipitates obtained from lysates of VEGF-A_{165} (50 ng/ml for 10 min)-stimulated quiescent PAE-KDR infected with empty PINCOS vector or PINCOS/Myc-Dgk-α. (**c**) Dgk enzymatic activity (upper panel), Western blot of Dgk-α protein (central panel) and Western blot of Src protein (lower panel), assayed in anti-Src immunoprecipitates obtained from lysates of either control or VEGF-A_{165} (50 ng/ml for 10 min)-stimulated quiescent PAE-KDR cells

complex formation was observed in anti-Src immunoprecipitates from PAE-KDR cells infected with empty vector (Figure 8b, upper panel). In addition, we have been able the observe the formation of the Src/Dgk-α complex with endogenous proteins, by detecting Src protein in anti-Dgk-α immunoprecipitates from VEGF-A_{165}-stimulated but not unstimulated PAE-KDR cells (Figure 8c).

In summary, these data suggest that Src tyrosine kinase mediates the activation of Dgk by VEGF in both PAE-KDR and HUVEC. In addition, VEGF induces the formation of an Src/Dgk-α complex in PAE-KDR, while in the HUVEC the paucity of Dgk-α expression impairs to address the issue. Although we had previously shown that Dgk-α can be regulated by tyrosine phosphorylation, no tyrosine phosphorylation of Dgk-α could be detected upon VEGF-A_{165} stimulation, as well as upon HGF stimulation (Cutrupi *et al*., 2000).

No Dgk activity or protein, either endogenous or myc-tagged, was detectable in anti-VEGFR-2 immunoprecipitates (data not shown). Consistent with similar data obtained with HGF-R, these data suggest that Dgk-α does not form a stable complex with activated tyrosine kinase receptors (Cutrupi *et al*., 2000).

Discussion

Angiogenesis is a complex phenomenon required for embryonic development, wound healing, female cycle, solid tumor growth and revascularization of ischemic tissues (Matsumoto and Claesson-Welsh, 2001). Upon exposure to angiogenic factors, such as FGF, VEGF-A and HGF, endothelial cells lining blood vessels are activated in a spatially and timely coordinated manner to degrade the vessel basement membrane, invade the extracellular matrix according a chemotactic gradient, proliferate and differentiate to form a new lumen-containing vessel (Matsumoto and Claesson-Welsh, 2001; Gerhardt *et al*., 2003). Although the signaling pathways involved in the transduction of angiogenic stimuli have been extensively investigated, the complete picture of such signaling network is still missing.

We had previously shown that Dgk-α, which phosphorylates DG to PA, is activated by HGF through an Src-mediated mechanism, and is required for HGF-stimulated cell movement in a porcine aortic-derived cell line, raising the hypothesis that it may be involved in the transduction of the angiogenic signaling (Cutrupi *et al*., 2000). However, in order to provide more compelling evidence for the requirement of Dgk-α in the transduction of angiogenic signaling, we have investigated the role of Dgk-α in the signal transduction of VEGF-A, the major angiogenic factor *in vivo*, in both HUVEC primary endothelial cells and in PAE-KDR cells, an endothelial-derived cell line stably expressing VEGFR-2.

Herein we showed that VEGF-A_{165} stimulates Dgk activity in endothelial cells. In PAE-KDR cells, by assaying Dgk activity in anti-Dgk-α immunoprecipitates, we provided compelling evidence that Dgk-α isoform is activated by VEGF. In addition, most of the total Dgk activity assayed from PAE-KDR whole-cell homogenates is inhibited *in vitro* by R59949, which inhibits selectively class-I Dgk enzymes, that is, the α-, β- and γ- isoforms, while it does not affect the activity of other Dgk isoforms (Jiang *et al*. 2000), as well as the activity of other lipid and protein kinases (De Chaffoy de Courcelles *et al*., 1989; Cutrupi *et al*., 2000). The finding that overexpression of wt Dgk-α reverts the ability of R59949 to impair HGF-induced cell movement and network organization on matrigel, respectively, in PAE and PAE-KDR cells provides further support to the selectivity of R59949 for Dgk-α (Cutrupi *et al*., 2000; Figure 4c). Conversely, in HUVEC, which express lower levels of Dgk-α than PAE-KDR, VEGF-stimulated Dgk activity is only partially inhibited by R59949, suggesting that also R59949-resistant Dgk activities are activated by VEGF.

In order to investigate whether Dgk-α plays a role in VEGF angiogenic signaling, we have inhibited it by three independent strategies, that is, pharmacological inhibition with R59949 in both PAE-KDR and HUVEC, expression of a Dgk-α dominant-negative mutant in PAE-KDR cells and specific downregulation of Dgk-α expression by RNA interference in HUVEC. *In vitro*, the ability of endothelial cells to move toward a

chemotactic gradient, to proliferate and to organize in a network of chord-like structures on matrigel reflects their behavior during the angiogenic process *in vivo*.

In PAE-KDR cells, the inhibition by both expression of Dgk-α dominant-negative mutant, and cell treatment with 1 μM R59949 severely impairs VEGF-induced chemotaxis and DNA synthesis, as well as network formation on matrigel, without affecting cell viability. Thus, the data clearly demonstrate that in PAE-KDR cells Dgk-α is activated by VEGF and that its catalytic function is required for VEGF-induced angiogenic signaling *in vitro*.

In HUVEC, R59949, which inhibits part of the VEGF-stimulated Dgk activity, impairs VEGF-induced chemotaxis and DNA synthesis, as well as network organization on matrigel. The higher concentration of the drug required to impair DNA synthesis may further suggest that other R59949-resistant Dgk isoforms may be involved in the transduction of VEGF proliferative signaling in HUVEC. Alternatively, R59949 may be sequestered by the serum required to obtain HUVEC proliferation in cultures (De Chaffoy de Courcelles *et al*., 1989). However, more direct evidence that Dgk-α is required for *in vitro* angiogenesis in HUVEC primary endothelial cells comes from the demonstration that specific downregulation of Dgk-α expression by RNA interference totally impairs the ability of HUVEC to form networks on matrigel, without affecting cell viability. The same results were obtained with three different dsRNA, which target respectively two sequences at the N-terminal of the Dgk-α and a sequence next to the ATP-binding site. The three sequences do not feature any homology with other Dgk isoforms or other proteins.

In summary, these experiments strongly suggest that activation of Dgk-α is required for angiogenic signaling in both PAE and HUVEC endothelial cells, although the possibility that in HUVEC, which express a low level of the α-isoform, other Dgk isoform may also be involved in VEGF signaling should be taken into account.

Dgk-α does not contain any domain suggesting a direct interaction with tyrosine kinase receptors, and indeed does not associate with either VEGFR-2 or HGF-R (data not shown). However, a Dgk activity is upregulated upon expression of v-Src in fibroblasts (Sugimoto *et al*., 1984); we and others have recently shown that Src stimulates Dgk-α activity *in vitro* and mediates HGF- and IL-2-stimulated Dgk-α activation in intact cells (Cutrupi *et al*., 2000; Cipres *et al*., 2003). Here, we reported that in both PAE-KDR and HUVEC, the VEGF-induced stimulation of Dgk activity is blunted by cell treatment, respectively, with PP2 and PP1, two Src family-specific inhibitors that do not affect Dgk activity *in vitro* (data not shown). These data imply that even if other Dgk isoforms are also stimulated by VEGF in these cells, their activation is completely Src family kinase dependent. Indeed, very recent data indicate that Dgk-α associates with Src upon GnRH stimulation of pituitary cells (Davidson *et al*., 2004). In addition, in PAE-KDR cells, we were able to show that VEGF stimulation induces the formation of a complex between Dgk-α and Src, but not between Dgk-α and the activated receptor, as Dgk-α does not co-purify in antireceptor immunoprecipitates (data not shown). This may depend on the low stoichiometry of the Src/Dgk-α complex, that is, only a small fraction of receptor-associated Src forms a ternary complex with Dgk-α. Alternatively, receptor-activated Src may come off the complex with the receptor and may associate with Dgk-α. Indeed according to current models of its function, Src is recruited to the active receptor through its SH2 domain, switches to the active open conformation, allowing complex formation with its substrates, for instance p130Cas and FAK, their processive phosphorylation and establishing a more stable complex with them through its SH2 domain, excluding the receptor (Nakamoto *et al*., 1996; Thomas *et al*., 1998; Scott and Miller, 2000; Pellicena and Miller, 2001).

Although these data indicate Dgk-α as a putative substrate of Src tyrosine kinase activity, no tyrosine phosphorylation of Dgk-α could be detected upon VEGF-A stimulation of either PAE-KDR or HUVEC. However, tyrosine phosphorylation of Dgk-α was previously shown in PAE cells upon treatment with sodium pervanadate, and in COS cells upon coexpression of both Src and Dgk-α (Cutrupi *et al*., 2000). In both cases, tyrosine phosphorylation correlates with an increase of Dgk-α enzymatic activity. We speculate that either tyrosine phosphorylation of Dgk-α occurs at very low stoichiometry or is a transient event leading to the switch of Dgk-α to an active conformation. Alternatively, Src may induce tyrosine phosphorylation of a regulator of Dgk-α. Indeed recent findings indicate that $PI(3,4,5)P_3$, the lipid product of PI 3-kinase, binds and stimulates Dgk-α activity and that active Lck stimulates Dgk-α in a PI 3-kinase-dependent manner (Cipres *et al*., 2003). Therefore, it is likely that multiple mechanisms may participate in the regulation of Dgk-α. The identification of the molecular determinants of the physical and functional interactions between the two proteins will shed light on such mechanisms.

VEGF-A-induced angiogenesis, both *in vitro* and *in vivo*, requires Src function: Src is required for VEGF-A-induced chemotaxis and proliferation of endothelial cells *in vitro* (Abu-Ghazaleh *et al*., 2001), while genetic evidences *in vivo* indicate that deletion of either Src or Yes gene in mice impairs VEGF-dependent increase of endothelial permeability, that is, edema formation (Eliceiri *et al*., 2002). Furthermore, retroviral-mediated overexpression of Src dominant negative in a subcutaneous model of angiogenesis severely impairs neo-vascularization *in vivo* (Eliceiri *et al*., 1999). Thus, the demonstration that VEGF-induced stimulation of Dgk activity depends on Src activity, and that Dgk-α is required for VEGF-A-induced angiogenetic responses *in vitro* suggest that it may act as a crucial Src effector in VEGF angiogenic signaling in endothelial cells. In addition, at least in HUVEC, the involvement of other Src-regulated Dgk isoforms in VEGF signaling is not ruled out.

The signaling pathways downstream to Dgk-α have not been extensively investigated yet. Dgk enzymes phosphorylate DG to generate PA. Indeed VEGF-A stimulates PLC-γ-mediated hydrolysis of $PI(4,5)P_2$ to generate DG, which then leads to activation of PKC (Matsumoto and Claesson-Welsh, 2001). PKC-α and -β isoforms are required for VEGF-stimulated cell proliferation and migration *in vitro*, as well as for neovascularization *in vivo* (Wellner *et al*., 1999; Wang *et al*., 2002).

