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ROLE OF DIACYLGLYCEROL KINASE ALPHA IN TUMOUR PROGRESSION OF DES-ACYL GHRELIN ON SKELETAL MUSCLE .

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Preface: an important initial note

In this final report I present the publications that resulted from the two main research lines I followed during my PhD course. I also briefly summarize related results still awaiting to be fully developed in order to be considered for publication as well.

PART I- **Role of Diacylglycerol Kinase-alpha in tumour progression and metastasis formation.**

When I started the Molecular Medicine PhD programme my first project was aimed to characterize the biological role Diacylglycerol Kinase-alpha (DGKalpha) in processes such as cell migration and invasion and, in particular, to investigate the potential link between DGKalpha and tumour progression.

Some of the results obtained during this PhD have been published in three different articles, which represent the outcome of this research line, while others, although encouraging, still need some experiments in order to be conclusive.

The first article to be published (Filigheddu et al. 2007) identifies Diacylglycerol Kinases as important mediators of cancerous phenotype in an higly aggressive breast cancer cell line, MDA-MB-231. Indeed, their pharmacological inhibition blocked anchorage-independent growth and HGF-induced invasion.

The second paper (Chianale et al. Mol Biol Cell 2007) demonstrates that DGKalpha is a specific regulator of epithelial cell migration downstream tyrosine kinase receptors and Src and that it mediates Rac activation and Rac-dependent remodelling of actin cytoskeleton and focal contacts in epithelial cells.

The third work (Baldanzi et al. Oncogene 2008) describes the molecular determinant essential for Src-mediated DGKalpha activation.

Finally, in the last paper (Porporato et al, in preparation) we demonstrate that DGKalpha is activated also upon GPCR activation. We show, indeed, that DGKalpha is essential for SDF1a-mediated invasion and metalloproteinase activity, and we suggest a novel molecular mechanism by which DGKalpha

mediates these activities, i.e. the regulation of cellular localization of an atypical PKC (PCKz) by DGKalpha-derived phosphatidic acid.

PART II- **Activity of ghrelin and des-acyl ghrelin on skeletal muscle.**

Although my first focus was Dgk, during my PhD course I got progressively involved in the other main research line of Graziani's laboratory, focused on the study of the biological effects of Des-acyl Ghrelin.
The first project I participated lead to the discovery of a direct effect of both Des-acyl Ghrelin and Ghrelin on skeletal muscle myoblasts, promoting their differentiation and fusion into multinucleated myotubes through a novel receptor not yet identified (Filigheddu et al. Mol Biol Cell 2007).
Then, we proceeded to further unveil the effect of Des-acyl Ghrelin on skeletal muscle, in particular, we undertook the analysis and characterization of a transgenic mouse wth high levels of circulating des-acyl Ghrelin and, by working on models of skeletal muscle atrophy, we identified a strong antia-trophic potential of Des-Acyl Ghrelin. This work lead to a patent and to a manuscript currently submitted to Nature Medicine.

PART I:
DIACYLGLYCEROL KINASE ALPHA AND TUMOUR PROGRESSION

Diacylglycerol (DAG) kinase (DGK) modulates the balance between two signaling lipids, DAG and phosphatidic acid (PA), by phosphorylating DAG to yield PA. (Sakane F et al, 2007). DAG is a membrane lipid which is produced by phospholipase C (PLC) and phospholipase D/PA phosphatase upon cell stimulation by several factors (Hurley JH et al 1997) . DAG is well known to regulate a wide variety of cellular functions through binding to several protein, including conventional PKC (cPKC) and novel PKC (nPKC), protein kinase D, chimaerin (Rac-specific GTPase-activating protein (GAP)) and Ras guanyl nucleotide-releasing protein (GRP) (Ron, 1999).

On the other hand, PA has important signaling role, as it has a mitogenig potential and it can drive cell transformation (Fang, 2001, Bar-Sagi, 2005). Furthermore, it has also been reported to regulate a number of signaling proteins such as phosphatidylinositol (PI)-4-phosphate 5-kinase (PIP5K) and, RasGAP, Raf-1 kinase, mammalian target of rapamycin (mTOR), atypical PKC, $p47^{phox}$, sphingosine kinase p21-activated kinase 1, transcriptional repressor Opi1p, DOCK2 and protein phosphatase-1 catalytic subunit (Delon I, 2004) (Fig. 1). It is notable that the list of PA-dependent protein members is still rapidly growing.

Although the bulk of PA is generated by two isoform of phospholipase D (PLD) through conversion of phosphatidylcholine, Dgks likely also contribute to its concentration by converting DAG in PA in specific cellular compartment.

Diacylglycerol Kinae Family

Mammalian Diacylglycerol kinase Family comprises ten different member, subdivided in five classes on the basis of their domain composition. DGKs numbers is even higher as many of these present different isoform produced by alternative splicing. (Fig.2).

In constrat with this variability in mammals one or a few Dgk isoforms have been identified in organisms such as Caenorhabditis elegans, Drosophila melanogaster, Dictyostelium discoideum and Arabidopsis thaliana. No DGK gene has been identified in yeast, and there is one bacterial diacylglycerol kinase that has little similarity to eukaryotic Dgks.

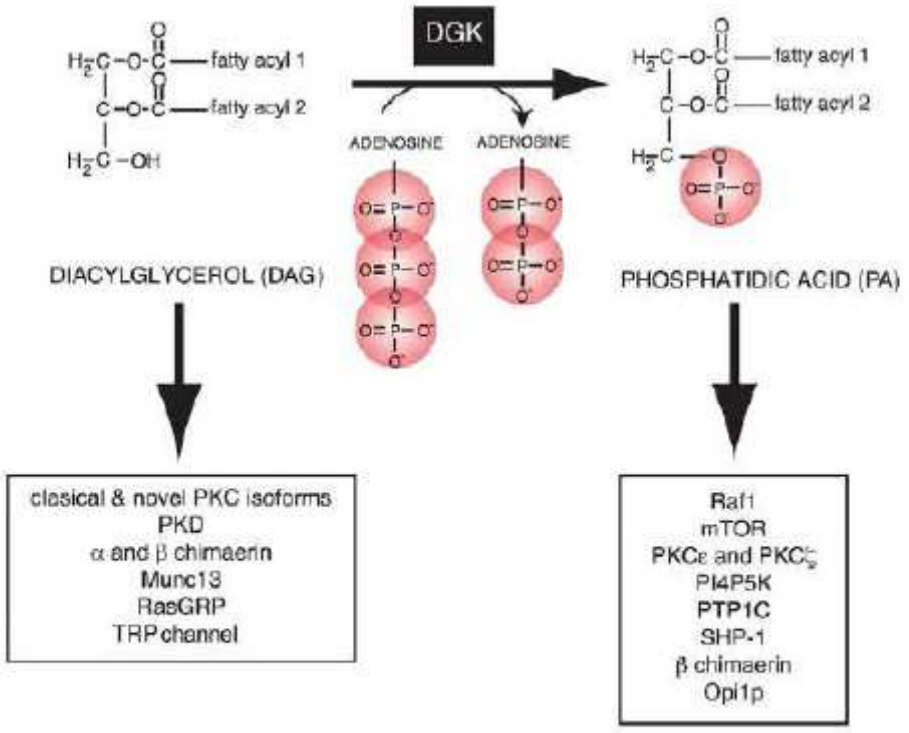


Fig1. DGK enzymatic activity regulates levels of DAG and PA at the membrane (Merida I, Biochem J 2008)

All of the eukaryotic Dgk isoforms identified to date are similar in having a kinase domain consisting of catalytic and accessory domains. All DGK family members contain at least two C1 domain simlar to the PKC phorbol ester/DAG-binding regions. The presence of C1 domains in the DGK sequence originally led to consideration of these motifs as being responsible for DAG binding. Nevertheless, sequence analysis indicated that the C1 regions lack the key residues that define a canonical C1-like phorbol ester-binding domain (Hurley et al, 2000), and none of them has been conclusively reported to bind DAG, while it has been proposed for some of them a role in protein-protein interaction. (Yakubchyk et al, 2004).

The three mammalian type I DGK (alpha, beta and gamma) have characteristic Ca2+-binding EF-hands and a recoverin-like motif in the N-terminus, whereas type II (delta, eta and kappa) have a PH (pleckstrin homology) and a SAM (sterile alpha motif) domain. ype III (DGK epsilon), present no recognizable regulatory domains except for the C1 domains and the catalytic region, but it does have an unusual specificity toward acyl chains of DAG, strongly preferring an arachidonoyl group at the sn-2 position. Members of the type IV group (DGKs zeta and iota) contain C-terminal ankyrin repeats and a PDZ-domain-binding sequence, as well as MARCKS (myristoylated alanine-rich C-kinase substrate) homology region upstream of the catalytic site. The single type V member, DGK theta, has a Rho-binding domain and present 3 C1 domain.

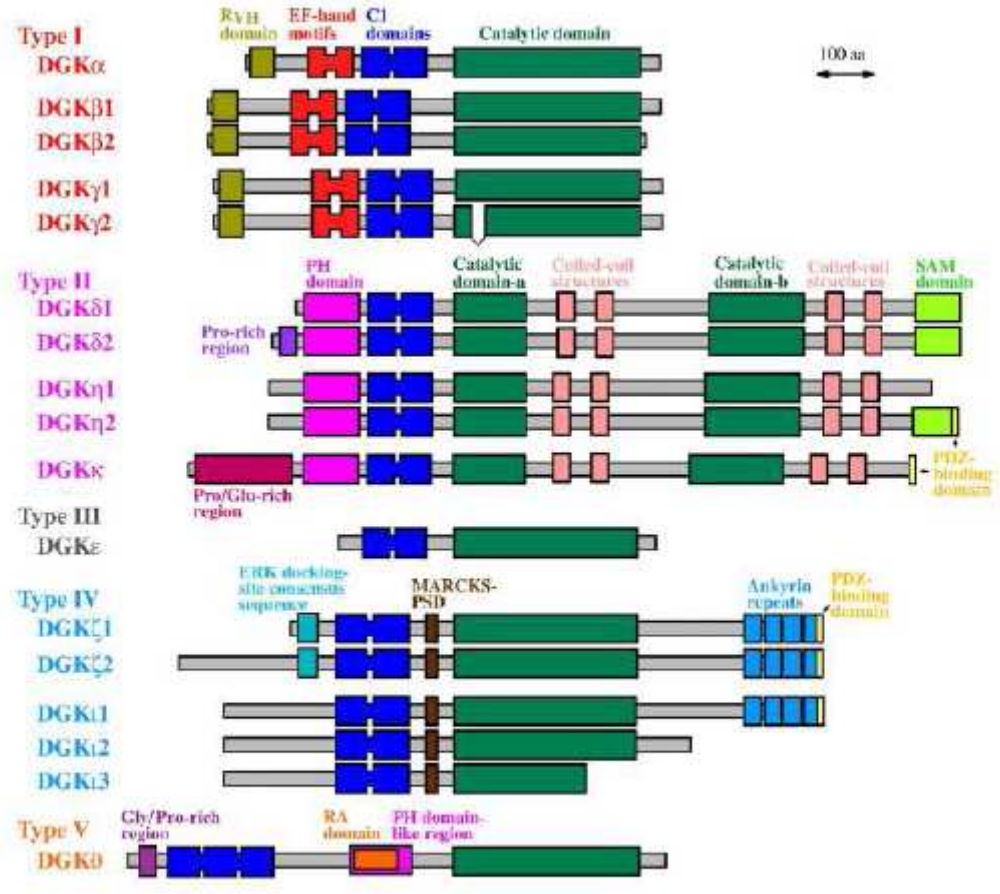


Fig 2: Schematic representation of DGK isozymes. (Sakane F 2007)

DGK family members are expressed ubiquitously, although isoforms display precise pattern of tissue distribution, and are particularly expressed in brain and

hemopoietic system, when different DGK isozymes are expressed in a cell type, they are usually from different subfamilies, strongly suggesting that each Dgk subfamily has a specific function.