In some cell signaling system, activation of Dgk-α, which removes DG to generate PA, contributes to terminate DG-mediated signaling, while its inhibition results in the accumulation of DG, leading to sustained DG-mediated signaling. However, in endothelial cells, the increased activity of PKCα, which indeed is activated by VEGF-A in a DG-mediated manner, is expected to enhance rather than inhibit VEGF-A-induced angiogenic response (Wellner *et al*., 1999; Matsumoto and Claesson-Welsh, 2001; Wang *et al*., 2002). Conversely, the role of activation of Dgk-α in VEGF-A angiogenic signaling may consist in the generation of PA-mediated signals. The role of PA in VEGF-A signaling still remains to be investigated. *In vitro*, PA is a regulator of several signaling proteins, some of which are involved in VEGF-A signaling (English, 1996; Gomez-Cambronero and Keire, 1998; Topham and Prescott, 1999). For instance, VEGF-A activates PKCζ, which is positively regulated by PA and Src tyrosine phosphorylation (Limatola *et al*., 1994; Seibenhener *et al*., 1999). Similarly, VEGF-A activates Raf, which is regulated by PA *in vitro* and in intact cells (Andresen *et al*., 2002). Activation of both PKCζ and Raf is required for VEGF-induced angiogenic signaling (Matsumoto and Claesson-Welsh, 2001). Among other putative targets of PA *in vitro*, Rho-GDI and PI(4)P 5-kinase might play a role in VEGF-A signaling, respectively, by mediating Rac activation (Del Pozo *et al*., 2002; Zeng *et al*., 2002) and by participating in the dynamic recruitment of focal adhesion proteins required for cell movement (Jenkins *et al*., 1994; Di Paolo *et al*., 2002). In addition, both *in vitro* and in intact cells, PA also regulates activation of mTor, a serine kinase involved in the transduction of growth factor-stimulated proliferative signaling (Fang *et al*., 2001). However, the role of mTor in VEGF-A signaling has not been investigated. Alternatively, PA generated through activation of Dgk-α may be further metabolized to lysosphosphatidic acid (LPA), through a phospholipase A2. Then LPA, through an autocrine loop, may stimulate its receptor(s), edg.2 and -4, whose expression has been reported in endothelial cells (Panetti, 2002). Indeed, exogenously added LPA stimulates endothelial cell proliferation and cell matrix-adhesion dependent cell migration (Panetti, 2002). Interestingly, in prostate carcinoma, bombesin, a growth factor for these cells, has been shown to activate an LPA autocrine circuit, which is required for bombesin signaling (Xie *et al*., 2002). However, no evidence of growth factor-stimulated synthesis of LPA has been reported in endothelial cells.

Finally we provided evidence indicating that Dgk-α is an essential component of VEGF-A signal transduction, which is required to convey both chemotactic and proliferative signals downstream from Src. Because tumor cells produce a wide array of angiogenic factors, it has been suggested that successful strategies to block angiogenesis in cancer patients may repress the ability of endothelial cells to participate in the angiogenic process. As Dgk-α activity is required for angiogenesis and quite specific inhibitors are available, Dgk-α may represent a new and promising target for pharmaceutical control of angiogenesis.

Materials and methods

Cells culture, reagents and antibodies

Porcine aortic endothelial cell line (PAE), a cell line derived from porcine aortic endothelium, and PAE-KDR, a PAE-derived cell line stably expressing VEGFR-2, were a kind gift of Waltenberger (Waltenberger *et al*., 1994), and were cultured in Ham's F12 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies). Human endothelial cells from umbilical cord veins (HUVEC) were prepared and characterized as previously described, and were grown in M199 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 20% FCS (Life Technologies), endothelial cell growth factor (100 μg/ml) (Sigma Chemical Co., St Louis, MO, USA) and porcine heparin (Sigma) (100 μg/ml). HUVEC were used at second passage and grown on a plastic surface coated with porcine gelatin (Sigma), otherwise specified. Recombinant $VEGF\text{-}A_{165}$ was purchased from R&D systems, and R59949, PP1 and PP2 were from Alexis. Anti-Src antibodies (N-16 for immunoprecipitation and SRC-2 for Western blotting) were from Santa Cruz Biotechnology, and 4G10 antiphosphotyrosine and 9E10 anti-myc tag antibodies were from UBI. Anti-Dgk-α antibodies were a mix of monoclonal obtained as described (Schaap *et al*., 1993). Anti-VEGFR-2 antibodies were from Santa Cruz Biotechnologies. ECL and secondary antibodies were from Perkin-Elmer. PINCOS, PINCOS/Myc-Dgk-α, PINCOS/Myc-Dgk-α-K^- and the PHOENIX retroviral system were previously described (Cutrupi *et al*., 2000). Unless specified, all other reagents were from Sigma-Aldrich.

Infection with retroviral vectors

Phoenix cells obtained by G Nolan (Swift *et al*., 1999) were transiently transfected by calcium phosphate (Cell Phect Transfection kit, Amersham-Pharmacia) with 20 μg PINCOS, PINCOS/Myc-Dgk-α or 20 μg PINCOS/Myc-Dgk-α-K^- in growth media containing chloroquin (25 μM). Cells were incubated for 8 h at 37°C (5% CO_2) and the precipitate was removed by washing with PBS. Then, cells were incubated with DMEM containing 10% FCS for 72 h; the retroviral supernatant was collected, the debris removed by 1500 *g* centrifugation, filtered by a 0.8 pore filter, and supplemented with Polybrene (8 μg/ml). Log-phase growing PAE-KDR cells were infected by addition of 5 ml retroviral supernatant. Following 16 h from infection, cells were placed into 10% FCS medium for 48 h and serum starved overnight in 0.5% FCS prior to further experimental manipulations. PINCOS-infected cells express both GFP and the protein of interest. Efficiency of infection was about 70% as measured by FACS analysis of GFP expression.

Preparation of cell lysate, homogenates, immunoprecipitation and Western blotting

Subconfluent PAE-KDR cultures were made quiescent by 16 h serum starvation. Subconfluent HUVEC cultures were made quiescent by incubating for 16 h in 5% FCS. Following cell stimulation, the cell lysates were prepared in buffer A (25 mM Hepes pH 8, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM ammonium molibdate, supplied before use with 1 mM sodium orthovanadate, 10 mM NaF, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin, 1 μg/ml soybean trypsin inhibitor) supplemented with 1% NP-40, as described previously (Cutrupi *et al*., 2000). Where indicated, cell extracts were prepared by collecting the cells with a rubber scraper in buffer A and homogenizing them in syringe–needle in buffer A. Protein concentration was determined by the BCA method (Pierce). Immunoprecipitation, SDS–PAGE and Western blots were performed as described previously (Cutrupi *et al*., 2000).

Dgk-α assay

Dgk-α was assayed in the immunoprecipitates essentially as described (Schaap *et al*., 1993): immunocomplexes were incubated for 5 min at 30°C with 1 mg/ml diolein (Fluka), 1 mM ATP, 30 μCi-[γ^{32}P]ATP (Amersham), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM EGTA in 25 mM Hepes pH 8, and the reaction stopped with 50 μl of 1 M HCl and 100 μl of chloroform : methanol 1 : 1. Lipids were then extracted, and separated by TLC in chloroform : methanol : water : 25% ammonium hydroxide (60 : 47 : 11 : 4). TLC was exposed on a autoradiographic film (Kodak Biomax), [^{32}P]PA was identified by co-migration with nonradioactive PA standards stained by incubation in an iodine chamber. PA spots were quantified using phosphor imager (Biorad). Dgk activity in homogenates (1–5 μl) was assayed as described above, except that ATP was 5 mM.

Cell migration assay

For migration on gelatin, endothelial cells were seeded (1×10^5 cells in 50 μl suspension) in the upper chamber of a modified Boyden chamber. The undersurface of PVDF filter (8 μm pores, Nucleopore) was coated with 0.1% gelatin. The lower chamber was filled with serum-free medium with or without VEGF-A_{165} and incubated at 37°C in air with 5% CO_2 for 6 h. Where indicated, 1 μM R59949 was added to the upper chamber with the cells. Filters were then removed, stained with Diff-Quik (Baxter Diagnostic AG) and the cells of five fields were counted at the inverted microscope with a high-power oil immersion objective (Zeiss). For migration on collagen I, endothelial cells were seeded (1×10^5 cells in 50 μl suspension) in the upper chamber of a chemotox chamber (Nuroprobe), coated with 20 ng/ml collagen I. The lower chamber was filled with serum-free medium with or without VEGF-A_{165} and incubated at 37°C in air with 5% CO_2 for 6 h. Filters were then removed, stained with crystal violet and the cells of five fields were counted at the inverted microscope (Zeiss axiovert).

Cell proliferation assay

PAE KDR cells were plated and after 6 h treated as indicated for 24 h. HUVEC cells were plated and after 24 h treated as indicated for additional 48 h. BrdU incorporation was measured with 'Cell Proliferation ELISA Biotrak System' from Amersham-Pharmacia as suggested by the manufacturer.

Matrigel morphogenetic assay

Matrigel (Collaborative Biomedical Products, Becton Dickinson, Milan, Italy; not purified from contaminant growth factors; 0.2 ml). was added to each well of a 48-well plate and incubated at 37°C for 30 min to allow gel formation. Endothelial cells (2×10^4/well) were plated onto Matrigel. After 48 h incubation in the presence or absence of the indicated treatments in 5% CO_2 humidified atmosphere at 37°C, the three-dimensional organization of the cells was examined under an inverted phase contrast photomicroscope (DM-IBM model; Leica Microsystems, Wetzlar, Germany) and then photographed. Not purified matrigel allows spontaneous *in vitro* angiogenesis (Marconcini *et al*., 1999).

RNA interference

HUVEC were transfected with siPORT lipid transfection agent (Ambion) as suggested by the manufacturer. Cell transfection procedures must be optimized for each HUVEC preparation using Silencer siRNA Transfection KIT (Ambion). siRNA used were chemically synthesized as double-strand RNA (Ambion). The sequence was designed using Cenix bioscience algorithm, which considers T_m, nucleotide content of the 3′ overhangs, nucleotide distribution over the length of the siRNA, and presence and location of mismatches to off target genes. A key step in the algorithm is a stringent analysis of each siRNA sequence to maximize the target specificity. Sequences were as follows: DGK1 sense GGUCA GUGAUGUCCUAAAGTT, antisense CUUUAGGACAU CACUGACCTT (target AAGGTCAGTGATGTCCTAAAG); DGK2 sense GGAUGGCGAGAUGGCUAAATT, antisense UUUAGCCAUCUCGCCAUCCTC (target AAGGATGGC GAGATGGCTAAA); and DGK3 sense GGAUUUAGA-GAUGAGUAAATT, antisense UUUACUCAUCUCUAAA UCCTT (target AAGGATTTAGAGATGAGTAAA). A GA PDH scramble siRNA was used as negative control (provided with Silencer siRNA Transfection KIT from Ambion).

Reproducibility and data analysis

All the experiments shown are representative of three or more independent experiments with similar results. In Figures 3c and 7a, data are mean ± s.e.m. of five independent experiments normalized for control.

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Activation of α-diacylglycerol kinase is critical for the mitogenic properties of anaplastic lymphoma kinase

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Oncogenic rearrangements of the tyrosine kinase receptor anaplastic lymphoma kinase (ALK), most commonly represented by the nucleophosmin/ALK fusion protein (NPM/ALK), are involved in the pathogenesis of anaplastic large-cell lymphomas (ALCLs). In an effort to identify new intracellular transducers operative in ALK-positive malignancies, we have investigated the potential involvement of diacylglycerol kinase (DGK). Here we show that αDGK is constitutively activated in the NPM/ALK-positive ALCL-derived cell line Karpas 299 and in NPM/ALK-infected 32D hematopoietic cells. These results were further validated in fibroblastic NIH-3T3 cells expressing a previously described chimeric epidermal growth factor receptor (EGFR)/ALK molecule that allows dissection of ALK enzymatic function under conditions of controlled ligand-induced activation. In this cell system, we also show that ALK-mediated αDGK activation is dependent on p60^{src} tyrosine kinase, with which αDGK forms a complex. The specific inhibition of αDGK, obtained by cell treatment with R59949, significantly reduced cellular growth in all cell lines. This result was further confirmed in Karpas 299 cells following specific down-regulation of αDGK by RNA interference. Overall, our data indicate that αDGK activation is involved in the control of ALK-mediated mitogenic properties. (Blood. 2005;106: 2175-2182)

Introduction

The anaplastic lymphoma kinase (*ALK*) gene, localized on chromosome 2p23, encodes for a large, glycosylated 200-kDa membrane-spanning tyrosine kinase receptor whose expression is highly tissue specific, being restricted to components of the nervous system; structural homology studies place ALK within the family of insulin receptor tyrosine kinases (RTKs), with the highest homology to the leukocyte tyrosine kinase (LTK).[1,2] Involvement of ALK in the pathogenesis of hematopoietic malignancies derives from the observation that constitutively active forms of ALK are detected with a high frequency in anaplastic large-cell lymphomas (ALCLs), a subgroup of non-Hodgkin lymphomas, predominantly of T or null type.[3] The oncogenic forms of ALK are the result of somatic chromosome translocations that fuse the ALK cytoplasmic domain to the 5′ region from different partner genes. The most frequent oncogenic version of ALK is represented by nucleophosmin (NPM)/ALK, an 80-kDa hybrid protein created by the t(2;5)(p23; q35) rearrangement.[4,5] The tumorigenic properties of NPM/ALK have been demonstrated in vitro in different cell systems[6-9] and confirmed in vivo by the generation of NPM/ALK-mediated tumor models.[10-13]

Despite several studies, the pathogenic mechanisms leading to ALK-mediated transformation remain still poorly defined. Thus far, several signaling molecules have been identified that associate and/or are activated by ALK, including growth factor receptor–bound protein 2 (Grb2), Src homology and collagen (Shc), insulin receptor substrate-1 (IRS-1), phospholipase C–γ (PLC-γ), p60^{src}, and phosphatidylinositol 3-kinase (PI3-K), although only a few have been shown to be strictly specific for ALK's transforming potential.[3] In particular, several lines of investigation have indicated that both PLC-γ and PI3-K are critical transducers of NPM/ALK-mediated oncogenesis through activation of mitogenic and/or survival signals,[8,14-15] while the exact role of the ras/mitogen activated protein kinase (MAPK) cascade needs further evaluation.[3] The contribution of p60^{src} has been recently evaluated in NPM/ALK-positive cell lines and demonstrated through the effects of p60^{src} down-regulation and pharmacologic inhibition on cellular proliferative rate.[16] Additional relevant effectors of NPM/ALK-mediated lymphomagenesis are represented by signal transducer and activator of transcription 3 (Stat3) and Stat5.[17]

Obviously, further investigations are needed to identify other pathways that are operative in ALK-positive malignancies and that could represent intracellular targets for a more appropriated therapeutic intervention in ALCL treatment.

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Diacylglycerol kinase (DGK) has attracted much attention in recent years since growing evidence indicates its direct involvement in the regulation of signal transduction processes. The family of mammalian DGKs comprises 9 isoenzymes classified in 5 distinct groups on the basis of their primary structure.[18] In particular, type I DGKs include α-, β-, and γ-isoforms. αDGK is abundant in the cytosol of T lymphocytes,[19] but it is also expressed in endothelial and epithelial cells, fibroblasts, and oligodendrocytes.[20-22]

It has been reported that αDGK activity is required for interleukin-2 (IL-2)–induced G_1- to S-phase transition in T cells.[23-24] In addition, more recent studies in nonlymphoid cells have highlighted the importance of αDGK activation for the transduction of migratory signals mediated by the hepatocyte growth factor receptor (HGFR)[21] and for vascular endothelial growth factor receptor-2 (VEGFR-2)–induced angiogenesis.[22] In these cell systems, it has also been demonstrated that receptor-mediated αDGK activation takes place through a $p60^{src}$-dependent mechanism, since it requires $p60^{src}$-tyrosine kinase activity and involves the formation of a $p60^{src}$/αDGK complex.[21-22]

On the basis of these data and on the requirement of $p60^{src}$ activation in NPM/ALK-induced signaling,[16] we sought to analyze the role of αDGK in ALK-mediated mitogenic properties. Here we report that the αDGK catalytic function is up-regulated by activated ALK through a $p60^{src}$-dependent mechanism and is critical for the proliferation of NPM/ALK-positive cell lines. These data indicate that αDGK is a downstream target of ALK signaling and suggest that the inhibition of its activity can interfere with NPM/ALK function in ALCL cells.

Materials and methods

Retroviral construct and infection

The NPM/ALK retroviral construct was obtained by cloning the open-reading frame (ORF) of pcDNA3-NPM/ALK into the PINCO vector encoding the enhanced green fluorescent protein (GFP), as previously described.[25] Recombinant polymerase chain reaction (PCR) was used to amplify the entire NPM/ALK sequence[4] using primers designed to yield the *NPM/ALK* cDNA flagged by unique *Eco*RI site at both ends of PCR products. The sequence of the primers used was 5′ primer GAATTCATGGAAGATTCGATGGACATGG and 3′ primer GAATTCCTCAGGGCCCAGGCTGG. Following *Eco*RI digestion, the PCR fragment was cloned into the *Eco*RI site of the PINCO retroviral vector. To infect 32D cells, the cells were cultured for 3 hours in the presence of 0.45 μM filtered viral supernatant collected from Phoenix cells 2 days after transfection with PINCO or PINCO-NPM/ALK vectors.[25] Two infection cycles were performed in the presence of 5 μg/mL polybrene (Sigma, St Louis, MO). GFP-positive infected cells were sorted following standard procedures by a fluorescence-activated cell sorter (FACS) scan (FACS Vantage; Becton Dickinson, Omaha, CA) with an excitation wavelength of 488 nm.

Cell lines and transfections

The murine growth factor–dependent 32D parental cell line and 32D cells infected with empty virus were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS; Invitrogen, Paisley, United Kingdom) and 10% WEHI-conditioned medium as a source of IL-3. NPM/ALK-infected 32D cells, the human NPM/ALK-negative T lymphoblastoid CEM cell line, the human NPM/ALK-positive Karpas 299 (kindly provided by B. Falini, University of Perugia, Italy), and TS (kindly provided by R. Piva, University of Turin, Italy) cell lines were cultured in RPMI 1640 containing 10% FBS. Genetically engineered NIH-3T3 cells overexpressing the chimeric epidermal growth factor receptor (EGFR)–ALK receptor (NIH-EGFR/ALK) were described previously[26] and maintained in Dulbecco modified Eagle medium (D-MEM; Invitrogen) containing 10% FBS. For epidermal growth factor (EGF) triggering experiments, subconfluent NIH-EGFR/ALK cell monolayers, grown in poly-L-lysine–coated dishes, were incubated for 18 hours in D-MEM medium supplemented with transferrin (5 μg/mL; Becton Dickinson, Franklin Lakes, NJ) and Na_2SeO_3 (10^{-8} M; Sigma) in the absence of serum (starvation conditions) before EGF treatment (Upstate Biotechnology, Lake Placid, NY). When indicated, 1 to 10 μM R59949 or 1 μM PP2 ([4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-di] pyrimidine]; both from Alexis Biochemicals, San Diego, CA) were added to the culture medium 30 minutes to 1 hour before the addition of EGF and maintained at the same concentration throughout the experiments. Stock solutions of the inhibitors were prepared in dimethyl sulfoxide (DMSO) and diluted so that the final concentration in culture medium never exceeded 0.02%.

In some experiments, NIH-EGFR/ALK cells were transiently transfected with pMT2-myc-αDGK expression vector[21] by the calcium-phosphate precipitation technique. Thirty-six hours from transfection, cells were cultured overnight under starvation conditions and then EGF stimulated.

Cell lysis and protein analysis

Total cell lysates were prepared at 4°C by using 1% Triton X-100 lysis buffer as previously described,[26] unless otherwise specified. For immunoprecipitation procedures, cellular lysates were incubated with the indicated antibody for at least 2 hours at 4°C and immunocomplexes were recovered by adsorption to Protein-G Sepharose beads (Amersham Biosciences, Piscataway, NJ). Lysates or immunocomplexes were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose filters, and immunoblotted according to previously described procedures.[26-27] Bound proteins were visualized with [^{125}I]-labeled anti–mouse immunoglobulin G (IgG, 0.2 μCi [0.0074 MBq]/mL; Amersham Biosciences) or by enhanced chemiluminescence (ECL) following incubation with the appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences).

The monoclonal antibody recognizing the intracellular domain of ALK[28] was kindly provided by B. Falini (University of Perugia, Italy) and P. G. Pellicci (European Institute of Oncology, Milan, Italy). A commercially available anti-$p60^{src}$ monoclonal antibody (clone GD11; Upstate Biotechnology) was used. The mouse monoclonal antibody, clone 9E10, recognizing the c-myc carboxy terminal domain (residues 408-439) was purchased either from Sigma or from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-αDGK antibodies were a mixture of monoclonal antibodies obtained as described.[29] The mouse monoclonal anti–α-tubulin (clone B-5-1-1) was from Sigma. Ab-1, the monoclonal antibody directed against the extracellular domain of EGFR, was purchased from Calbiochem (San Diego, CA).

The pGEX-$p60^{src}$/Src homology 2 (SH2) vector, used for the in vitro association experiments described in Figure 5A, was kindly provided by R. M. Melillo (Department of Cellular and Molecular Biology and Pathology, University Federico II, Naples, Italy) and G. Superti-Furga (Cellzome, Heidelberg, Germany). Glutathione S-transferase (GST) fusion proteins were induced in log-phase growing bacteria upon treatment with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 hours. Bacteria were recovered by centrifugation, resuspended in 1/100 volume lysis buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 8.0, 500 mM NaCl, 0.5% nonidet P-40 [NP-40], 1 mM EDTA [ethylenediaminetetraacetic acid], 1 mM dithiothreitol [DTT], 2 mg/mL lysozyme, 2 mM phenylmethylsulfonylfluoride [PMSF], and 50 μg/mL aprotinin) and lysed on ice by sonication. Lysates were clarified by centrifugation and the resulting supernatant was incubated with glutathione-agarose (Amersham Biosciences) overnight at 4°C with gentle rotation. The resin was washed with excess phosphate-buffered saline (PBS) and then resuspended in lysis buffer before storing at −70°C. For in vitro binding assays, cell lysates (2 mg) were incubated with 2 μg recombinant protein immobilized onto glutathione-agarose for 1.5 hours at 4°C under gentle rotation. After several washes, proteins were eluted in SDS-PAGE sample buffer and analyzed by immunoblotting.

p60^{src} kinase assay

Following in vivo treatment with 100 ng/mL EGF for the indicated time, NIH-EGFR/ALK cells were lysed with Staph A buffer (10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, and 50 μg/mL aprotinin) and immunoprecipitated with the indicated antibody. Half of each sample was analyzed directly by Western blot; the other was subjected to in vitro kinase assay. To this end, immunocomplexes were resuspended in 50 μL kinase buffer (0.1% Triton X-100, 6 mM $MnCl_2$, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid], pH 7.5) containing 10 μCi (0.37 MBq) [γ-^{32}P] adenosine triphosphate (ATP, ~ 3000 Ci [111 000 GBq]/nmol; Amersham Biosciences) and 5 μg acid-denaturated rabbit muscle enolase (Sigma)[30] for 10 minutes at room temperature. Kinase reactions were stopped by adding SDS-PAGE sample buffer; samples were then analyzed directly by SDS-PAGE and autoradiography. Spots were quantified using a PhosphorImager apparatus (Molecular Dynamics, Sunnyvale, CA), and background was subtracted from each sample using the local median algorithm from the Image Quant program (version 1.2; Molecular Dynamics).