DGKs interactors, localization and specificity of action

As mentioned before ten DGK isozymes exist, that, along with the known splicing variants account for 17 different isoform.

The specific action of any DGK isotype is granted, along with a specific pattern of expression, by a precise intracellular localization and specific interactor/regulators. (Cai 2009). In line with this model is the evidence that specificity in signal transduction is obtained by gathering together signaling proteins in common pathways along with their regulators (Pawson T 1996). Indeed, different DGKs interact with different pools of DAG and binds unique subset of interactors and DAG- or PA- interactiong protein leading to different, sometimes opposed, effects. A typical example of this complexity derives from two higly related type IV DGKs, zeta and iota, that, interacting with different Ras guanyl-releasing protein (RasGRP1 and 3, respectively) have opposing effects on Ras signaling, while DGKzeta while DGKζ deficiency enhances Ras activity, DGKι deficiency reduces it (Topham et al 2001; Regier et al 2006). The already characterized interaction are reported in TableI.

Moreover, the subcellular localization where DGKs are active indicate how Dgks tune DAG- and PA-mediated signals in a strictly spatial-regulated manner.

The presence of nuclear localization signals (NLSs) in type IV DGKs, allow them to regulate DAG/PA levels in the nucleus (Topham, 1998).

Most DGKs are cytosolic in unstimulated cells and translocate to specific membrane compartment upon stimulation, for example, the presence of EFhands and recoverin regions confer Ca2+-sensitivity to type I DGKs, coupling their activation to receptors that elicit PLC (phospholipase C)-mediated Ca2+ elevation, similarly DGKzeta translocate to the plasma membrane upon PKC phosphorylation (Santos 2002).

Table1

Upstream regulator	DGK isoform	Interaction region in DGK	Effect on DGK
Tyr kinase			
Src*	DGKα	?	Tyr phosphorylation, Activation
Src	DGKα	–	Phosphorylation at Tyr-334, Activation
Src*	DGKζ	?	Activation
SFK (Src)	DGKκ	–	Phosphorylation at Tyr-78
Ser/Thr kinase			
cPKC (PKCα)	DGKδ1	–	Phosphorylation at Ser-22/Ser-26 (within the PH do Inhibition of translocation to the plasma membrane
PKCα*	DGKζ	aa 467–605 within C-terminal half of CD	Phosphorylation at MARCKS-PSD (Ser-258/Ser-26 Ser-270/Ser-271), Inactivation, Inhibition of the nuclear localization Enhancement of translocation to the plasma membra Inhibition of interaction with Rac1
PKCγ*	DGKγ	C-terminal half of CD	Phosphorylation at Ser-776/Ser-779, Activation
ERK	DGKζ	ERK docking-site consensus sequence?	Phosphorylation at Ser-768/Ser-773/Ser-776, Inhibition of translocation to the actin cytoskeleton
Second messenger			
Ca^{2+}*	DGKα	EF-hands (K_d=0.3 μM)	Activation
Ca^{2+}*	DGKβ	EF-hands (K_d=~10^{-8} M)	Activation
Ca^{2+}*	DGKγ	EF-hands (K_d=<10^{-8} M)	?
PI3K products ($PI(3,4)P_2$ $PI(3,4,5)P_3$)	DGKα	?	Activation, Translocation to the plasma membrane
PIP5K product ($PI(4,5)P_2$)	DGKβ	?	Activation
Phorbol ester* (DAG?)	DGKβ	First C1 domain (C1A domain)	?
Phorbol ester* (DAG?)	DGKγ	First C1 domain (C1A domain)	Translocation to the plasma membrane?
Receptor			
Leptin receptor* (long form)	DGKζ	Ankyrin repeats	?
Cytoskeleton-related protein including small GTPase			
Syntrophin* (α1 and γ1)	DGKζ	PDZ-binding domain	Recruitment to the actin cytoskeleton
RhoA* (active)	DGKθ	?	Inactivation
Inhibitor of cell-cycle progression			
pRB*	DGKζ	?	Activation
Adaptor			
RACK1*	DGKδ	aa 1015–1097 (DGKδ1)	?
DGK			
DGKs δ1*, δ2* and η2*	DGKδ1	SAM domain	Oligomer formation, Inhibition of translocation to the plasma membrane

Downstream effector	DGK isoform	Interaction region in DGK	Mediator	Effect on effector
Ser/Thr kinase				
PKCα*	DGKζ	aa 467–605 within C-terminal half of CD	DAG	Inhibition
cPKC (α*) and nPKC (δ*, ε*, η*)	DGKδ	?	DAG	Inhibition
PKCβI	DGKβ	–	DAG	Inhibition
PKCγ*	DGKγ	C-terminal half of CD	DAG	Inhibition
mTOR	DGKζ	–	PA	Activation
Regulatory protein of small GTPase				
RasGRP1*-Ras	DGKζ	aa 467–605 within C-terminal half of CD	DAG	Inhibition
RasGRP1-Ras	DGKα	–	DAG	Inhibition
RasGRP3*-Rap1	DGKι	?	DAG	Inhibition
β2-Chimaerin* (RacGAP)	DGKγ	?	PA?	Activation
Small GTPase				
Rac1*	DGKγ	?	–	Inactivation (via β2-chimaerin?)
Rac1*	DGKζ	C1 domains	–	Activation? (via PIP5K Iα?)
Lipid kinase				
PIP5K Iα*	DGKζ	?	PA	Activation
Transcription factor				
NF-κB	DGKα	?	DAG/PA?	Activation (indirectly
Channel				
TRPC2	Type I DGK (α, β, γ)?	?	DAG	Inhibition

Diacylglycerol Kinase Alpha

DGKalpha in the Immune system

One of the system in which DGKalpha is best characterized is the T-Lymphocyte, in which are present high levels of this protein.

in the T-Cell lymphocyte, TCR triggering initiate to a complex signalling network that leads to PLCgamma1 activation, whose DAG is required for localizing and activating PKCalpha and theta, along with PKD ad RasGRP1 (Mathews 2006). This signalling network is tightly regulated in order to avoid an excess of activation, that would lead to lymphoproliferative disordes, to this aim, following

TCR triggering, a number of negative regulators is required for guarantee an adequate control of the immune response.

Part of this negative response is provided by negative regulators of DAG, whose one of these is DGKalpha.

DGKalpha, upon TCR triggering, translocates to the membrane following DAG production and acts as a “switch off” signal for Ras activation, mediated by an impaired localization of the DAG-interacting protein RasGRP1.

At the same time, DGKalpha reduces the membrane localization of another mediator of T-cell activation DAG-dependent, PKC theta.

Negative TCR regulation is not only required for normal T cell activity, but it is also important for another T lymphocyte process, essential for avoiding autoimmune disease, called clonal anergy, which is a state of antigen unresponsiveness induced by TCR stimulation in absence of costimulatory signals.

Anergic T cells present multiple defect in TCR signalling, as defective Ras activation upon TCR stimulation, which is an essential requirment for anergy maintence (Sanjuan 2001).

Accordingly with the model in which DGKalpha functions as a negative regulator of RasGRP, DGKalpha is found to be upregulated in anergic T cells, while in cycling T-cell DGKalpha levels drop sharply. These results correlate with the identification of DGK*α* as an anergy-induced gene (Macian, 2002).

Coherently, DGK*α*-deficient mice are refractory to anergy induction, while primary T-cells overexpressing DGK*α* resemble anergic cells (Olenchock, 2006).

The following step, after TCR activation, is IL-2-dependent clonal expansion, intriguingly, in contrast with its negative role in the regulation of TCR-dependent responses through phosphorylation of PLC-derived DAG, DGK*α*-dependent PA generation by DAG phosphorylation is necessary for IL-2-dependent proliferation.

On the other side, DGKalpha is also required for secretion FasL-bearing lethal exosomes during AICD (activation-induced cell death), through a mechanism that apparently relies on a reduction in the DAG levels needed for vesicle fusion

and transport from the *trans*-Golgi network. This suggests distinct DGK*α* functions depending on its subcellular localization. Whereas down-regulation of early TCR responses depends on DGK*α* plasma membrane localization, regulation of exosome secretion requires enzyme association to internal membrane compartments (Alonso, 2005).

Beside DGKalpha, T cells express the DGK isoforms zeta and delta, but their function appears to be non-redundant, as they have structurally distinct regulatory domain and differ in their subcellular localization.

Anaplastic large-cell lymphomas (ALCLs) are frequently characterized by the presence of a fusion protein bearing the active forms of the anaplastic lymphoma

kinase gene product (ALK). 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. 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), Src kinases, and phosphatidylinositol 3-kinase. The contribution of Src has been recently evaluated in NPM/ALK-positive cell lines and demonstrated through the effects of Src down-regulation and pharmacologic inhibition on cellular proliferative rate. Additional relevant effectors of NPM/ALKmediated lymphomagenesis are represented by signal transducer and activator of transcription 3 (Stat3) and Stat5. Bacchiocchi et al., in collaboration with my laboratory, demonstrated that DGKalpha is constitutively activated, in a Src-dependent manner, downstream of NPM/ALK and is involved in ALK-mediated mitogenic properties.

Defective DGK*α* function is linked to LAP (localized aggressive periodontitis), a genetic disorder characterized by destruction of the supporting structures for dentition. A recent study correlated this pathology with low levels of DGKalpha, that brings to decreases in neutrophil transmigration and increases in

superoxide generation, as superoxide production relies on the phosphorylation of p47phox protein by a DAG-sensitive PKC (Fontaine, 2002).

DGKalpha in epithelial and endothelial cells

Since at lower levels, respect to T cells, DGKalpha is also expressed in epithelial and endothelial cell.

My laboratory was the first to report an involvement of DGKalpha downstream to tyrosine kinase receptors signaling in epithelial cells.

Hepatocyte Growth Factor (HGF), through binding to its tyrosine kinase receptor MET, induces an high number of biological effect, such cellular migration, proliferation, protection from apoptosis and cancer cell invasion.

By stimulating with Hepatocyte Growth Factor (HGF) Cutrupi et al discovered that DGKalpha is activated both in epithelial and in endothelial cells and that its blockade impairs HGF induced motility in endothelial cell.

Consistently with its activation downstream HGF, it has been shown that DGKalpha is also activated in response to VEGF, again in a Src-dependent manner (Baldanzi et al, 2004). DGKalpha activation in endothelial cells downstream VEGF is required for chemotaxis, proliferation and *in vitro* angiogenesi

Taken together, the data produced in both epithelial and endothelial cells suggest that the role of Dgkα downstream of tyrosine kinase receptors accomplishes several biological functions.

While in lymphocyte DGKalpha levels justify the potential role of this enzyme as terminator of DAG- mediated signal in T-cell (Merida, 2007), this way of action has never been reported in endothelial and epithelial cell, where DGKalpha is expressed at low level.

For this reason, in many work on epithelial and endothelial cells it has been proposed a role of DGKalpha as local producer of PA, but only recently target of DGKalpha-derived PA are emerging, such atypical PKC, a class of PKC DAG-

insensitive, but capable to bind PA, thus providing the first molecular mechanism for DGKalpha action in epithelial cell.

In the following part of this thesis will be presentated papers addressing some molecular clue of DGKalpha regulation of DGKalpha, i.e. how it is regulated by Src, and its main targets downstream HGF and v–Src stimulation.