DGK assay

Immunocomplexes, obtained as described, were washed once with buffer containing 0.1% Triton X-100, 20 mM HEPES (pH 7.5), 10% glycerol, and 150 mM NaCl; once with 0.5 M LiCl and 25 mM Tris, pH 8; and twice with TNE (25 mM Tris, 150 mM NaCl and 1 mM EDTA, pH 8). Immunocomplexes or cellular extracts (10 μg) were incubated for 10 minutes at 30°C with 0.45 μg/μL diolein (Sigma), 1 mM ATP, 30 μCi (1.110 MBq) [γ-^{32}P]-ATP (Amersham Biosciences), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, and 1 mM Na_3VO_4 in 25 mM HEPES, pH 8. Reactions were stopped and lipids extracted by adding 1 N HCl and chloroform-methanol (1:1). Phosphatidic acid (PA) was separated by thin-layer chromatography (TLC) in chloroform–methanol–water–25% ammonium hydroxide (60:47:6:6), followed by exposure to autoradiographic film (Kodak, Rochester, NY). [^{32}P]-PA was identified by comigration with nonradioactive PA standards (Sigma) stained by incubation in a iodine chamber. PA spots were quantified using a PhosphorImager apparatus (Molecular Dynamics). Background was subtracted from each sample as indicated in the previous section.

Cell proliferation assay

[^{3}H]-thymidine incorporation assay was performed as previously described.[26] For MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) proliferation assay, cell lines were seeded into 96-well plates at a concentration of 2×10^4 cells/well in 100 μL RPMI supplemented with 0.1% FCS and 0.2 mg/mL bovine serum albumin (BSA) in the presence of 10 μM R59949 or DMSO as control. After 24 or 48 hours, a volume of 15 μL MTT dye (Sigma) was added to each culture. Reaction was stopped following 3 hours of incubation at 37°C by adding to each well 150 μL of a solution containing 50% dimethyl formamide and 10% SDS. The absorbance of converted water-insoluble MTT-formazan was measured at a wavelength of 540 nm. Bromodeoxyuridine (BrdU) incorporation was measured by the Cell Proliferation ELISA Biotrak System (Amersham Biosciences) according to the manufacturer's directions.

RNA interference

Karpas 299 cells were transfected by electroporation with siPORT siRNA Electroporation Buffer (Ambion, Austin, TX) using a Gene pulser apparatus (Bio-Rad Laboratories, Hercules, CA) as suggested by the manufacturer. siRNAs used were chemically synthesized as double-strand RNA (Ambion). An interfering RNA, which has been previously shown to efficiently down-regulate the expression of human αDGK in endothelial cells,[22] was used. Sequences were as follows: sense, GGAUUUAGAGAUGAGUAAATT and antisense, UUUACUCAUCUCUAAAUCCTT (target AAGGATTTAGAGATGAGTAAA); a scramble siRNA was used as negative control.

Results

Coupling of ALK with DG kinase activity

In vitro DGK activity was initially evaluated in whole-cell lysates obtained from the human NPM/ALK$^+$ ALCL-derived cell line Karpas 299. DGK activity was determined by quantifying the in vitro phosphorylation of exogenously added diacylglycerol (DG) to generate [^{32}P]-PA. As shown in Figure 1A (left panel), this cell line revealed a high constitutive DGK activity. Similar results were observed in the murine hematopoietic 32D cell line infected with a retrovirus expressing a GFP-NPM/ALK fusion protein (32D-NPM/ALK). 32D is a committed-undifferentiated cell line, absolutely dependent for growth and survival on IL-3. As it has been previously shown for other growth factor–dependent hematopoietic cell lines,[8] the expression of NPM/ALK in 32D cells is sufficient to induce a growth factor–independent phenotype, a marker of cellular transformation (data not shown). In 32D-NPM/ALK cells, the total DGK activity was significantly higher compared with that measured in the parental 32D cells infected with the empty vector, indicating that a DGK activity could be directly related to the expression of NPM/ALK (Figure 1A).

In addition, pretreatment of both cell lines with 10 μM R59949, a specific inhibitor of DGK type I,[31] resulted in a dramatic decrease of radioactive PA (approximately 70% of the control value), suggesting that the observed DGK activity was mainly due to the enzymatic action of the α-isoform (Figure 1A). In order to demonstrate more directly the activation of the α-isoform, the in vitro DGK assay was performed in anti-αDGK immunocomplexes obtained from Karpas 299 cells or CEM, a NPM/ALK-negative human T-lymphoblast cell line. Both cell lines express αDGK at similar levels, but DGK activity in anti-αDGK immunoprecipitates from Karpas 299 was considerably higher than from CEM cells (Figure 1B, left panel). Similar results were observed in an additional human NPM/ALK-positive cell line derived from a different patient[32] (Figure 1B, right panel).

To further validate our findings, we took advantage of a previously described model system represented by a chimeric

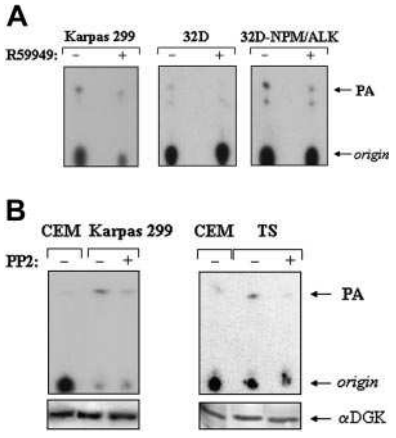

Figure 1. αDGK activity in NPM/ALK-positive cells. Cell lines were cultured for 30 minutes to 1 hour at 37°C in the absence (−) or presence (+) of either 10 μM R59949 or 1 μM PP2 and lysed thereafter. Total cell lysates (A) or anti-αDGK immunocomplexes (B, upper panel) were analyzed for in vitro DGK enzymatic activity in the presence of [γ-^{32}P] ATP and diolein as exogen substrate; lipid phase was separated by TLC. An aliquot from each anti-αDGK immunoprecipitate was analyzed by SDS-PAGE to ensure that equal amounts of αDGK were present during in vitro kinase assay (B, bottom panel). PA indicates phosphatidic acid.

molecule encompassing the extracellular and transmembrane domain of the EGFR and the entire cytoplasmic portion of ALK. We have previously shown that in this chimera the biochemical and biologic properties of ALK are controlled by EGF stimulation, thus making it possible to dissect ALK enzymatic function under condition of controlled ligand-induced activation.[26] Therefore, fibroblastic NIH-3T3 cells expressing the chimera EGFR/ALK (NIH-EGFR/ALK) were cultured at 37°C for different time periods in the absence or presence of EGF prior to lysis and subsequent in vitro DGK assay on total homogenate. As shown in Figure 2, an increase in total DGK activity was observed after EGF treatment, further supporting a direct correlation between ALK activation and stimulation of a cellular DGK enzymatic function. The effect was rapid, reached a maximum in 10 minutes (about 2-fold over control), and went back to the basal level within 30 minutes.

Similarly to Karpas 299 and 32D-NPM/ALK cells, the DGK activity observed in EGF-treated NIH-EGFR/ALK cells was very sensitive to R59949 treatment, again suggesting the involvement of the α isoenzyme in ALK-mediated signal transduction (data not shown).

Limitations of the anti-αDGK antibodies used in this study do not allow efficient immunoprecipitation of the murine isoform. Therefore, to assess our results more directly and provide further support to the hypothesis that αDGK activity is stimulated upon activation of ALK tyrosine kinase, we have transiently transfected NIH-EGFR/ALK cells with a myc-tagged αDGK construct and determined αDGK activity in anti-myc immunoprecipitates obtained from lysates of either untreated or EGF-stimulated cell cultures. As shown in Figure 3 (upper panel), αDGK activity was significantly higher (around 3-fold over control) in immunocomplexes obtained from EGF-stimulated NIH-EGFR/ALK cell lysates, despite a comparable amount of immunoprecipitated myc-αDGK (lower panel).

These results indicate a specific activation of αDGK by ALK under conditions in which this molecule promotes mitogenic and transforming signals.

ALK-induced αDGK activation is mediated by a p60^{src}-dependent mechanism

Evidence is increasing that members of the Src family of tyrosine kinases provide regulatory signals relevant for DGK activation. Indeed recent studies obtained in both lymphoid and non-lymphoid cells have indicated the requirement of p60^{src} activity for αDGK[21-22,33] and ζDGK[34] activation.

In Karpas 299 and TS cells, we have initially observed that αDGK activity was strongly reduced by 1 hour of cell pretreatment with the specific Src inhibitor PP2 (Figure 1B), suggesting that the elevated αDGK activity in these NPM/ALK-positive cells is maintained through Src tyrosine kinase activity. Similar results have been observed in 32D-NPM/ALK cells (data not shown).

This possibility was further tested in NIH-EGFR/ALK cells. To this end, we have first analyzed p60^{src} kinase activity following in vivo stimulation of NIH-EGFR/ALK cells with EGF for different time periods. Lysates were immunoprecipitated with a p60^{src}-specific antibody, and kinase assay was performed in the presence of [γ-^{32}P]ATP and 5 μg acid-denaturated enolase added as exogenous substrate. Under our experimental conditions, p60^{src} autophosphorylation was increased within 2 minutes of ligand exposure, reached a maximum in 5 minutes (about 2.2-fold stimulation), and

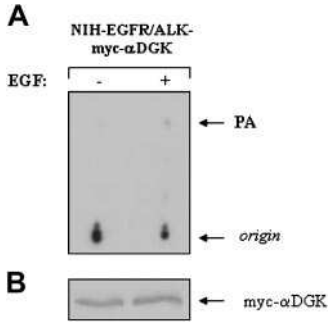

Figure 3. EGF-induced αDGK activation in NIH-EGFR/ALK cells. NIH-EGFR/ALK cell line was transiently transfected with the expression vector pMT2-myc-αDGK according to the calcium-phosphate technique. Cells were serum-starved for 18 hours and then either mock-treated (−) or stimulated (+) for 10 minutes at 37°C with 100 ng/mL EGF and lysed thereafter. Lysates were immunoprecipitates with appropriate concentrations of agarose-conjugated anti–c-myc antibody. (A) Immunocomplexes were subjected to in vitro kinase assay as described in Figure 1. (B) An aliquot of each sample was analyzed by Western blot to control that equal amounts of myc-αDGK were present during in vitro kinase assay.

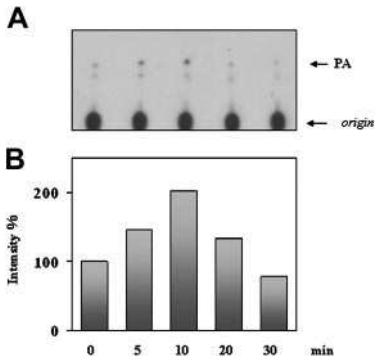

Figure 2. DGK activity in NIH-EGFR/ALK cells following EGF stimulation. NIH-EGFR/ALK cells were serum-starved for 18 hours and then either mock-treated or treated with 100 ng/mL EGF for the indicated time at 37°C. (A) Cell lysates were analyzed for in vitro DGK enzymatic activity as described in Figure 1. (B) PA spots were quantified by densitometric scanning of the autoradiograms and results are expressed as percent of control (signal obtained from medium-treated cells). Results are representative of 3 independent experiments.

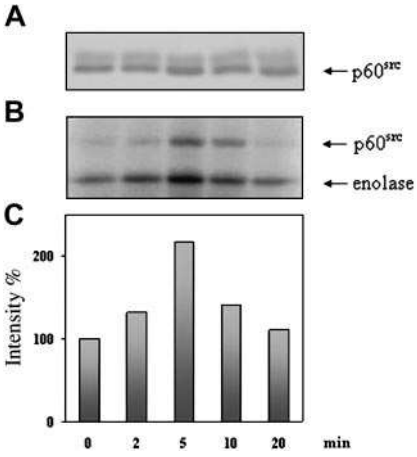

Figure 4. p60^{src} kinase activity in NIH-EGFR/ALK cells following EGF stimulation. Quiescent NIH-EGFR/ALK cells were either mock-treated or treated with 100 ng/mL EGF for the indicated time at 37°C. Cell lysates were immunoprecipitated with an anti-p60^{src} antibody. (A) An aliquot from each immunoprecipitate was directly analyzed by immunoblotting to evaluate p60^{src} content. (B) The remainder of each immunoprecipitate was subjected to in vitro kinase assay in the presence of [γ-^{32}P]ATP and acid-denaturated enolase. Samples were then analyzed by SDS-PAGE and autoradiography. (C) The ^{32}P content of bands corresponding to enolase shown in panel B was quantified by densitometric scanning of the autoradiograms and results are expressed as percent of control (signal obtained from medium-treated cells).

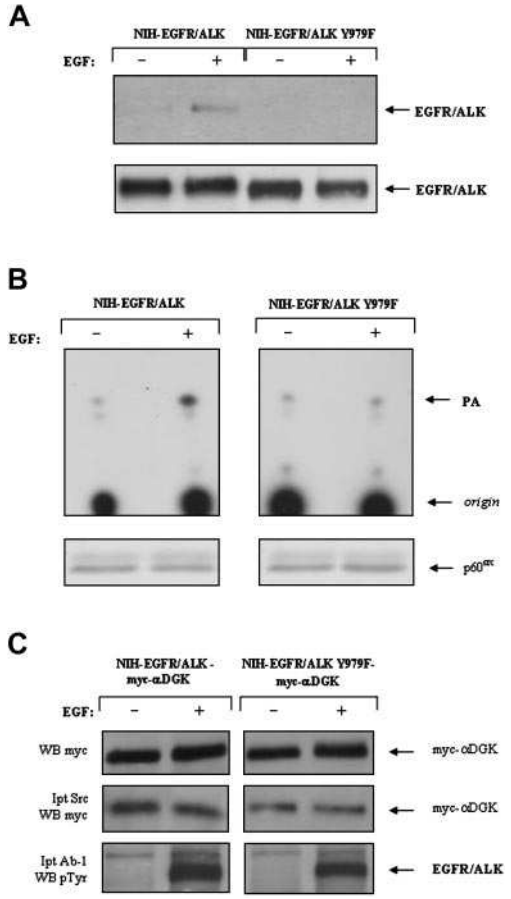

Figure 5. Association of αDGK protein and activity with p60src in NIH-EGFR/ALK and NIH-EGFR/ALK-Y979F cells. (A-B) NIH-EGFR/ALK and NIH-EGFR/ALK-Y979F cells, expressing comparable levels of receptors, were serum-starved for 18 hours and then either mock-treated or stimulated for 5 minutes at 37°C with 100 ng/mL EGF and lysed thereafter. (A) A 2-mg aliquot of total cellular proteins from each lysate was incubated with GST-p60src/SH2 (10^{-7} M) immobilized onto glutathione-agarose for 1.5 hours at 4°C. After extensive washing, proteins were electrophoresed on SDS-PAGE and immunoblotted with the anti-ALK monoclonal antibody (top panel). The same lysate (100 μg) was directly analyzed by immunoblot with the anti-ALK antibody (bottom panel). (B) Total cellular proteins (3 mg) were immunoprecipitated with an anti-p60src–specific antibody. Two thirds of each immunoprecipitate was subjected to in vitro DGK enzymatic activity as described in Figure 1 (top panel). The remainder of each immunoprecipitate was analyzed by immunoblot with p60src antibody (bottom panel). Comparable results were obtained in 3 different experiments. (C) NIH-EGFR/ALK and NIH-EGFR/ALK-Y979F cells were transiently transfected with the expression vector pMT2-myc-αDGK. Cell treatment and lysis were performed as described in Figure 3. Control (top panel) and p60src immunoprecipitates (middle panel) were analyzed for myc-αDGK protein by Western blot with anti-myc antibodies. Each lysate was also analyzed for proper receptor activation by immunoprecipitation with Ab-1 antibody, which recognizes the EGFR extracellular domain and subsequent Western blot with anti–phospho-Tyr antibodies (bottom panel). WB indicates Western blot.

returned to basal levels after 20 minutes (Figure 4B). The increased autokinase activity of p60src was accompanied by an increase in phosphorylation of the exogenous substrate enolase (Figure 4B-C). These data indicate that p60src kinase activity is stimulated by ALK activation, according to a recent report that describes the association/activation of p60src with NPM/ALK.[16] In vivo association of p60src with the cytoplasmic domain of activated ALK was also confirmed in our model system by coimmunoprecipitation (data not shown) and further validated in vitro by pull-down experiments using a bacterially expressed GST-p60src/SH2 fusion protein (Figure 5A).

Tyrosine 418 of NPM/ALK (numbered according to Morris et al[4]) has been identified as the residue responsible for NPM/ALK-p60src interaction.[16] Indeed, tyrosine to phenylalanine substitution at the corresponding position in the EGFR/ALK sequence (EGFR/ALK-Y979F, according to Piccinini et al[26]) completely abolished p60src-EGFR/ALK binding, even when lysates were prepared from EGF-stimulated cells (Figure 5A). Although the Y979F mutation did not alter the in vivo autophosphorylation activity of the EGFR/ALK chimera (Figure 5C), the mutation impaired significantly the chimera's ability to trigger an efficient proliferative response to EGF (data not shown), supporting the importance of p60src activation for ALK-mediated mitogenicity.[16]

In NIH-EGFR/ALK cells, the time course of DGK activation following EGF stimulation correlates with the period of p60src activity, in line with the possibility of a tight regulation of DGK activity by p60src. Therefore, to evaluate whether the catalytic activation of αDGK was dependent on ALK-induced p60src activation, we have analyzed DGK activity copurified with p60src from either control or EGF-treated NIH-EGFR/ALK cells. As shown in Figure 5B (left panel), DGK activity in anti-p60src immunocomplexes from treated cells was significantly higher than control cells. p60src-associated DGK activity was completely abrogated by pretreatment of NIH-EGFR/ALK with 1 μM R59949 (data not shown). Furthermore, in EGF-treated NIH-3T3 cells expressing the EGFR/ALKY979F mutant (NIH-EGFR/ALKY979F), the amount of DGK activity coprecipitated in complex with p60src remained at the basal level (Figure 5B, right panel). Taken together, these data are consistent with the existence of a signaling pathway in which ALK activation modulates the αDGK catalytic function through a p60src-dependent mechanism.

Results shown in Figure 5B suggest a physical interaction in vivo between αDGK and p60src. However, under our experimental conditions, only endogenous DGK activity but not endogenous αDGK protein could be detected in p60src immunocomplexes obtained from NIH-EGFR/ALK, because of the poor recognition of the murine protein by the anti–human αDGK antibodies. Therefore, to provide further evidence for an in vivo p60src/αDGK association and to establish whether this complex is induced by EGF cell treatment, coimmunoprecipitation experiments were performed in NIH-EGFR/ALK cells transiently transfected with the myc-tagged αDGK construct previously described (Figure 3). Under these conditions, αDGK was easily detected in anti-p60src immunoprecipitates; however, the amount of recovered αDGK was not dependent on EGF cell stimulation (Figure 5C), suggesting that the p60src/αDGK interaction does not require p60src activation. Indeed, similar results were observed in NIH-3T3 cells expressing the EGFR/ALKY979F mutant in which p60src signaling is specifically abrogated (Figure 5C).

Role of αDGK in ALK-mediated signal transduction

To establish the importance of αDGK in ALK-mediated signaling, we first examined the effect of R59949 treatment on EGF-induced proliferation of NIH-EGFR/ALK cells. As shown in Figure 6A, 10 μM R59949 markedly inhibited the EGF responsiveness of NIH-EGFR/ALK cells (maximal effect, about 40% inhibition). Notably, in the same cells, αDGK inhibition by R59949 did not influence the mitogenic response of EGFR/ALK to 1% FBS (Figure 6B), indicating that αDGK is involved in a pathway that is specifically required for ALK signaling but dispensable for serum-induced mitogenesis. The presence of 10 μM R59949 also resulted in a significant inhibition of 32D-NPM/ALK cell growth, determined at 24 (data not shown) and 48 (Figure 6C) hours using the MTT proliferation assay. Similar results have been obtained in Karpas 299 cells (Figure 6D). We have observed no toxic effect on all cell

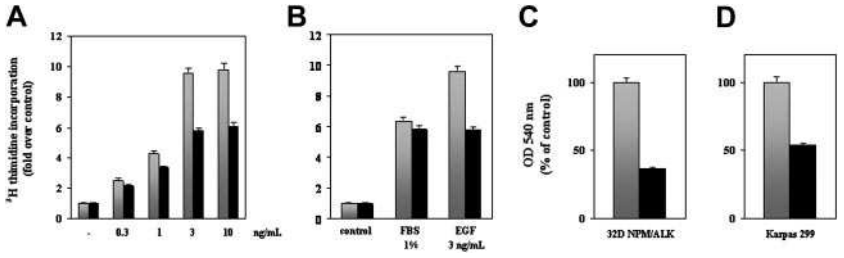

Figure 6. Effect of the R59949 inhibitor on NIH-EGFR/ALK, 32D-NPM/ALK, and Karpas 299 cell growth. (A-B) NIH-EGFR/ALK cells were serum starved for 72 hours and then pretreated for 30 minutes with 1 μM R59949 (■) or DMSO (▨) as control, before stimulation with the indicated dose of EGF (A) or 1% FBS (B) in the presence of 4 μCi (0.148 MBq) [*methyl*-^{3}H] thymidine/well for 22 hours. Results are expressed as percent of control (radioactive incorporation obtained from medium + DMSO-treated cells). (C-D) 32D NPM/ALK and Karpas 299 cells were cultured for 48 hours in medium containing FBS 0.1% and BSA 0.2 mg/mL in the presence of 10 μM R59949 (■) or DMSO 0.02% (▨) as control. Cell proliferation was determined using the MTT proliferation assay. The values represent the means ± SD (n = 3). OD indicates optical density.

lines maintained for 96 hours in the presence of 10 μM R59949 as measured by trypan blue exclusion.

Biologic effects following αDGK-specific RNA interference

To obtain further evidence that αDGK is involved in NPM/ALK mitogenic activities, we have down-regulated αDGK expression by RNA interference in Karpas 299 cell line. Transfection with a specific interfering RNA lowered but did not abolish the expression of αDGK, as detected by Western blot on whole-cell lysates (Figure 7A). BrdU incorporation, carried out 72 hours after transfection with this siRNA, showed a marked inhibitory effect on cell proliferation (Figure 7B), comparable with that obtained following treatment with 10 μM R59949 (Figure 6D). On the contrary, transfection with control siRNA did not result in any cell growth alteration. In conclusion, these data indicate that either inhibition of αDGK catalytic activity or down-regulation of its expression severely impair the ability of NPM/ALK-expressing cells to proliferate, providing evidence that the catalytic function of αDGK is required for the transduction of mitogenic and transforming signal promoted by ALK.