CANCER pathology

Tumours derive from a complex and multi-step evolutionary process that allow cells to escape from multiple levels of tumour suppression mechanisms.
Several of these protective mechanisms are cell intrinsic, such as the genotoxic stress induced by oncogenes, the expression of growth inhibitory, apoptotic and senescence pathways, and telomere attrition; bypassing this tumour suppression strategy is an hallmark of primary tumour development (Massaguè, 2006).
However, an entirely distinct class of inhibitory pressures comes from cell extrinsic mechanisms that act on transformed cells. Limiting factors in the microenvironment of tumour cells are extracellular matrix components, basement membranes, reactive oxygen species, the limited availability of nutrients and oxygen, interstitial pressure, tensional forces and attack by the immune system.
Major alterations in genomic DNA were once viewed as an exclusive trait of advanced cancers. However, it is now recognized that DNA damage and genomic instability are underlying features of human cancer from the earliest stages of tumorigenesis (Feinberg et al., 2006).
Usually, tumour cells develop early genetic instability in order to rapidly evolve to bypass and, paradoxically, take advantage of these extrinsic suppression mechanisms.
For example, hypoxia, in a tumoural environment, becomes a strong selective pressure that can lead to the promotion of the outgrowth of malignant cells with an increased resistance to apoptosis, through hypoxia inducible factor-1a (HIF1-a) stabilization and the subsequent expression of its targets, such as CXCR4 and MET, which are the receptors for two strong inducers of the invasive phenotype, SDF1a and HGF, respectively. Even reactive oxygen species (ROS), that are generated by infiltrating inflammatory cells, can contribute to the genomic instability of cancer cells by inducing the expression

of metastogenic genes (Hussain, 2003). Tumors also exert physical pressures that are different than those of well-organized tissues. For example, tensional forces on mammary epithelial cells during tumorigenesis may result in clustering of mechanotransducing integrins and subsequent downstream activation of ERK and Rho-GTPase, thus promoting cell proliferation and disrupting cell-polarity (Paszek et al, 2005).

A hallmark of cancer malignancy is the acquired ability of cancer cells to invade into neighbouring tissues and survive in these ectopic sites. In the process of invasion, cancer cells enter the circulation from where they can reach distant organs and eventually form secondary tumors, called metastases.

Metastases represent the end point of an exceedingly complex process, which occurs through a series of sequential steps that include the invasion of adjacent tissues, intravasation, transport through the circulatory system, arrest at a secondary site, extravasation and growth in a secondary organ.

In many cases cancer cells hijack the strategies by which the embryo grows and develops. Morphogenesis and metastasis seem to arise from the same genetic programme that instructs cells to detach from a primary colony, cross tissue boundaries, adhere to and migrate through extracellular matrices, and escape death caused by an unfamiliar tissue context (a process known as anoikia).

As stated before, metastasis is a very complex process and, fortunately, highly inefficient, since really few cells in proportion to primary tumour possess all the prerequisites to act as “cancer-initiating cells”, thus successfully completing all the steps listed above (Fig 3) and producing a distant metastasis.

In this introduction I will describe only a few steps of this process, the ones necessary to contextualize and understand the articles concerning DGKalpha presented in this section.

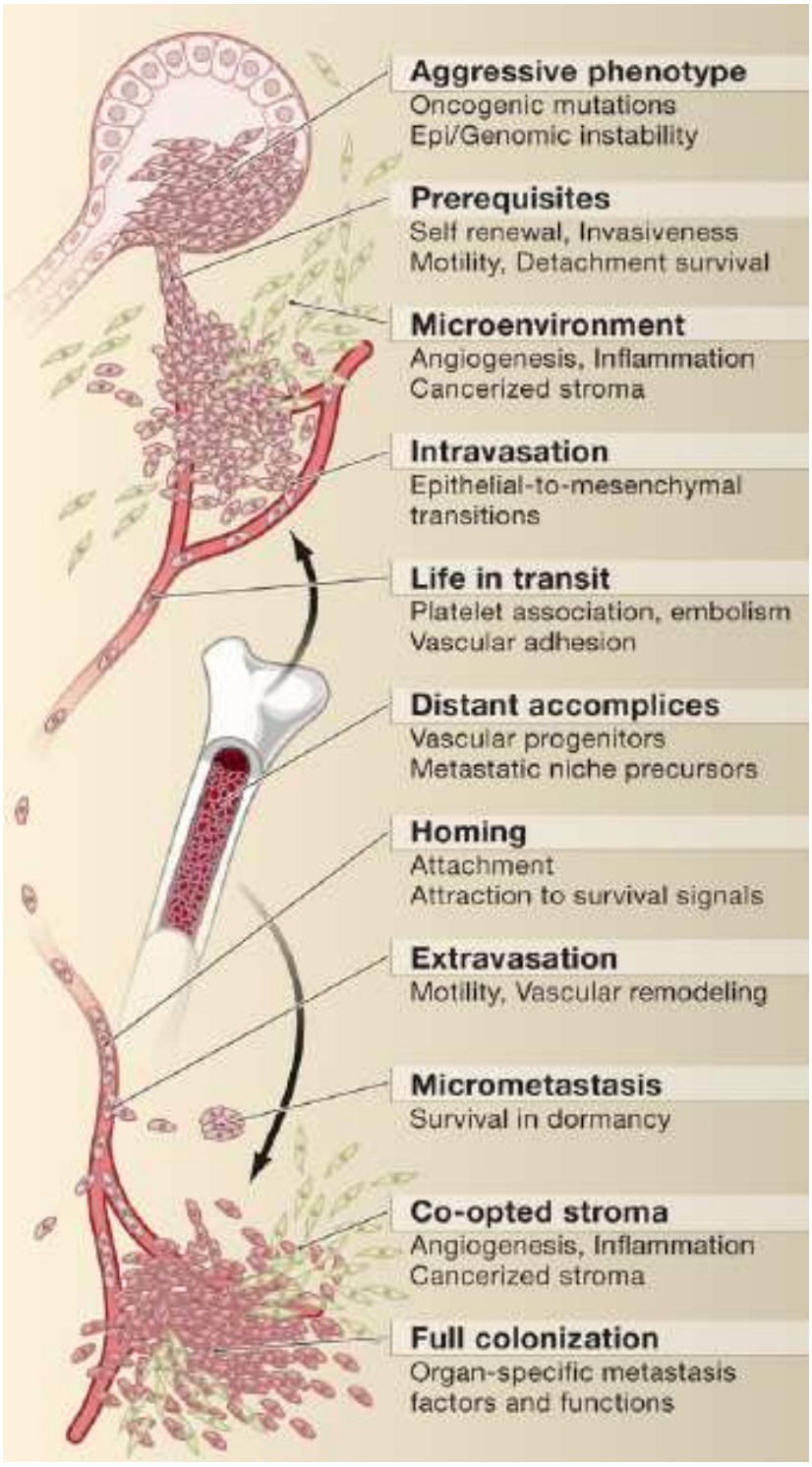


FIG 3. Stages of metastatic progression (from Massaguè, 2006).

Oncogenes and tumour suppressor: v-Src as prototype

A series of alterations in the genome of the original cell population forms the basis for tumor development. The genes of interest are classified as oncogenes (or tumor-promoter genes), one allele of which is activated leading to gain-of-function events, and tumor-suppressor genes (or antioncogenes), both alleles of which are inactivated leading to loss-of-function events. The products of these genes belong to various classes of protein families, such as cytokines, cell surface receptors, signal transducers, and transcription factors. The list of oncogenes encoding cell surface receptors of the protein-tyrosine kinase family alone counts more than forty members. Mechanisms of activation of oncogenes include mutation, gene amplification and promoter activation. Mechanisms of tumor-suppressor inactivation are exemplified by loss of heterozygosity plus silencing of the second allele either genetically, through mutation, or epigenetically, through methylation. In familial cancers, one mutation is carried with the germline.

The viral protein v-Src, the first recognized tyrosine kinase, was the first defined oncogene, and its cellular couterpart, c-Src, , was the first protooncogene to be discovered in the vertebrate genome (Martin, 2001).

The Src protein is anchored to the plasma membrane through myristoylation, is target of various signals, in its turn signals directly and indirectly to many substrates, and is implicated in numerous cellular functions, including proliferation, motility, as well as cell-cell and cell-substrate adhesion (Fig 4).

Cell Migration

Cell migration plays a key role in both normal physiology and various disease processes.

Metastasis process fundamentally involves the movement of cells from one site to another. A molecular description of cell migration in “in vitro” models is constituted by dynamic cytoskeletal changes, cell-matrix interactions, localized proteolysis, actin-myosin contractions, and focal contacts disassembly (Friedl and Wolf, 2003).

Key point of this process is the regulation of integrin-containing focal adhesion assembly and disassembly, polymerization of actin, formation of actin stress fibres, secreted and plasma membrane-tethered proteases, and the actomyosin contractile machinery.
Many forms of motility are dependent on the small GTPases Rho, Rac, Cdc42 and Ras, which are essential for the control of the actin assembly/disassembly regulating cell movement. Through their multiple target proteins, the function of these GTPases is not restricted to migration but also involves adhesion and proliferation. Whereas Rac controls protrusion of lamellipodia and forward movement, Cdc42 maintains cell polarity and Rho mediates the cell-substrate adhesion needed for migration and stabilizes microtubules that are oriented toward the leading edge.

Basement membrane and beyond: chemokine, growth factors and metalloproteinases

For epithelial structure integrity, it is essential a well-organized basement membrane, which provides both a physical boundary, as well as a signaling substrate to orient cells through integrin-based adhesions. For early-phase epithelial tumors, the basement membrane acts as a barrier to the invasion of transformed cells into the subjacent stroma. The critical event of tumor invasion is the interaction of the neoplastic cells with the basement membrane (BM). The BM is composed of type IV collagen, laminin, and heparan sulfate proteoglycan as its major components and tumour cell orchestrates degradation of these components by proteases, in particular metalloproteinases.
Matrix metalloproteinases (MMPs) have long been associated with many types and stages of cancer, and were thought to be essential for BM penetration during metastasis. The strong causal relationships between MMPs overexpression and tumour progression have been generally supported by more recent mouse models of cancer, such as mice that transgenically overexpress MMPs or MMPs-knockout mice.

However, considering MMPs only as matrix corroders is misleading , because it has emerged that them promote tumour progression not only through BM degradation, , but also through signalling functions. MMPs may indeed generate many bioactive peptides through proteolysis that promote angiogenesis, modulate immunity and stimulate tumour growth (McCawley, 2001; Overall, 2006).

During the process of invasion, cancer cells need the collusion of many not-transformed cells recruited to the tumor stroma, such as cancer-associated fibroblast and macrophages.

These cells are responsible for the secretion of many metastogenic factors, such as HGF and SDF1a (Condeelis, 2006). These factors are well-known powerful inducers of metastasis, since they induce the EMT and MMPs secretion. In addition, the signalling pathways elicited by HGF and SDF-1a are required to sustain tumour growth at different sites. (Corso, 2007). In particular, it has been reported that the expression of CXCR4, i.e. SDF1a receptor, along with other chemokine receptors, is required to sustain breast cancer outgrowth and metastasis to lung and bone (Muller, 2001).

Introduction to

"Diacylglycerol Kinase is Required for HGF-induced Invasiveness and Anchorage-independent Growth of MDA-MB-231 Breast Cancer Cells"

by

Filigheddu N, Cutrupi S, **Porporato PE**, Riboni F, Baldanzi G, Chianale F, Fortina E, Piantanida P, De Bortoli M, Vacca G, Graziani A, and Surico N.

Anticancer Research (2007) 27

ER-negative breast cancer tumour represent an open challenge in tumour therapy, as they have a worse prognosis respect to ER-positive cancer.

Usually, ER-negative breast cancer, rely on other stimuli for survival and tumour progression, among these Hepatocyte growth factor (HGF) plays a well-known role in the process of tumor invasion and metastasis. HGF stimulates proliferation, dissociation, migration, invasion and resistance to anoikis in a wide variety of tumor cells, and is a potent angiogenic factor (Di Renzo MF, 1991; Bocaccio 2006).

In the following paper, by using the pharmacological inhibitor R59949 we impaired MET signalling by inhibiting HGF induced invasion, intriguingly, pharmacological treatment inhibit also growth without anchorage, an essential requirement for tumour dissemination.

Unfortunately to identify the DGK isoform involved in this process has not been determined.

However, the data presented, beside underscoring the biological relevance of Dgk signaling in growth factor-elicited cell migration, suggest that DGKs might be suitable for the development of novel molecular strategies to selectively target cancer progression.

Introduction to

"Diacylglycerol Kinase-α mediates HGF-induced epithelial cell scatter by regulating Rac activation and membrane ruffling"

by

Chianale F*, Cutrupi S*, *Rainero E, Baldanzi G,* ***Porporato PE****, Traini S, Filigheddu N, Gnocchi VF, Santoro MM, Parolini O, van Blitterswijk WJ, Sinigaglia F and Graziani A.*

***equal contribution of the two Authors**

Molecular Biology of the Cell *18 (**2007**) 4859-71*

Epithelial-mesenchymal transition (EMT) is a well characterized process required for physiological embryonic development, as well as in tissue repair, in which epithelial cell line transiently acquire high motility. Transformed epithelial cell commonly undergo EMT in order to develop tumour progression and became metastatic. *In vitro*, epithelial cells colonies, in presence of growth factors or activated oncogenes, can acquire motility and disperse themselves with a process called cell scatter, which recapitulates the early phases of EMT.

HGF and activated Src induce *in vitro* cell scatter, while their uncontrolled activation, *in vivo*, is associated to progression and acquisition of a metastatic phenotype in several epithelial-derived cancer (Avizienyte, 2005). We have previously identified DGKalpha as required for epithelial cell motility upon HGF stimulation, but without identifying a specific signalling pathway. Due to the demonstration of the crucial role played by DGKalpha downstream of tyrosine kinase receptors in the contest of cell migration, we analyzed the effect of DGKalpha inhibition on several well-characterized morphological changes and signaling cascades upon HGF- and vSrc-induced migratory signal.

In the present work we show that DGKalpha inhibits HGF- and oncogenic Src-mediated cell scatter and we demonstrate for the first time that DGKalpha is involved in the specific subset of events triggered by HGF contributing to

spreading and early protrusion of membrane ruffles, while it is not involved in the disruption of E-cadherin-mediated cell-cell adhesions.

Finally, we found that DGKalpha inhibition leads to defective HGF-induced Rac activation and localization to the plasma membrane which is an essential hallmark of cell migration thus identifying DGKalpha as a link between tyrosine kinases receptors to small GTPases regulation in the context of cell migration.

Introduction to

"Diacylglycerol kinase-a phosphorylation by Src on Y335is required for activation, membrane recruitment and Hgf-induced cell motility"

by

G Baldanzi, S Cutrupi, F Chianale, V Gnocchi, E Rainero, **P Porporato**, N Filigheddu, WJ Blitterswijk, O Parolini, F Bussolino, F Sinigaglia and A Graziani

Oncogene. 2008 Feb 7;27(7):942-56

It has been reported that HGF- VEGF- ALK- induced activation of DGKalpha is mediated by Src Kinase (Cutrupi, 2000; Baldanzi, 2004; Bachiocchi, 2005) and that this Src-mediated activation is required for factors or oncogene- induced cell scatter and proliferation, but, despite this previous knowledge the details of such activation were missing for a long while.

In the present paper (Baldanzi et al, 2004), we analyzed the molecular determinant of DGKalpha required its Src- mediated activation.

Through DGKalpha sequence analysis we identified a tyrosine, Y335, as potential target of Src phosphorylation and a proline-rich C-terminal region that, along with the phosphorylated tyrosine 335, would interact with SH3 and SH2 domain respetively.

By generating mutant construct for these two site, named respectively Myc-DGKalpha-Y335F and Myc-DGKalpha-DP, we characterized their biological role.

Firstly we demonstrated that DGKalpha Tyrosine 335 and proline-rich C-terminal sequence are required for Src-induced tyrosine phosphorylation of DGKalpha, and for interaction respectively with Src-SH2 and -SH3 domain and then that both sequence are required for HGF- and v-Src-induced enzymatic activation of DGKalpha. Then we showed that mutant lacking these sequence are unable to translocate to the plasma membrane, but, intriguingly, both mutant remain associated with intracellular vescicles.

Finally, phosphorylation of DGKalpha on tyrosine 335, its translocation to the plasma membrane and its activation are shown to be required for HGF-induced cell migration, while constitutive recruitment of DGKalpha at the plasma membrane by myristoylation is sufficient to trigger spontaneous cell motility in absence of HGF.

Thus, in this paper are provided significant progress in the understanding the mechanism that regulate DGKalpha, individuating two DGKalpha domain required for the transduction of migratory signals mediated by Src activation. (Baldanzi et al, 2008)

Preview of

"Diacylglycerol Kinase-alpha mediates SDF1a- and HGF-induced breast cancer cell invasion and MMPs activity by atypical PKCζ/ι"

by

Porporato P, Rainero E, *Chianale F, Ranaldo G, Locatelli I, Gaggianesi M, Filigheddu N,Baldanzi G, Sinigaglia F and Graziani A.*

In this part I also briefly summarize related results still awaiting to be fully developed in order to be considered for publication as well.

As reported before, DGKalpha is known to be connected to many hallmarks of tumour development such as angiogenesis (Baldanzi, 2004), proliferation (Bachiocchi, 2005) and migration (Chianale, 2007), albeit the belief that DGKalpha is required for tumour progression still lacks a definitive proof.

In order to investigate this hypothesis, we decided to analyze whether DGKalpha was involved in the signalling pathway downstream SDF1a, a chemokine which has been identified as a potent inductor of metastasis in many cancer, and specifically required in breast cancer pathology (Muller, 2000; Lapteva, 2005).

By transfecting Myc-DGKalpha in HeLa, in MDA-mb-231 cells and MDCK, we discovered that it is potently activated and that it translocates to the plasma membrane upon SDF1a stimulation. These observations prompted us to investigate the role of DGKalpha in this signalling pathway.

As SDF1a-mediated invasive phenotype is particularly potent in breast cancer, we decided to study DGKalpha biological role in a well known and highly aggressive breast cancer model derived from pleural effusions in a patient with widely metastatic tumour, i.e. MDA-MB-231 cell line.

Through the generation of a lentivirus expressing a Short Hairpin RNA (ShRNA) specific for human DGKalpha, we produced two cell lines derived from MDA-

MB-231: one mock-infected (CTR231) and the other one characterized by the constitutive knock down of DGKalpha (Sh231). Along with this approach of inhibition we used also a pharmacological Dgk inhibitor (R59949), which allows inhibition of Dgk enzymatic activity without depleting cells from the protein itself, and transient expression of specific siRNA directed toward DGKalpha isoform, which may avoid from compensatory mechanisms potentially induced upon stable knock-down of a specific isoenzyme.

We thus unveiled that DGKalpha is required for SDF1a- and HGF- induced metalloproteinases (MMPs) secretion and invasion across a matrigel basement, thusa first strong indication that DGKalpha is required for tumour invasion, at least in an in vitro model.

Morover, we showed that DGKalpha is required for the localization at the plasma membrane of atypical PKC zeta, a well-known mediator of SDF1a -induced invasion and MMPs secretion.

Intriguingly, it has been reported that PKCzeta is activated upon binding to phosphatidic acid. Thus we investigated the hypothesis that PA produced by DGKalpha may be a crucial lipid signal mediating the targeting of PKCzeta to specific membrane compartment at the leading edge, where PKCzeta could promote SDF1a-induced secretion of MMPs. Indeed, DGKalpha inhbition, by ShRNA,SiRNA or pharmacological inhibitor, impahirs SDF1a and HGF induced PKCζ plasma membrane localization. These results, for the first time, unveil a PA-dependent molecular mechanism by which DGKalpha might exert its pro-invavive activity.

Finally, encouraging, even preliminary, results comes from in vivo experiment in which MDA-MB-231 cells have been injected in the mammary fat pad of nude mice in order to detect spontaneous metastasis. In a first pilot study, we found that Sh231 and CTR231 did not display significative differences in primary tumor growth. However, Sh231 cells failed to produce lung metastasis in contrast to CTR231, which conversely displayed a strong metastatic phenotype.

PART II:
DES-ACYL GHRELIN, GHRELIN AND SKELETAL MUSCLE

Ghrelin, GHSR1a, and Ghrelin-related peptides

In the 70s, several new compounds that stimulate and amplify pulsatile growth hormone (GH) secretion, independently from growth hormone releasing hormone (GHRH), were synthesized. The family of these molecules, named growth hormone secretagogues (GHSs; Smith RG, 1993), act through binding and activation of the GHS receptor (GHS-R1a), a G protein-coupled receptor with seven transmembrane domains identified several years later (Howard, 1996). Finally, the endogenous ligand of GHSR-1a was identified by Kojima and coworkers who named it ghrelin (Kojima, 1999).

Ghrelin is a peptide of 28 amino acids with the sequence highly conserved among various species.

Although the X/A-like cells within the oxyntic glands of the gastric fundus mucosa are mainly responsible for the production of ghrelin (Date et al, 2000), its expression at low levels is present in many other tissues, such as heart, pancreas and brain, suggesting a local paracrine and/or autocrine activity on cells throughout the body.

Ghrelin is synthesized from prepro-ghrelin, a 117 amino acids precursor, through proteolytic cleavage.

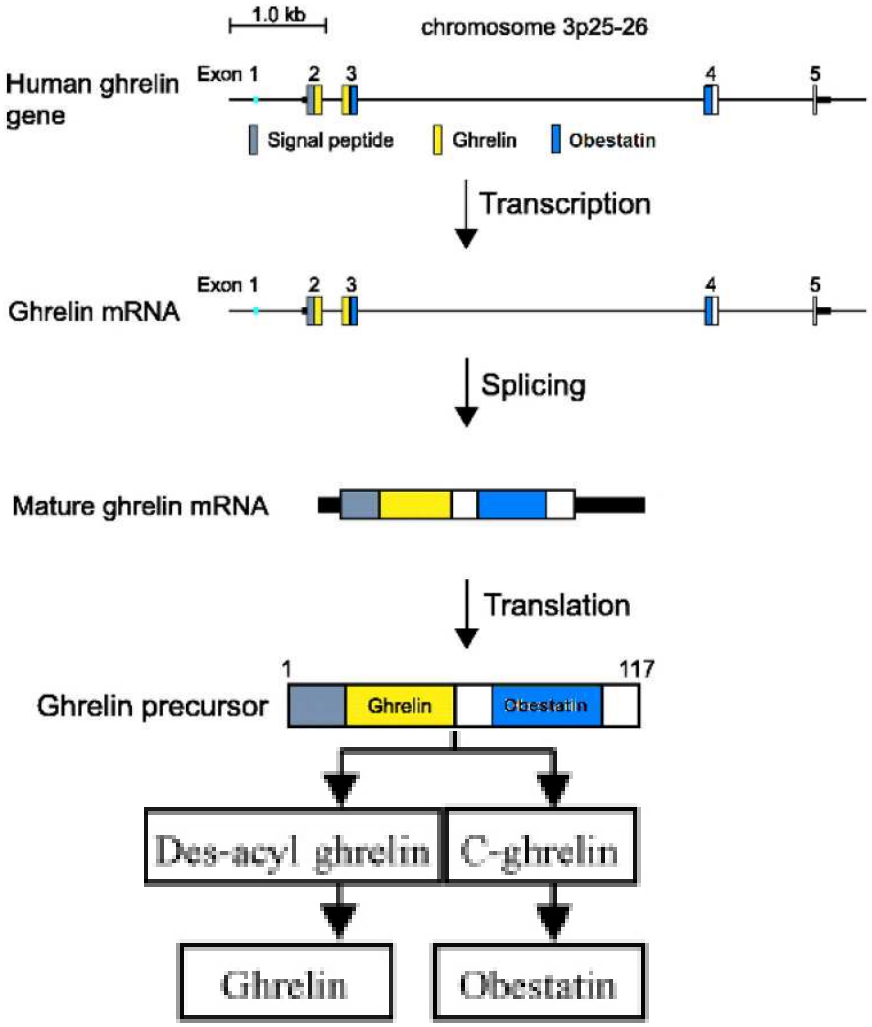
Ghrelin exists in two different isoforms: the first one has an unique post-translational modification, an acylation with octanoic acid on the Ser3 (GHR); the second isoform lacks the octanoylation and is therefore called des-acyl ghrelin (des-acyl GHR) (Fig 1). Acylation of Ser(3) permits ghrelin to cross the blood-brain barrier and is essential for binding its receptor. (Kojima, 1999).