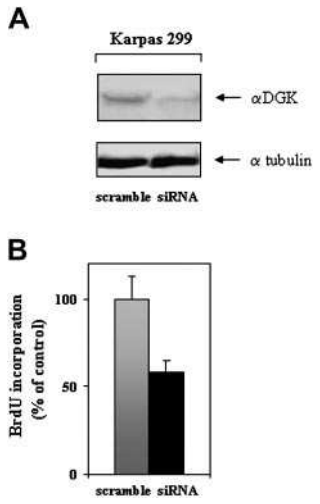

Figure 7. Down-regulation of αDGK by RNA interference on Karpas 299 cell proliferation. (A) siRNA-transfected Karpas 299 cells were lysed 72 hours from transfection, and their αDGK content was assayed by Western blot with anti-αDGK antibodies (top panel). As an internal control, the same blot was reprobed with an anti–α-tubulin antibody without prior stripping (bottom panel). (B) BrdU incorporation of siRNA-transfected Karpas 299, 72 hours following transfection. Results are expressed as percent of control (BrdU incorporation obtained from untransfected cells). Upon our experimental conditions, 60% to 70% of Karpas 299 cells resulted transiently permeabilized, as measured by trypan blue staining immediately after electroporation. The values represent the means ± SD (n = 3).

Discussion

Clonal abnormalities of the ALK receptor are typically associated with ALCLs and most frequently represented by the t(2;5) translocation.[3] This chromosomal rearrangement leads to the expression of the NPM/ALK fusion protein, which possesses a high constitutive tyrosine kinase activity and can potently transform in vitro a wide array of different cell types.[6-9] The oncogenic properties of NPM/ALK in vivo have been initially supported by a murine bone marrow retroviral transduction/transplantation-based approach[10] and more recently confirmed in transgenic murine models.[11-13]

Efforts aimed at the molecular characterization of the signaling pathways relevant for NPM/ALK-mediated oncogenesis have led to the identification of different intracellular effectors. These include PLC-γ, PI3-K, Stat3-5, and p60^{src}.[8,14-17] However, current knowledge of the signaling events promoted by ALK are still preliminary, and therefore further studies are required to identify additional critical transducers of NPM/ALK-mediated transformation. In this study, we have investigated the potential involvement of αDGK, since cumulative evidence indicates a central role for this enzyme family as mediators of various cellular responses.[35]

For this purpose, we have used 2 different NPM/ALK-positive cell systems represented by the murine NPM/ALK-transformed 32D cell line and the human ALCL-derived Karpas 299. However, a comparison of the biologic and biochemical properties of the activated versus a ligand-inducible ALK receptor is necessary to gain insight into its function as a transforming gene. Although pleiotrophin and midkine have been proposed as potential high-affinity ligands for human ALK,[36-37] the physiologic relevance of these receptor/ligand pairs has not been undoubtedly demonstrated and is presently under discussion.[3,38] Moreover, these data have not found correspondence in *Drosophila,* where the ALK homolog binds to and is activated by jelly belly (Jeb), a protein structurally unrelated to both pleiotrophin and midkine.[39] Therefore, to analyze the different aspects of ALK-mediated mitogenic signal under conditions of controlled ligand-induced activation, we made use of a previously described EGFR/ALK chimera in which the ALK enzymatic function could be modulated by EGF stimulation.[26]

In the current study, we demonstrate that activation of ALK correlates with an up-regulation of αDGK catalytic function. The identification of αDGK as the isoform mainly involved in ALK-mediated signaling is based on (1) sensitivity to R59949, a class I αDGK-specific inhibitor; (2) immunoprecipitation of endogenous αDGK with specific antibodies in Karpas 299 cells; and (3) immunoprecipitation of myc-tagged αDGK transfected in NIH-EGFR/ALK cells.

Our data also indicate that ALK-induced activation of αDGK takes place through a p60^{src} tyrosine kinase–dependent mechanism.

Indeed, R59949-sensitive DGK activity copurified with p60^{src} in anti-p60^{src} immunoprecipitates was obtained from EGF-stimulated but not from unstimulated NIH-EGFR/ALK cells. Furthermore, only basal levels of DGK activity were revealed in anti-p60^{src} immunocomplexes obtained from EGF-treated NIH-EGFR/ALKY979F cells, in which p60^{src} signaling is specifically abrogated. The inhibition of radioactive PA formation from exogenous DG in NPM/ALK-positive cells by the PP2 p60^{src} inhibitor is in agreement with these results and with previous data obtained in other receptor signaling systems in both lymphoid and nonlymphoid cells.[21-22,33]

Surprisingly, in NIH-3T3 fibroblasts the complex between p60^{src} and myc-αDGK is preformed, as the 2 proteins efficiently coprecipitate from both unstimulated or EGF-treated NIH-EGFR/ALK and NIH-EGFR/ALKY979F cells. This finding contrasts with previous reports that indicated ligand-induced p60^{src}/αDGK association in COS and PAE cells expressing HGFR and VEGFR-2, respectively.[21,22] However, when EGFR/ALK and αDGK were coexpressed in COS cells, p60^{src}/αDGK protein complex formation was dependent on receptor and p60^{src} activation (data not shown). Taken together, these data suggest that while activation of αDGK is tightly regulated by p60^{src} tyrosine kinase activity, its association in a complex with p60^{src} might be either dependent on or independent of receptor-induced p60^{src} activation, according to different cell types. This observation might reflect different status of basal p60^{src} activity, which is also regulated by cell adhesion in different cells. Alternatively, it is possible to speculate that αDGK might associate with p60^{src} through multiple interactions, either dependent on or independent of p60^{src} activation.

The molecular details of ALK-mediated αDGK activation and the functional relevance of αDGK/p60^{src} association in living cells are still unclear. Since different p60^{src} interactors, such as p130Cas, are tyrosine phosphorylated by p60^{src} upon binding to its SH3 domain and given the requirement of p60^{src} catalytic function for αDGK activation, the more obvious possibility is that αDGK acts as a substrate of p60^{src}. Indeed, 2 potential Src phosphorylation sites are present on αDGK amino acid sequence, and in vivo αDGK tyrosine phosphorylation has been observed following its transient coexpression with p60^{src} in COS cells or upon cell treatment with pervanadate.[21] However, at present, no evidence exists for αDGK tyrosine phosphorylation under more physiologic experimental conditions, and we also were unable to reveal any in vivo αDGK tyrosine phosphorylation in the cell systems used in this study. Although it cannot be excluded that tyrosine phosphorylation of αDGK occurs at very low stoichiometry or under the specific control of a tyrosine phosphatase, alternative possibilities should be taken into consideration and further studies are required to elucidate this issue.

Finally, in the present study we have demonstrated the importance of αDGK in ALK-mediated signaling. Indeed, we have shown that pharmacologic inhibition of αDGK significantly impairs the mitogenic responsiveness of NIH-EGFR/ALK to EGF, as well as the growth rate of NPM/ALK-positive cells. The importance of αDGK in ALK-mediated mitogenic activity has been further validated in Karpas 299 cells by using a siRNA approach.

By converting DG to PA, DGKs modulate the intracellular levels of 2 important second messengers and thus can participate in the regulation of different intracellular events. Because there is evidence of multiple DGK isoforms within the same cell type, it is currently believed that the different isoenzymes perform specialized roles, probably by recognizing DG pools generated in response to different stimuli and that are localized in specific membrane compartments. Indeed, membrane translocation appears to be an effective process for regulating DGK activity and subtype-specific functions.[40-41] Up-regulation of these enzymes results in a transient increase of the intracellular amount of PA. The precise role of αDGK-generated PA has not been elucidated, although several studies linked PA to the modulation of different molecules known to control cell proliferation, including protein kinase Cϵ (PKCϵ)[42] and PKCζ,[43] c-Raf,[44] and phosphatases.[45-46] The potential role of DGK-generated PA in the mammalian target of rapamycin (mTOR) pathway has been analyzed in a recent study and indicates that mTOR activity interacts with and is regulated by ζ- rather than the α-DGK.[47]

Intriguingly, ALK-mediated mitogenic properties involve activation of p60^{src},[16] which activates αDGK and PLC-γ,[8] which provides the lipid substrate to it and PI3-K,[14-15] for which a direct role in regulating αDGK activity has recently been described in lymphocytes.[33] It is therefore becoming evident that the turnover of bioactive lipids plays a pivotal role in ALK-mediated signal transduction, and further studies are needed to explore in greater detail how the lipid enzymes involved in their generation are functionally linked upon ALK activation to extend our knowledge into the oncogenic mechanisms involved in NPM/ALK-positive ALCL.

In conclusion, our data indicate that αDGK activation is involved in ALK-mediated mitogenic signaling and suggest that the inhibition of this activity can interfere with the pathogenic NPM/ALK function in ALCL cells.

Acknowledgments

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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*.,

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

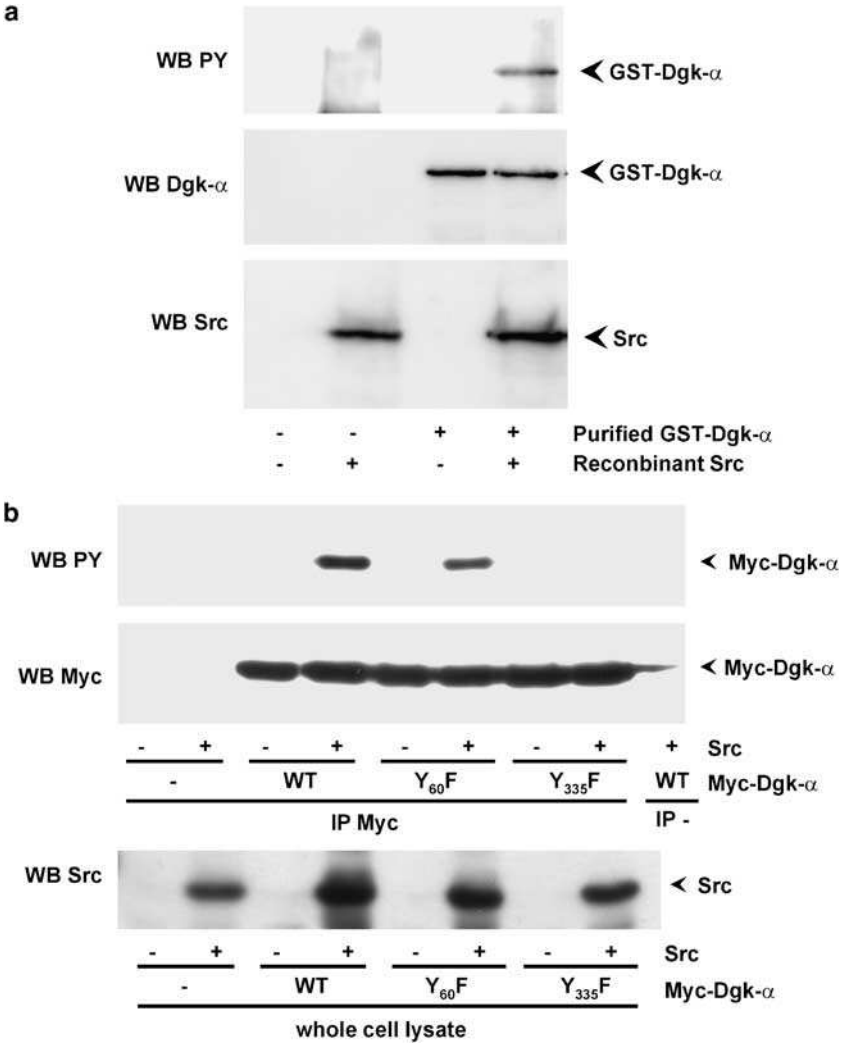

Figure 1 Dgk-α phosphorylation by Src on Y335. (**a**) Partially purified GST-Dgk-α or GST were incubated with or without recombinant Src in kinase buffer for 10 min at 30°C. Samples were split and analysed by western blot with antibodies against phosphotyrosine (upper panel), Dgk-α (middle panel) or 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.