The enzyme responsible for the octanoylation of GHR has been recently identified (Gutierrez et al, 2008; Yang J. et al, 2008). It is a hydrophobic intracellular membrane-bound acyltransferase, named “GOAT” (Ghrelin-O-AcylTransferase). It has been verified that in vitro this enzyme acylates, in intracellular compartments, des-acyl GHR to ghrelin in various endocrine cells. However, its expression in vivo was detected only in the stomach, intestine, and testis.

Figure 1. Human ghrelin primary structure, octanoylation at Ser3 (modified from Kojima M. and Kangawa K., 2005).

Ghrelin, moreover, has been shown to exist in other forms, rarely, it may be synthethized a variant form lacking of a Gln at position 14, or it may be cleaved to a 27 aa form. More frequently, however, it may be acylated by decanoyl (C10:0) or decenoyl (10:1) groups istead by the common octanoic acid. Additionally, ghrelin, both in its acylated and unacylated forms, is not the only product of ghrelin gene. Indeed, it has been recently found that from the same pre-pro peptide, named obestatin, is obtained (Zhang et al, 2005 Fig 2).

Obestatin is 28 aminoacids peptide in the pre-pro peptide sequence and it was initially reported to bind to GPR39, an orphan G protein coupled receptor, and to counteract ghrelin effects on food intake. However, the finding of its receptor was not confirmed by further investigation from other groups (Holst et al, 2006). In addition, even its capability to inhibit food intake has been matter of controversy, as some studies reported no activity on food intake, recent studies have partially clarified this issue. In one of these studies, intraperitoneal obestatin suppressed food intake and body weight gain in rodent in an U-shaped dose–response relationship. This relationship may explain the difficulties in reproducing the effects of obestatin on feeding reported by some groups (Lagaud, 2007).


1.0 kb
chromosome 3p25-26
Human ghrelin gene
Exon 1
2
3
4
5
Signal peptide
Ghrelin
Obestatin
Transcription
Ghrelin mRNA
Splicing
Mature ghrelin mRNA
Translation
1
117
Ghrelin precursor
Ghrelin
Obestatin
Des-acyl ghrelin
C-ghrelin
Ghrelin
Obestatin

Ghrelin and Des-acyl Ghrelin functions

Local actions of ghrelin on the digestive system include the control of acid secretion and gastric motility (Masuda et al, 2000; Kohno et al, 2003) and influences on pancreatic activity (Arosio et al, 2003). Through binding to the GHSR-1a in the hypothalamus and the pituitary GHR induces the release of GH, stimulates food intake and adiposity (Kojima et al, 1999; Kohno et al, 2003; Reimer et al, 2003). The studies on activity of partially digested ghrelin and its derivatives revealed that the N-terminal portion, consisting of first 4-5 residues, is the active core of the molecule (Matsumoto, 2001) able to bind and activate GHSR-1a.

Circulating ghrelin is higher during starving and its level decreases soon after food intake; interestingly though, ghrelin levels are low in obese humans and rodents (Ogawa et al, 1999; Ariyasu et al, 2002).

Despite different mechanisms of action, the overall GHR activities highlight a general positive contribution to the whole body metabolism, i.e. GH release stimulation, appetite induction and fat accumulation. Moreover, GHR is capable of inducing hyperglycaemia and inhibit insulin secretion in humans, and directly modulate glucose metabolism at the hepatic level, as it is able to induce glucose output from pig hepatocytes. (Granata, 2007)

For all those reasons GHR is considered an inductor of positive energy balance (Asakawa, 2005).

Moreover, ghrelin, besides the GH-releasing activity, elicits several actions other central activities, including inhibitory effects on the gonadotropic axis, interfering with LH pulsatile secretion, acceleration of colonic motility and increase of pancreatic protein secretion (reviewed in Soares et al, 2008).

In addition to its metabolic and endocrine actions, in vivo GHR features several activities in the cardiovascular and muscular systems, as it improves left ventricular function in patients with Chronic Heart Failure (CHF) (Nagaya N. et al, 2001 and Nagaya N. et al, 2004), ameliorate CHF-, chronic kidney disease- and arthritis-induced cachexia and its plasma concentration is increased in

cachectic patients (Nagaya N. et al, 2001; Deboer M.D. et al, 2008; Granado M. et al, 2005 and Nagaya N. et al, 2001).

Interestingly, the circulating des-acyl ghrelin (D-GHR) is far more abundant than GHR (10:1 ratio). Des-acyl GHR, not binding the GHSR-1a and not releasing GH, was initially considered as a circulating reservoir of inactive GHR. However, an increasing body of evidence indicates that D-GHR shares with GHR many biological activities and common binding sites on several peripheral tissues and cell types.

However, both GHR and D-GHR recognize common binding sites in H9c2 cardiomyocytes and in C2C12 skeletal myoblasts, where they respectively stimulate anti-apoptotic and differentiative signaling (Baldanzi et al., 2002; Filigheddu et al. 2007). Moreover, GHR and D-GHR common binding sites have been detected in breast and prostate carcinoma cells as well as HIT-T15 pancreatic beta cells (Jeffery et al., 2002; Cassoni et al., 2001; Granata et al. Endocrinol 2007). In addition, both GHR and D-GHR elicit a wide range of common biological responses such as *i)* activating anti-apoptotic signaling in several cell types, including cardiomyocytes, endothelial cells, cortical neurons, pancreatic beta cells (Baldanzi, 2002, Granata, 2007), *ii)* stimulating neurogenesis and proliferation of osteoblasts and adipocytes precursors (Fukushima, 2005; Maccarinelli et al., 2005; Delhanty et al., 2006; Nanzer et al., 2004) Kim SW et al., 2005; Choi et al., 2003; Zhang et al., 2005), *iii)* inducing growth arrest and terminal differentiation of skeletal myoblasts and Leydig cells (Barreiro et al., 2004; Filigheddu et al., 2007).

Up to date, no receptor for these activities has been identified, but it has been reported that its activity requires GalphaS function (Granata 2007).

Skeletal Muscle Differentiation

Skeletal muscle is composed by many myofibres bundled together and wrapped in a connective tissue covering.

The myofibre is the functional, contractile unit of the skeletal muscle. It is a syncytial cell with a high number of post-mitotic myonuclei derived from the massive fusion of myoblasts during development and muscle formation. Skeletal muscles vary considerably in size, shape, and arrangement of fibres. The skeletal muscle has a typical striated appearance when observed through a microscope, due to the peculiar organization of its major components, actin and myosin, in the myofibres. The coordinated slipping of actin and myosin one on the other is a highly regulated and energy (ATP) consuming process that allows the contraction and decontraction of the muscle and, thus, its function. Moreover, different muscles have different isoforms (or combination of isoforms) of the protein myosin, depending on the function they have to exert. Based on the myosin isoforms expressed and the consequential contractile characteristics, the muscles can be classified as slow, fast or combination of the two (reviewed in Wigmore and Evans, 2002).

In normal conditions, the number of fibres in an adult, completely developed skeletal muscle does not change significantly, while its protein content, and therefore its mass, may vary significantly during adult life, depending on several factors such as use, nutrients, and signalling molecules, leading up to hypertrophy or atrophy.

Although in physiological condition skeletal muscle does not undergo massive turnover, it has a certain degree of regenerative potential, which is required in particular conditions, e.g. muscle injury.

Many cell types have been proposed to be mediators of muscle regeneration, such as mesoangioblasts (Sampaolesi et al, 2006), pericytes (Dellavalle et al, 2007) or bone marrow-derived stem cells (LaBarge and Blau, 2002), but the

main physiological source of new myofibres during regular turnover or following a muscle injury, are satellite cells.

Satellite cells are the resident precursor cells of the skeletal muscle. Lying quiescent on the myofibre, under the basal lamina, they are able of massive regeneration when required (Zammit et al, 2002).

Satellite cells are in a reversible quiescent state: upon specific triggering signals, they can activate, proliferate, differentiate and fuse together or with pre-existing myofibres allowing the tissue to renew. The processes of activation, proliferation and differentiation of the satellite cell pool are highly efficient; despite satellite cells account for only the 1-4% of the total nuclei on a single myofibre (Bischoff, 1994), following a muscular injury they can fully regenerate the damaged tissue in a time that depends upon the type and the extension of the injury, but in the order of a few days (Zammit et al, 2002). Although the specific signals able to trigger the satellite cells response have not been fully elucidated yet, the major steps leading to regeneration have been defined: first, the satellite cell exits the mitotic quiescence and become activated.

A first division takes place within the first 24 hours from activation and a massive proliferation follows. Next, the newly formed myoblasts have to arrest their proliferation again in order to undergo terminal differentiation. The final step is fusion, which allows the production of new, multinucleated myofibres (reviewed in Zammit et al, 2006).

In the past years, a valuable model to study skeletal muscle differentiation, i.e. C2C12 cell line, has been extensively used. C2C12 are proliferating mouse myoblasts, which still retain a certain degree of multipotency, as treatment with bone morphogenic protein 2 (BMP-2) causes a shift in the differentiation pathway from myoblastic to osteoblastic (Katagiri et al, 1994). By allowing them to get confluent and switching the medium to low serum, they undergo terminal muscular differentiation (Blau, 1985). Firstly, they express early differentiation markers, such as MyoD and myogenin, then irreversible cell cycle withdrawal follows. Finally, C2C12 monocytes fuse together, giving rise to multi-nucleated myotubes (Andrés and Walsh, 1996), closely mimicking the myofibre (Fig 3).

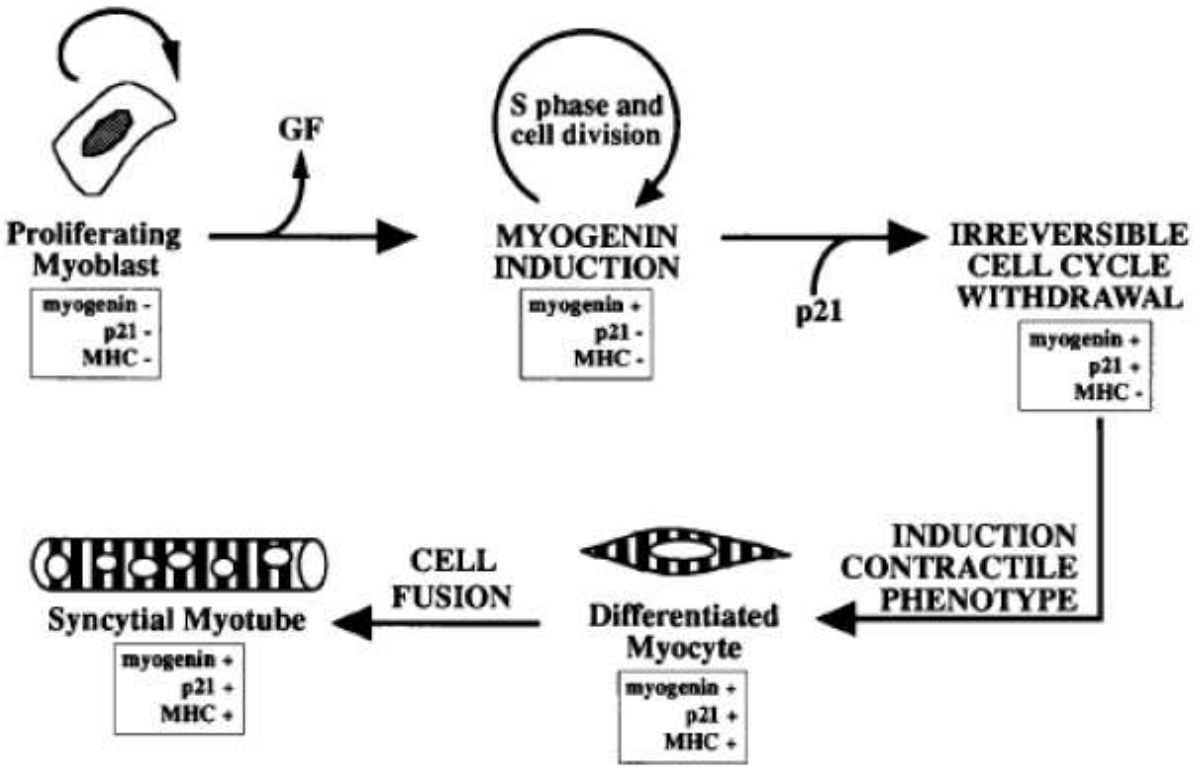


Fig. 3. C2C12 differentiation steps that give rise to terminal-differentiated myotubes. Growth factors (GF) withdraw and low-in-serum medium addition induce C2C12 differentiation (from Andres V. and Walsh K., 2007).

Indeed, C2C12 muscular differentiation process recapitulates the physiologically occurring events in satellite cells following a triggering signal and have been widely used to study the skeletal muscle *in vitro*.

Skeletal Muscle Atrophy

Skeletal muscle atrophy, i.e. the loss of skeletal muscle mass and strength, is a hallmark of several cachectic syndromes, including congestive heart failure, chronic obstructive pulmonary disease (COPD), severe burns and cancer. Moreover, muscle atrophy has been associated to other conditions, such as prolonged fasting, limb immobilization and accidental denervation, and it is also a dose-limiting side effect for the treatment with synthetic glucocorticoids.

Skeletal muscle atrophy has been often associated to a worse prognosis and lower quality of life. Accordingly, it has been reported that in cancer patients 20% of deaths is imputable to severe muscle waste (Tisdale, 2002).

Skeletal muscle mass and functionality are strictly dependent on the balance between atrophic and hypertrophic signals. In the presence of systemic diseases, the balance is shifted towards an atrophic condition, characterized by loss of myonuclei (apoptosis), accelerated proteolysis and reduction of fiber cross sectional area (CSA), which, consequently, causes muscle weight decrease and loss of functionality. By contrast, upon the prevalence of hypertrophic factors, the balance is shifted to an opposite state, characterized by myonuclear addition through satellite cells activation and augmented protein synthesis, which increases muscle mass and myofiber CSA (Fig 4).

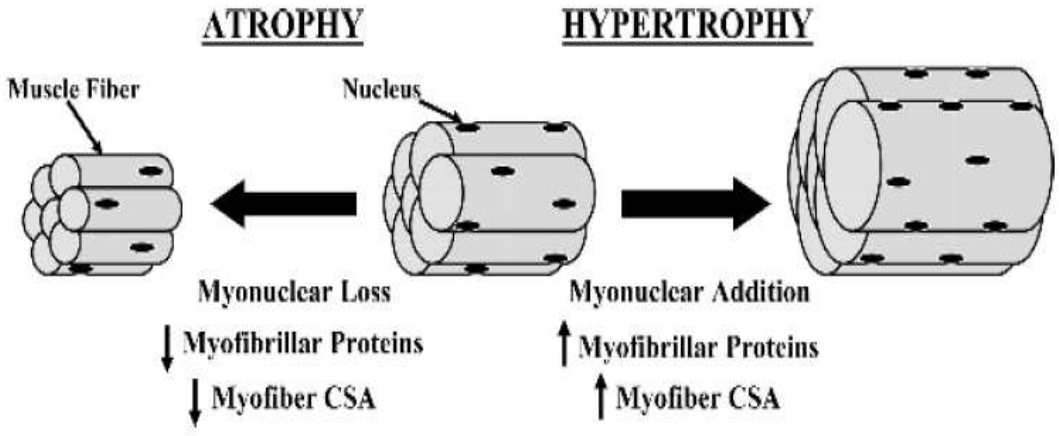


Fig. 4 Skeletal muscle mass homeostasis as balance between atrophic and hypertrophic signals (Powers S.K., 2007)

One of the major contribution to the characterization of the atrophic process in skeletal muscle comes from a work published in 2001 by David J Glass and coworkers, who, through the comparison of genes expression between normal and atrophying skeletal muscle, identified a common subset of genes that are up- or down- regulated.

More recently, the molecular changes occurring during atrophy have been deeply investigated by cDNA microarrays experiments comparing four different, not-correlated, atrophy-inducing conditions in mice or rats (fasting, cancer, renal failure, diabetes mellitus). This study revealed that in all types of atrophy-inducing conditions, a common set of 120 genes is co-ordinately regulated (Lecker et al, 2004). These genes, believed to regulate the loss of muscle components, or at least to be indicative of the atrophic process, were called atrophy-related genes or “atrogenes”.

Among these atrogenes, the two mainly up-regulated are two muscle-specific ubiquitin ligases, atrogin-1 (also known as MAFbx) and MuRF1, that are responsible for the increased protein degradation through the ubiquitin-proteasome system.

Intriguingly, knockout mice for either atrogin-1 or MuRF1 are partially resistant to denervation-induced atrophy (Bodine, 2001), while their over-expression is sufficient to induce muscle atrophy. Thus, these two genes are the best markers for the ongoing muscle atrophy process and can be considered as master genes of muscle wasting. However, several other genes among the atrophy-related genes are of potential interest, including genes coding for transcription factors, lysosomal degradation, regulators of protein synthesis, and enzymes of metabolic pathways, but their particular role in muscle wasting has to be fully elucidated, yet.

In particular, recent works directed to better understand the other main mediators of atrophy along with the ubiquitin-proteasome pathway, i.e. autophagy, found common regulators of both pathways (Mammuccari, 2008).

Muscle Atrophy Regulation

Several extracellular signals have been associated to the modulation of skeletal muscle atrophy, as shown in figure 5.

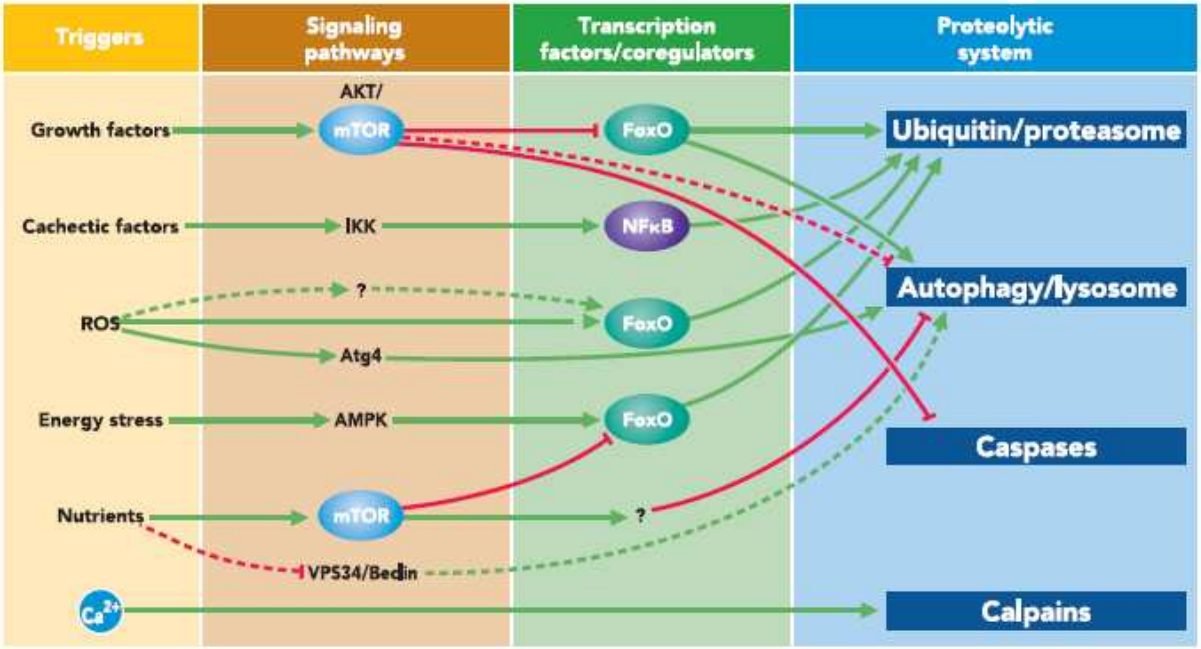


Fig 5 Schematic representation of the main regulator of muscle atrophy (Sandri et al, 2008)

Moreover, glucocorticoids are elevated in many pathological conditions associated to muscle wasting, and indeed, they are able to induce MuRF-1 and Atrogin-1 induction, thus leading to muscle atrophy, both in vitro and in vivo (Sandri 2007). However, the mechanism triggered by glucocorticoids is still unclear, since, none of the atrophy-related genes have been found to be directly regulated by glucocorticoids, and no glucocorticoid response elements on

atrogenes promoters have been identified as critical for their expression (Lecker, 2004).

However, although not directly, glucocorticoids have been reported to decrease IGF1 circulating levels, thus providing a molecular explanation for the induced atrophic process.

IGF1 has been indeed identified as one of the principal player in controlling muscle mass homeostasis, since, while promoting muscle growth, it is able to suppress protein breakdown (Musaro 2001; Stitt, 2004). Indeed, IGF1 transgenic mice are resistant to muscle atrophy induced either by angiotensin treatment, or in a mouse model of cardiac cachexia, and local IGF1 injection is sufficient to block disuse-induced atrophy. In these models of muscle loss, IGF1 completely suppressed the induction of the two critical ubiquitin-ligases, i.e. Atrogin1 and MuRF1 (Song,2005; Stitt, 2004).

The upregulation of Atrogin-1 and MuRF1 may be completely blocked by Akt activation which, in turn, negatively regulates the activity of FoxO family of transcription factors, and indeed this is the molecular mechanism trough which IGF-1 exerts its anti-atrophic function. The FoxO family in skeletal muscle is represented by three isoforms: FoxO1, FoxO3, and FoxO4. Akt phosphorylates FoxOs, blocking its translocation to the nucleus and promoting its degradation through ubiquitination (Fig 6).

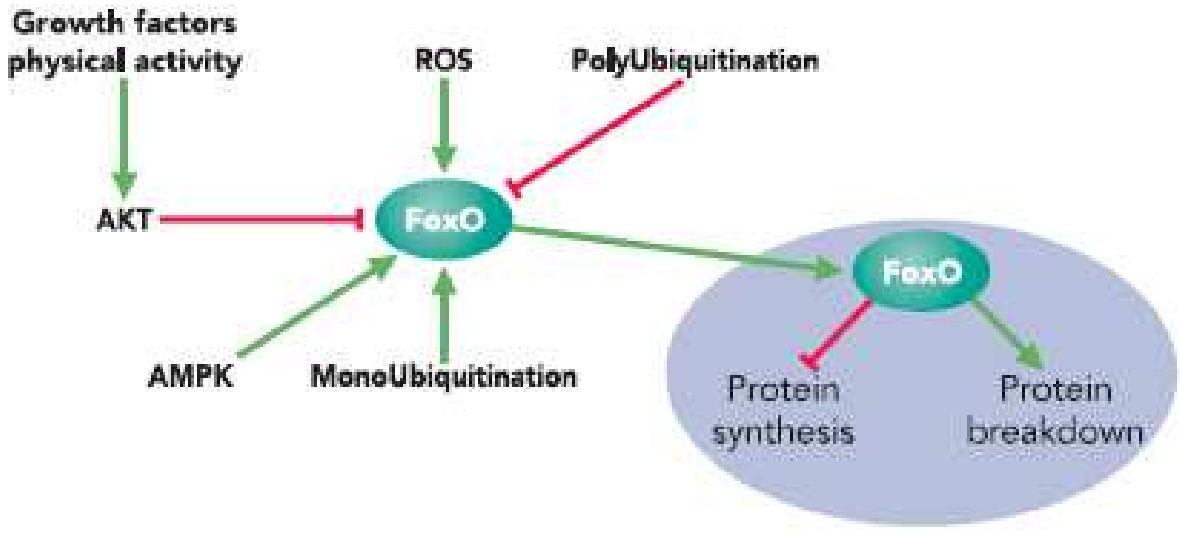


Fig 6. FoxO transcription factor regulation and role in the atrophy program

Accordingly, the reduction in the activity of the Akt pathway observed in different models of muscle atrophy results in decreased levels of phosphorylated FoxO in the cytoplasm and in a strong increase of nuclear FoxO protein. The translocation and activity of FoxO members is required for the upregulation of atrogin-1 and MuRF1, and FoxO3 was found to be sufficient to promote atrogin-1 expression and muscle atrophy when transfected in skeletal muscles in vivo. Moreover, FoxO3 has been identified as absolutely required for the induction of autophagy during atrophy.