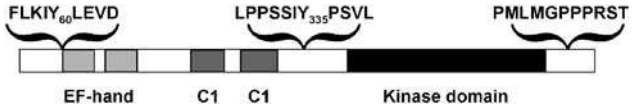

Figure 2 Dgk-α structure. The structure of Dgk-α contains three structurally defined domains: 2 EF-hand motifs, a double atypical C1 domain and a catalytic domain. Y60 lies upstream of the EF-hand motif, Y335 between the second C1 domain and the catalytic domain and the proline-rich region at the C-terminal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

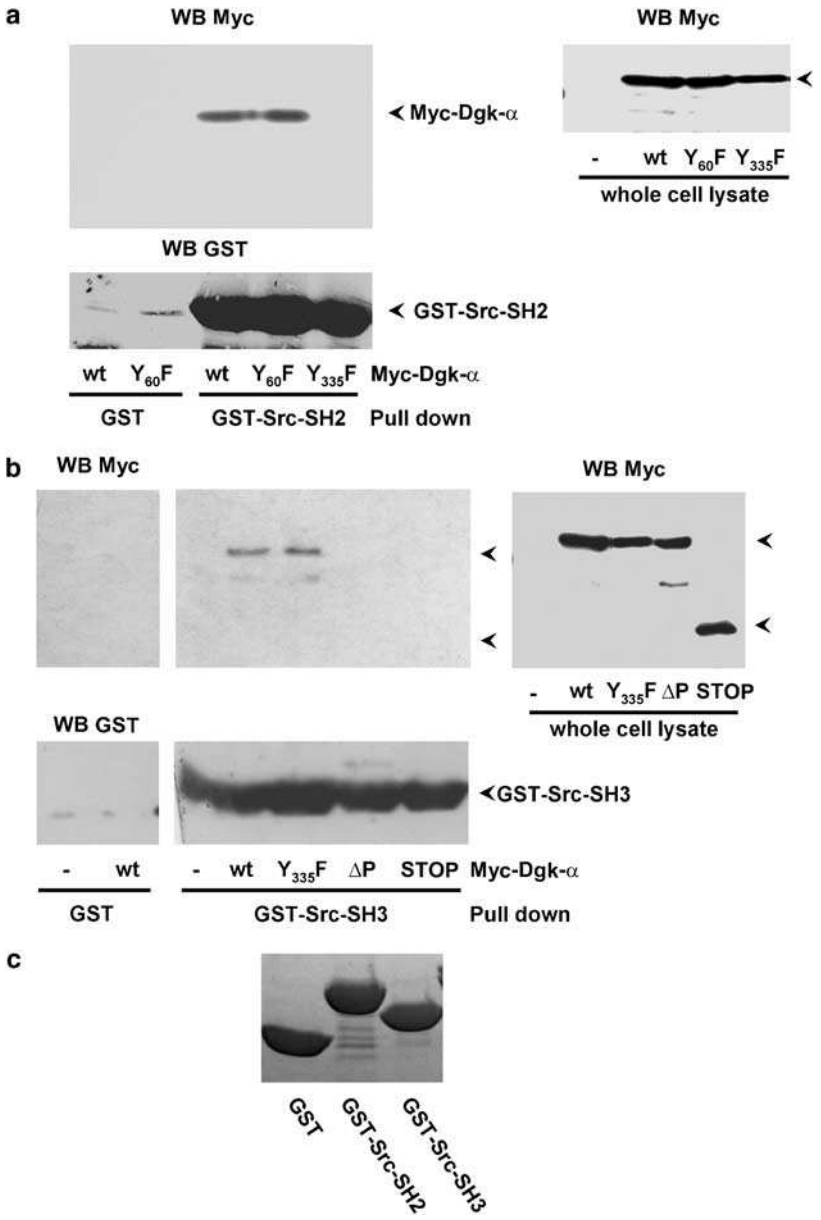

Figure 3 Dgk-α interaction with Src-SH2 and Src-SH3 domains. (**a**) Growing COS-7 cells, transfected with indicated Myc-Dgk-α constructs, were lysed in buffer A. Cell lysates were incubated with agarose-bound purified GST or GST-Src-SH2 for a pull-down assay. Pulled down Myc-Dgk-α (left panel) and Myc-Dgk-α 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.

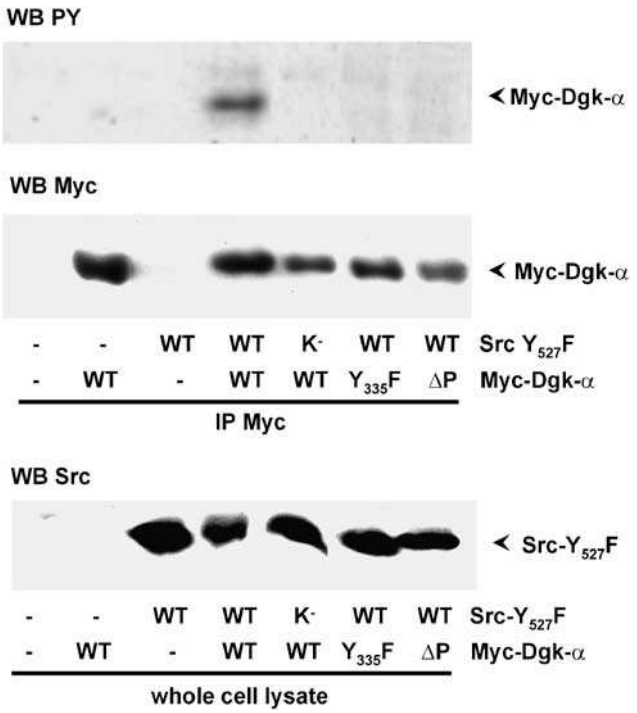

Figure 4 Dgk-α phosphorylation by Src requires Y335- and proline-rich C-terminal sequence. Growing HEK 293T co-transfected with the indicated Myc-Dgk-α and Src-Y527F constructs were lysed in detergent-containing buffer A. Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed by western 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).

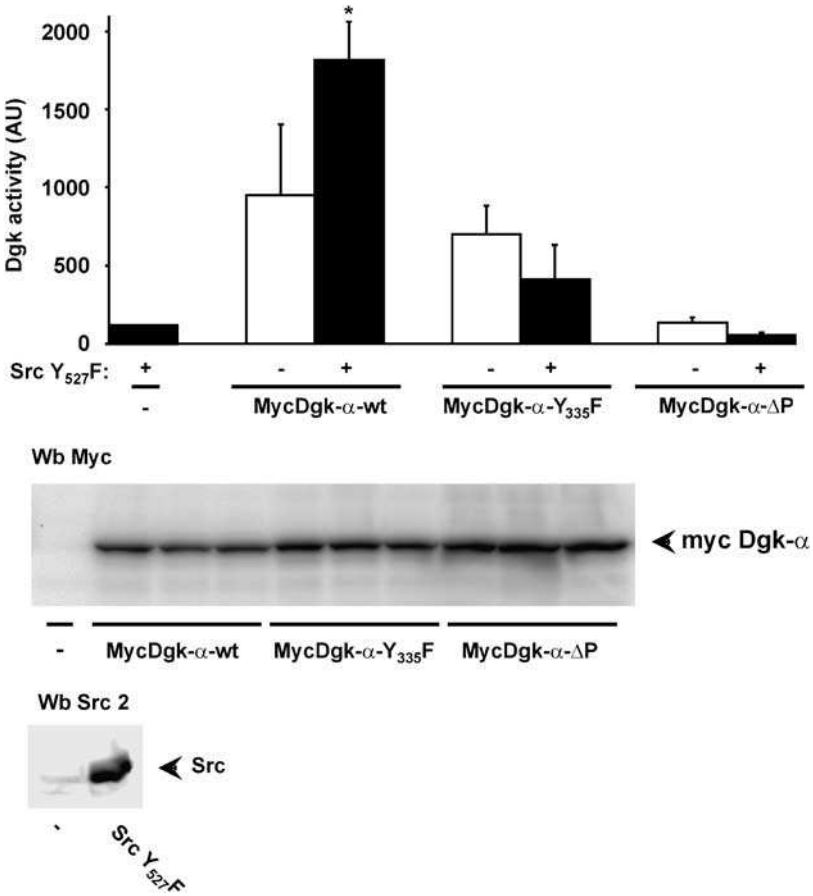

Figure 5 Dgk-α activation by c-Src *in vitro* requires Y335- and proline-rich C-terminal sequence. COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP or Src were homogenized with buffer B in absence of detergent. Cell extracts were mixed as indicated in presence 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

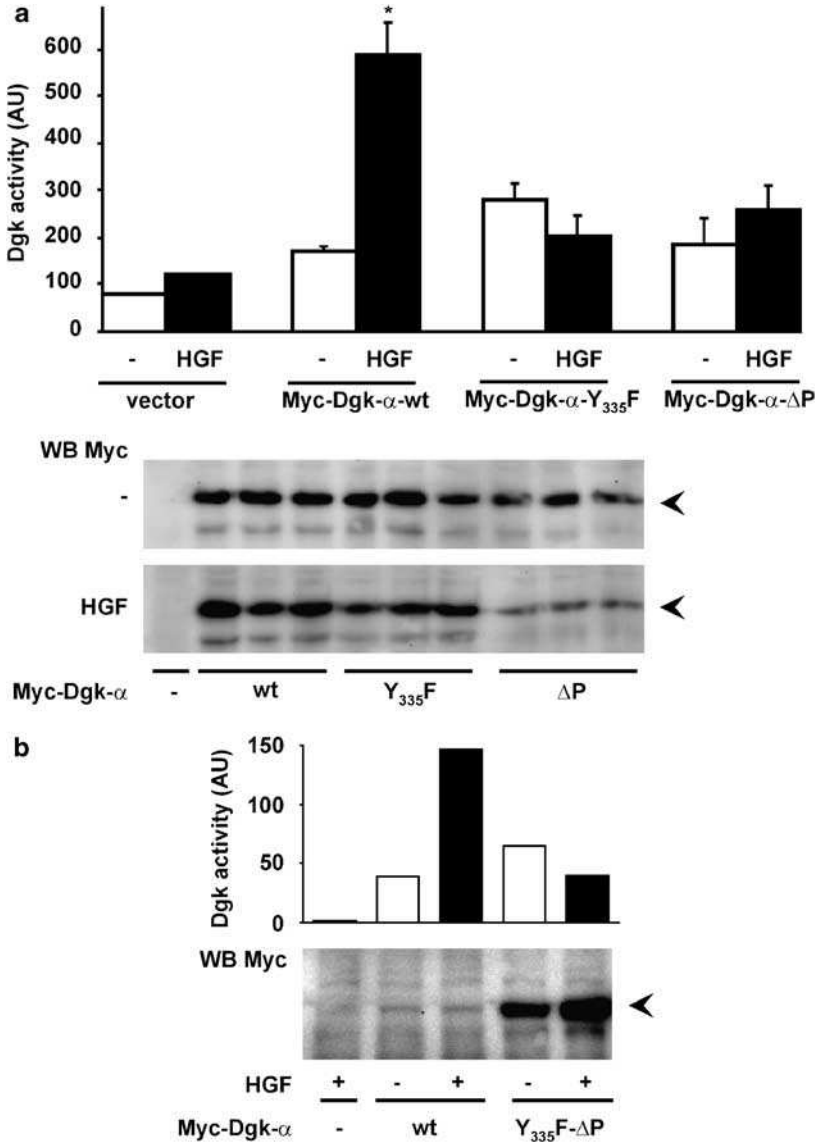

Figure 6 Dgk-α activation by HGF *in vivo* requires Y335- and proline-rich C-terminal sequence. (**a**) COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP were stimulated with HGF (100 μg/ml, 15 min), homogenized with buffer B in absence of detergent and analysed 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