In conclusion, FoxO family members, and especially FoxO3, are one of the principal mediators of atrophy, while Akt is one of the principal inhibitors of this process (Fig 6; Sandri, 2004; Bodine, 2001b).

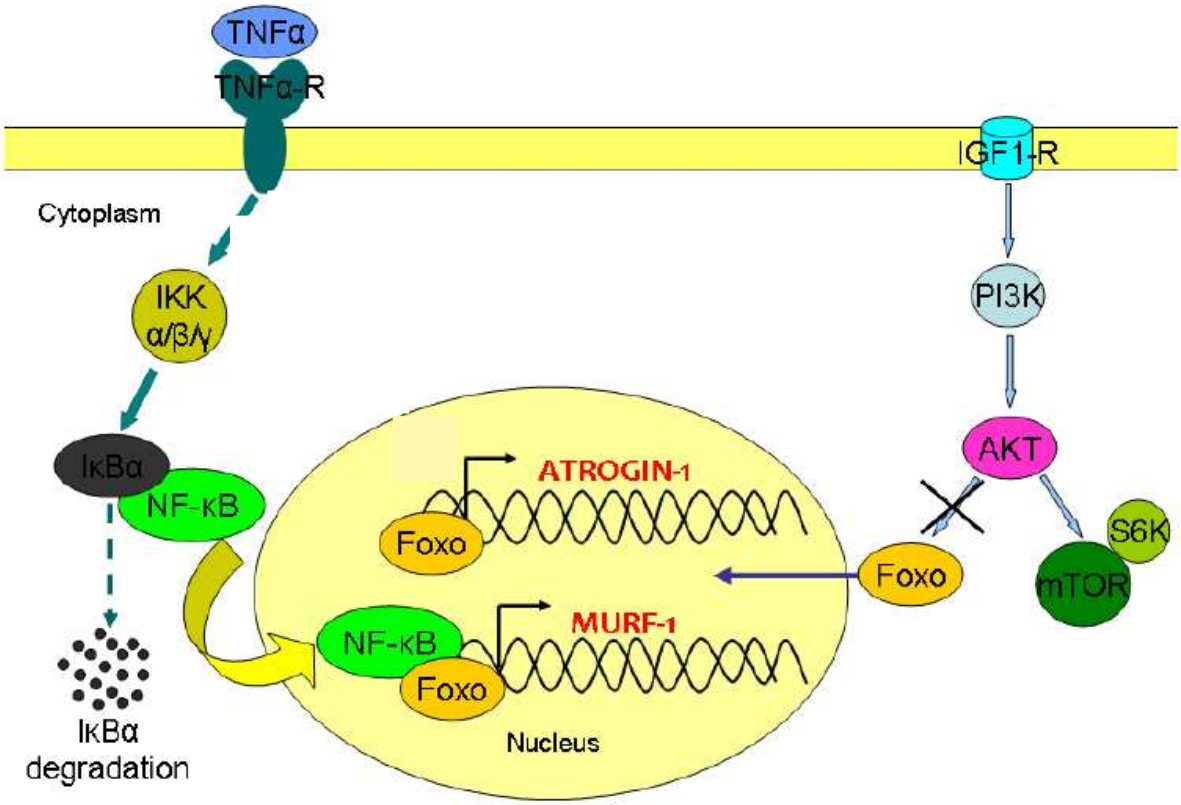


Fig 7 Signaling in atrophying skeletal muscle.

Besides regulation of Akt activity, e.g.by growth factors and mTOR, another important pathway of regulation of the atrophic process is mediated by inflammatory factors, in particular TNFalpha and prostaglandis, which are able to induce Nf-kB activation and translocation to the nucleus. Nf-kB induces MuRF1 transcription, thus mediating expression of atrophy-specific ubiquitin ligases in a FoxO-independent manner (Cai, 2004).

Other factors that mediate atrophy are ROS and the energy sensor AMPK, both acting through FoxOs activation, the first by acetylating specific lysines residues, the second by a specific activatory phosphorylation (Huang, 2007).

Introduction to

"Ghrelin and Des-Acyl Ghrelin Promote Differentiation and Fusion of C2C12 Skeletal Muscle Cells"

by

Filigheddu N, Gnocchi VF, Coscia M, Cappelli M, **Porporato PE**, Taulli R, Traini S, Baldanzi G, Chianale F, Cutrupi S, Arnoletti E, Ghè C, Fubini A, Surico N, Sinigaglia F, Ponzetto C, Muccioli G, Crepaldi T, and Graziani A.

Molecular Biology of the Cell (2007) 18: 986-994

When the skeletal muscle is affected by a disease such as a dystrophy or it is injured, an extensive production of new myofibres is required, in turn to reconstitute normal functionality.

Skeletal muscle satellite cells are mononucleated myoblasts that sustain this regenerative process by proliferating and eventually differentiting and fusing together in order to form new muscle fibres.

In vivo, differentiation of skeletal muscle involves first the growth factor-sustained expansion of the population of skeletal myoblasts, and then cell cycle exit and initiation of terminal differentiation, which involves expression of contractile proteins and formation of multinucleated syncitia by myocytes fusion. The extracellular signals triggering growth arrest and the molecular mechanisms involved in the induction of myoblasts differentiation and fusion still remain to be fully elucidated (Lluis, 2006).

In vitro, a valuable model to study the late steps of skeletal muscle regeneration, namely differentiation and fusion, are C2C12 myoblast, a murine cell line which still retain a certain degree of multipotency, as treatment with bone morphogenic protein 2 (BMP-2) causes a shift in the differentiation pathway from myoblastic to osteoblastic (Katagiri et al, 1994).

C2C12 cells are actively proliferating in condition of high nutrient availability (10% foetal bovine serum, growth medium, GM). By allowing them to get

confluent and switching the medium to low serum, typically 2% horse serum (differentiation medium, DM), they undergo terminal muscular differentiation.

In this article, we demonstrated that both ghrelin and des-acyl ghrelin stimulate proliferating C2C12 skeletal myoblasts to differentiate and to fuse into multinucleated myotubes in vitro through activation of p38 MAP kinase. Consistently, both ghrelin and des-acyl ghrelin inhibit C2C12 proliferation in growth medium. In addition, we demonstrated that ectopic expression of ghrelin in C2C12, by a lentiviral vector, enhances the differentiation and fusion of these myoblasts in differentiation medium. Finally, C2C12 cells were analyzed for expression of GHSR-1a, the canonical receptor for acyl Ghrelin, and are shown not to express its mRNA. Indeed, C2C12 contain a common high-affinity binding site recognized by both acylated and des-acylated ghrelin, suggesting that the described activities on C2C12 are likely to be mediated by a novel yet unidentified receptor for both ghrelin forms.

Introduction to

"DES-ACYL GHRELIN PROTECTS FROM SKELETAL MUSCLE ATROPHY"

by

Paolo E. Porporato*, Nicoletta Filigheddu*, Viola F. Gnocchi, Simone Reano, Giulia Bettas Ardisson, Michele Fornaro, Sharmila Fagoonee, Federica Chianale, Gianluca Baldanzi, Roy G. Smith, Yuxiang Sun, Stefano Geuna, Andrea Graziani

*Equal contribution of the two authors

Submitted to Nature Medicine and to Italian Patent Office

Skeletal muscle atrophy consists in a massive loss of muscle mass and functionality. Skeletal muscle atrophy is induced by muscle denervation and disuse, and it is also the key component of cachexia, a catabolic, debilitating response to several physio-pathological conditions, such as fasting, cancer, diabetes mellitus, AIDS, renal failure. Cachectic patients not only sustain a decreased quality of life, but also face a worse prognosis of the underlying disease, making cachexia an important target for treatment Muscle atrophy is mediated by induction of a common set of genes, named atrogenes, which execute a genetic program resulting in protein catabolism, and it is prevented by the PI-3-kinase/Akt signaling pathways activated by IGF-1 (Bodine 2001a, Bodine 2001b).

Ghrelin (GHR) and des-acyl ghrelin (D-GHR) are, respectively, a circulating peptidyl hormone acylated on Ser3, and its un-acylated precursor. Through binding to its receptor, GHSR-1a, GHR acts on the pituitary and the hypothalamus to stimulate growth hormone (GH) release and food intake (Kojima,1999; Kohno, 2003). Although D-GHR does not bind GHSR-1a and is devoid of any endocrine activity, an increasing body of evidence indicates that D-GHR and GHR share some biological activities, not mediated by GHSR1a, including inhibition of apoptosis of cardiomyocytes through activation of PI-3-

kinase/Akt pathway and differentiation of skeletal myoblasts (Baldanzi, 2002; Filigheddu, 2007) .

Inhere we show that D-GHR and GHR inhibit muscle atrophy *in vitro* on C2C12-derived myotubes treated with dexamethasone independently of GHSR-1a and via PI-3-kinase/Akt/mTOR pathway, as seen as maintenance of myotubes morphology and reduced induction of atrogenes such as Atrogin-1.l

Then skeletal muscle atrophy was induced *in vivo* either by food deprivation or by sciatic denervation in both wild type and transgenic mice with high levels of circulating D-GHR. In both models of atrophy D-GHR transgenic animals show reduced skeletal muscle loss and reduced induction of atrogenes compared to wild type animals. Noteworthy, anti-atrophic activity of D-GHR is not mediated by GH/IGF-1 axis, as increased D-GHR circulating concentration does not affect GHR and IGF-1 circulating levels, nor body mass index, tibial length and muscle mass, three major indicators of GH and IGF-1 activity.

Moreover, subcutaneous treatment of wild type mice with D-GHR induces Akt and mTOR phosphorylation in mouse gastrocnemius, indicating that, *in vivo*, D-GHR activates Akt/mTOR signaling pathway as it does in C2C12 myotubes. Indeed, D-GHR pharmacological treatment partially rescues skeletal muscle from atrophy induced by either starvation or denervation, similarly to what observed in D-GHR transgenic mice.

Furthermore, by showing that both des-acyl ghrelin and ghrelin activate Akt and mTOR in the skeletal muscle of GHSR-1a deficient mice, we provide the first genetic evidence that ghrelin and des-acyl ghrelin activate anti-atrophic signaling independently of GHSR-1a.

Altogether these findings provide the first demonstration that D-GHR directly activate in skeletal muscle *in vivo* a genetic program to inhibit atrophy induced by two very different kind of stimuli, such as starvation and denervation. Moreover, as D-GHR, differently from GHR, does not activate the GH/IGF-1 axis, this finding provides a new therapeutic strategy alternative to IGF-1 for the treatment of muscle wasting.

CONCLUDING REMARKS

The articles presented collect the outcome of the research work of my laboratory whose lines I contributed to develop during my PhD.

In the first part of this thesis, I introduce the works concerning the characterization of Diacylglycerol Kinase alpha biological functions and regulation.

In the presented articles DGKalpha emerges to be required for a specific subset of molecular events induced by HGF stimulation in epithelial cell and leading to cell migration, i.e. early protrusion of membrane ruffles and for the proper formation of new focal adhesions. In particular, DGKalpha emerges as an upstream regulator of Rac small GTPase signaling, whose role in cell migration is presently well established (Chianale et al, 2007).