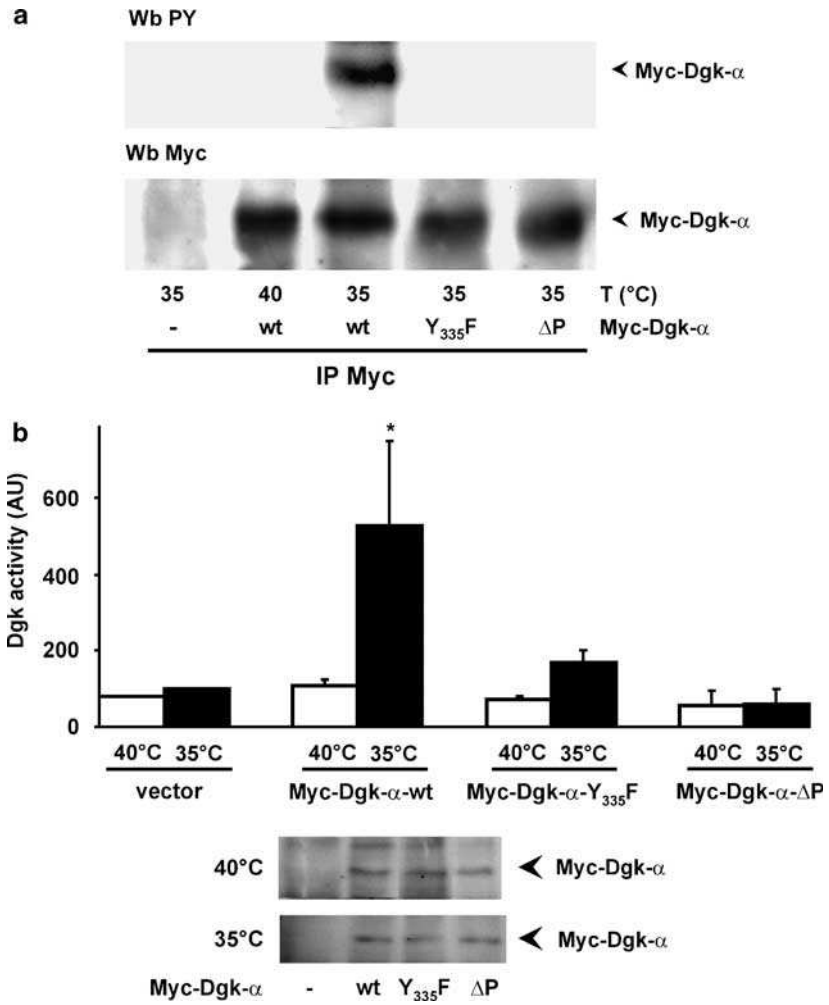

Figure 7 Dgk-α phosphorylation and activation by v-Src requires Y335- and proline-rich C-terminal sequence. Ts-v-Src/MDCK cells transfected with the indicated Myc-Dgk-α constructs, were cultured at nonpermissive temperature (40°C), and, where indicated, shifted at the permissive temperature (35°C) for 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

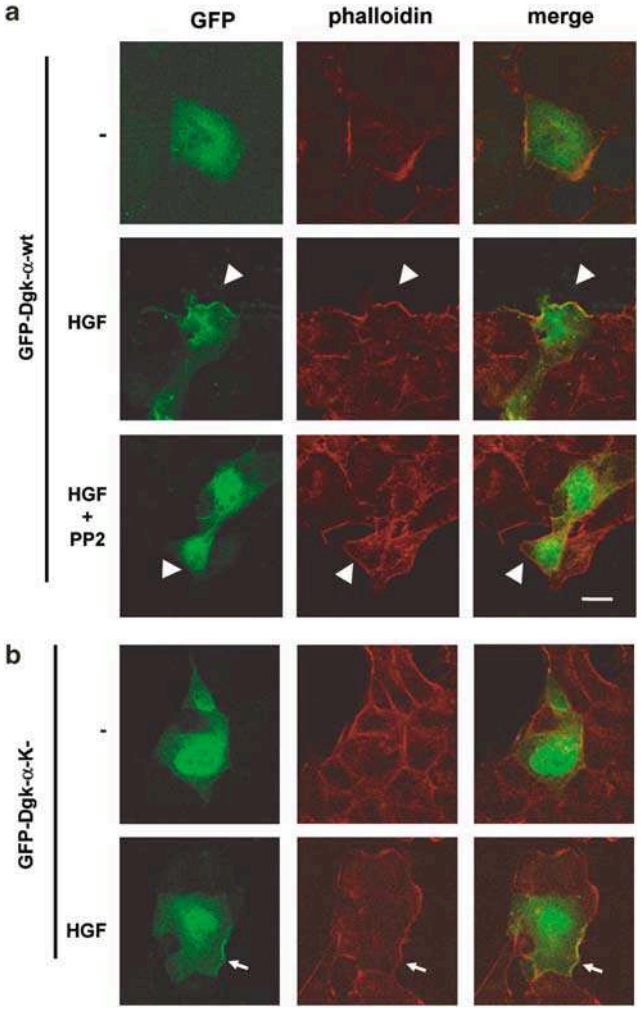

Figure 8 Dgk-α is recruited at cell membrane upon HGF treatment. MDCK cells transfected with GFP-Dgk-α wt or GFP-Dgk-α-K− were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with PP2 (10 μM PP2 for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal 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

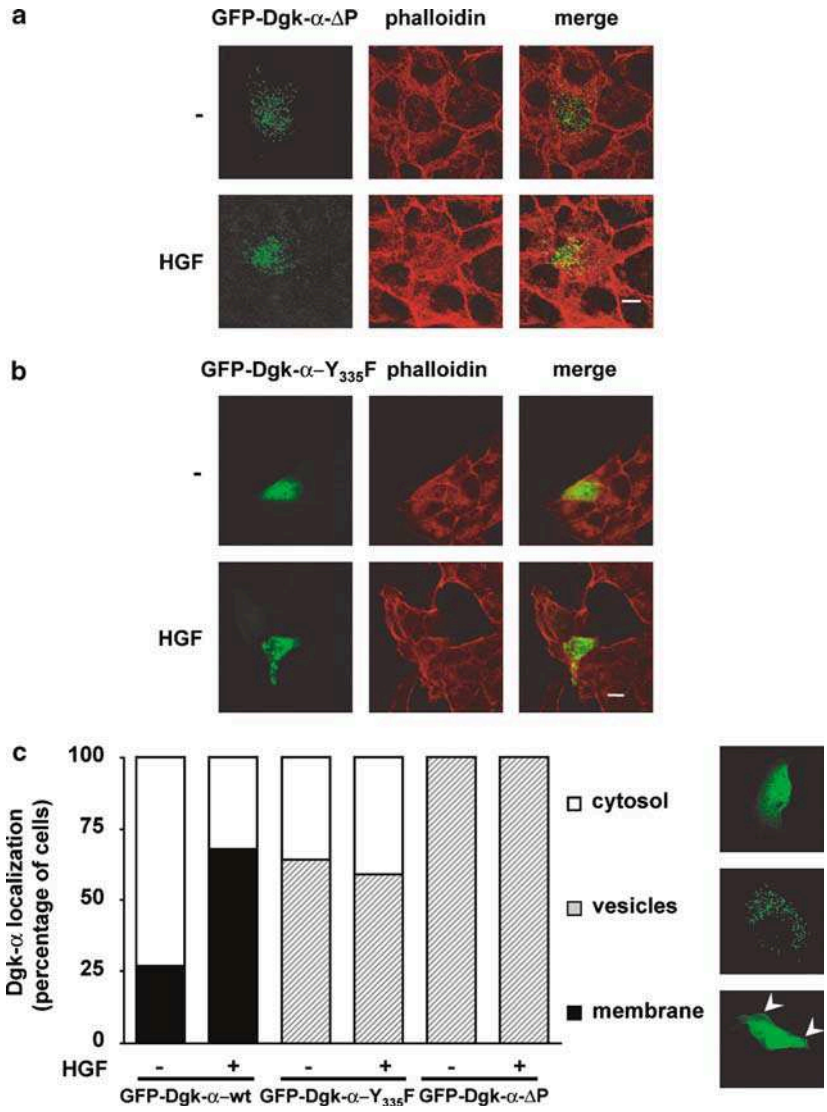

Figure 9 Recruitment of Dgk-α at cell membrane requires Y335- and proline-rich C-terminal sequence. MDCK cells tranfected with either GFP-Dgk-α-wt, GFP-Dgk-α-ΔP (**a**) or GFP-Dgk-α-Y335F (**b**) were stimulated with HGF (50 ng/ml, 15 min). Cells were stained with phalloidin-TRITC and images acquired 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

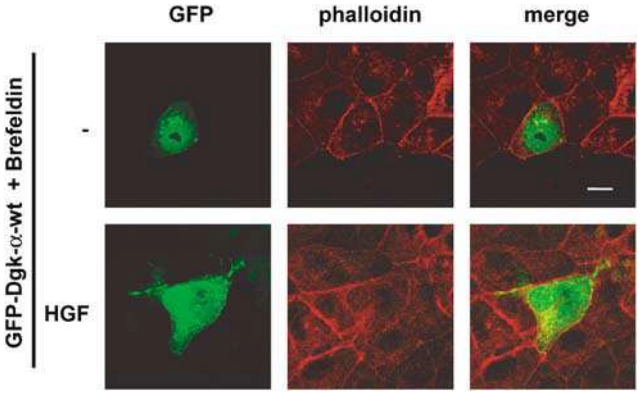

Figure 10 Brefeldin causes accumulation of Dgk-α on cytoplasmic vesicles. MDCK cells transfected with GFP- Dgk-α wt were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with BFA (10 μM BFA for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal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

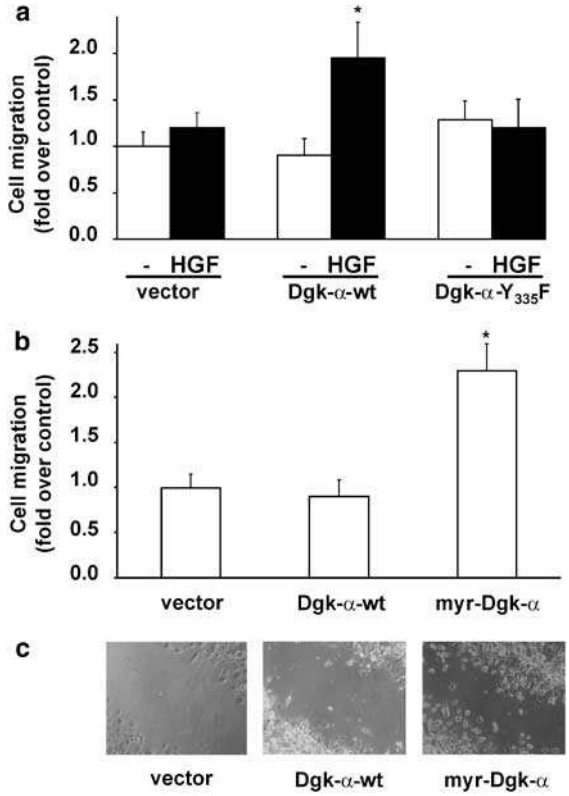

Figure 11 Membrane recruitment of Dgk-α is necessary and sufficient for cell motility. (**a**) COS cells transfected with either Dgk-α wt, or Y335 mutant or empty vector were stimulated to migrate by HGF (100 ng/ml) in a transwell chemotaxis assay. Data are expressed as fold increase over control, values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