In parallel, the determinant required for proper activation and localization of DGKalpha upon growth factors stimulation have been identified (Baldanzi et al, 2008). Indeed, DGKalpha phosphorylation on Tyr^{335}, mediated by Src in physio-pathological conditions or by oncogenic Src in a cancer context, is shown to mediate its enzymatic activation and to be required for HGF-induced cell migration. In addition, both Tyr^{335} and proline-rich sequence of DGKalpha result critical for DGKalpha phosphorylation, ability to interact with Src and proper targeting to the plasma membrane upon HGF stimulation.

Then, another article suggest how pharmacological inhibition of many members of Diacylglycerol Kinase family by the molecule R59949 may impairs HGF-induced invasiveness and anchorage-independent growth of the breast cancer carcinoma cell line MDA-MB-231 (Filigheddu et al, 2007).

Finally, I present an intriguing body of evidence indicating that DGKalpha might represent a novel target for the development of molecular strategies to selectively target cancer progression. In this paper (Porporato et al, in prep.) we show that DGKalpha is activated also upon GPCR activation and it is required for HGF- and SDF1alpha- induced invasion and metalloproteinase activation. Moreover, in this work DGKalpha has been demonstrated to be required for

proper localization of a major determinant of invasion and MMPs activity, i.e. the atypical PKC zeta/iota. Intriguingly, it has been shown that this protein is regulated by phosphatidic acid. Indeed, we show that the expression of a constitutively active form of DGKalpha is sufficient to recruit PKCzeta at the plasma membrane thus suggesting a novel molecular mechanism connecting DGKalpha to tumour progression.

In the second part of my PhD thesis I present the works regarding Ghrelin and Des-acyl Ghrelin.

In the presented works are shown outstanding advantages in the comprehension of the biological activity of both peptide on skeletal muscle.

In the first paper (Filigheddu et al, 2007b) we demonstrate that both GHR and D-GHR are sufficient to induce skeletal muscle differentiation of C2C12 myoblast in growth medium and that their supplementation enhance differentiation and fusion induced by differentiation medium.

From these data, we may speculate that GHR and D-GHR would contribute to skeletal muscle plasticity, promoting the differentiation and fusion of myoblasts in the damaged muscles. If this hypothesis would be proved, the activation of the receptor mediating GHR and D-GHR differentiative activity as well as the overexpression of the hormone may provide novel therapeutic strategies for the reduction or retardation of several skeletal pathologies characterized by chronic muscle injury, such as genetic dystrophies.

In the second paper (Porporato et al, submitted) we show that D-GHR and GHR have strong anti-atrophic activity on skeletal muscle through activation of Akt/mTOR pathway *in vitro*.

Moreover, we demonstrated that D-GHR impairs skeletal muscle atrophy *in vivo* and induces phosphorylation of Akt and mTOR through a novel, unidentified receptor.

Although the identity of the putative novel GHR/D-GHR receptor is yet unknown, our findings have important biological and therapeutic implications, since they provide the proof of concept that D-GHR, even upon pharmacological treatment, by activating a direct anti-atrophic signaling within

the skeletal muscle, has a strong and specific potential for the prevention or treatment of atrophy without inducing release of GH, nor of IGF-1.

In conclusion, the data presented in the reported articles constitute a outstanding advance in the knowledge of both DGKalpha and D-GHR biological activities, and lay the foundation for the future research directions.

Ringraziamenti

Un altro ciclo di studi si conclude con questa tesi, sebbene non sappia dove tutto ciò mi porterà resto fiducioso.
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Diacylglycerol Kinase is Required for HGF-induced Invasiveness and Anchorage-independent Growth of MDA-MB-231 Breast Cancer Cells

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Abstract. *Background: Estrogen receptor (ER)-negative breast cancers have a worse prognosis than ER-positive cancers, being more aggressive and overexposed to stimuli leading to their progression. Hepatocyte growth factor (HGF) has been associated with proliferation, migration and invasion of tumor cells, and several tumors, including those of breast cancer, produce HGF and overexpress its receptor. Diacylglycerol kinases (Dgks), which phosphorylate diacylglycerol to phosphatidic acid, are key regulators of cell signaling. Our research was focused on their role in HGF-induced invasion of MDA-MB-231 cells, a model of ER-negative breast cancer. Materials and Methods: Dgk activity was evaluated with a kinase assay, MDA-MB-231 cell invasion via culturing of cells in matrigel-coated transwells, and anchorage-independent growth was assessed using a soft agar assay. Results: HGF induces Dgk activation in MDA-MB-231 cells that is required for cell invasiveness. Moreover, Dgks are involved in MDA-MB-231 anchorage-independent growth. Conclusion: Dgks could be a target for ER-negative breast cancer therapy.*

Breast tumors expressing estrogen receptors (ER) are generally responsive to therapeutic strategies using selective ER modulators like tamoxifen and have a better prognosis than ER-negative ones (1). ER-negative breast cancer represents about 30% of invasive breast cancer and is more aggressive (2). The ER-negative tumors, unresponsive to anti-estrogens, are often overexposed to stimuli leading to their progression.

Hepatocyte growth factor (HGF) plays a well-known role in the process of tumor invasion and metastasis. HGF stimulates proliferation, dissociation, migration and invasion in a wide variety of tumor cells, and is a potent angiogenic factor (3, 4). Stromal fibroblasts are the main source of HGF, however, several tumor cells have been shown to produce HGF (5, 6). HGF elicits its biological functions through binding to its specific receptor, c-Met. The role of HGF and c-Met in human cancer metastasis is well established: the presence of c-Met has been reported in tumors of the thyroid, ovary, pancreas, breast, prostate and gastrointestinal tract (7-10) and its level of expression has been correlated with tumor progression and poor outcome in breast cancer patients (11, 12). In addition, c-Met has also been reported to be an independent prognostic factor in breast cancer (13).

Diacylglycerol kinases (Dgks), which phosphorylate diacylglycerol to generate phosphatidic acid (PA), comprise a family of ten distinct enzymes, grouped in five classes each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich C1 domains (14). It has been shown that Dgk-α, an isoform of class I Dgk, is activated by several growth factors: HGF and VEGF in epithelial and endothelial cells (15, 16), and IL-2 activation in T-cells (17). Inhibition of Dgk-α activity, obtained either pharmacologically or by expression of dominant negative mutant or by RNA interference, impairs

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HGF-, VEGF- and ALK-induced chemotaxis and proliferation in several cell types (15, 16, 18), as well as angiogenesis in endothelial cells (16). Similarly, in T-cells, pharmacological inhibition of Dgk-α severely impairs IL-2-induced G1- to S-phase transition (19).

Here we investigated whether HGF stimulation could induce activation of class I Dgks in the MDA-MB-231 breast cancer cell line and whether Dgk is involved in the invasiveness of MDA-MB-231 cells and their anchorage-independent growth.

Materials and Methods

Reagents. Cell culture medium and reagents were from Gibco (Invitrogen, Carlsbad, CA, USA), recombinant HGF was from Peprotech (London, UK), class I Dgk pharmacological inhibitor R59949 was from Sigma (St. Louis, MO, USA). All reagents were from Sigma, unless otherwise indicated.

Cell cultures. MDA-MB-231 (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco), penicillin (100 u/ml), streptomycin (100 μg/ml) and an antimycotic (0.25 μg/ml).

Dgk assay. Cells were starved in 0.2% FCS for 24 h, treated with 100 ng/ml HGF for 15 min and homogenised with a buffer containing 25 mM Hepes (pH 8), 10% glycerol, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM ammonium molybdate, 10 mM NaF, 1 mM sodium orthovanadate and Protease Inhibitor Cocktail. Homogenates were collected, passed through a 23 G syringe and centrifuged at 500 xg for 15 min at 4°C. Protein concentration was determined using the BCA method (Pierce, Rockford, IL, USA). Homogenates were incubated for 5 min with a saturating substrates concentrate (1 mg/ml diolein, Fluka), 5 mM ATP, 3 μCi/μl (γ^{32}P)-ATP (GE Healthcare), 10 mM $MgCl_2$, 1 μM $ZnCl_2$, 1 mM EGTA in 25 mM Hepes (pH 8) in the presence or absence of 1 μM R59949. Lipids were extracted as described elsewhere (20), and PA was separated using 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 dried. (^{32}P)-PA was identified by co-migration with non-radioactive PA standards stained by incubation in an iodine chamber. Radioactive signals were detected and quantified with the GS-250 Molecular Imager and Phosphor Analyst Software (Bio-Rad, Hercules, CA, USA).

Cell invasion assay. MDA-MB-231 cells were plated in the upper part of a transwell, the surface of which was coated with Matrigel (BD Biosciences, Erembodegem, Belgium). The lower chamber was filled with DMEM 0.2% FCS in the presence or absence of 1 μM R59949. After 15 min, 50 ng/ml HGF were added and cells were incubated for further 16 h. Non-migrated cells were removed from the upper part of the filters, while cells which migrated through the Matrigel in the lower part of the transwell were stained with Diff-Quik (Baxter, Deerfield, IL, USA) and counted.

Soft agar assay. MDA-MB-231 cells were suspended in 0.35% agar in DMEM 1% FCS and then plated ($5x10^4$ cells/well, 12-well plate) on a layer of 0.7% agar in DMEM 1% FCS in presence of 1 or 10 μM R59949. Upon 20 days of treatment, the colonies of living cells were stained with MTT and counted with the Quantity One software (Bio-Rad).

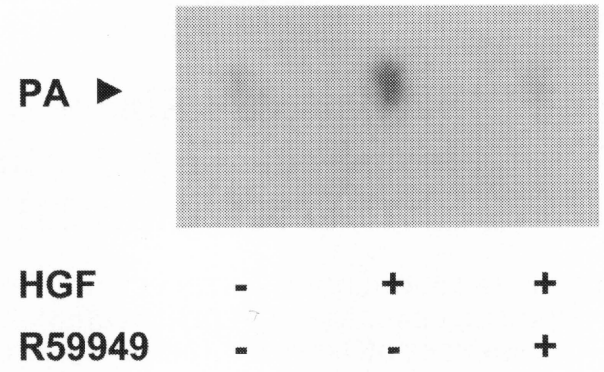

Figure 1. *HGF activation of Dgk in MDA-MB-231 breast cancer cells (representative TLC plate) (^{32}P)-phosphatidic acid (PA) was separated using TLC and identified by co-migration with non-radioactive PA standards. HGF: hepatocyte growth factor; R59949: Dgk inhibitor.*

Statistical analysis. Where appropriate, data are presented as the mean±SEM and the statistical significance was assessed using Student's *t*-test.

Results

HGF induced the activation of class I Dgk in the MDA BD-231 breast cancer cell line. The MDA-MB 231 human breast cancer cell line is often used as a model of ER-negative breast cancer. This cell line is considered particularly suitable for pre-clinical studies since it is highly aggressive both *in vitro* and *in vivo* (21).

As we previously demonstrated that HGF activated Dgk-α in epithelial cells (15), we investigated whether HGF would induce the activation of Dgk in MDA-MB-231 breast cancer cells (Figure 1). Indeed, 100 ng/ml HGF stimulated the activation of Dgk, as measured by its specific kinase activity *in vitro*. The Dgk activity up-regulated by HGF was inhibited when assayed in the presence of 1 mM R59949, a pharmacological inhibitor of class I Dgks.

HGF-induced invasiveness of the MDA-BD-231 breast cancer cell line. One of the peculiarities of aggressive tumors is their ability to metastasize. MDA-MB-231 cells, both spontaneously and upon growth factor stimulation, are able *in vitro* to pass through a matrigel layer, mimicking their ability to invade extracellular matrices *in vivo* (22).

In order to provide evidence that Dgk may be involved in the HGF-induced invasiveness of MDA-MB-231 cells, we investigated whether the pharmacological inhibition of Dgk impairs HGF invasive activity in these cells. Indeed, 50 ng/ml HGF in the lower chamber of the transwells promoted the

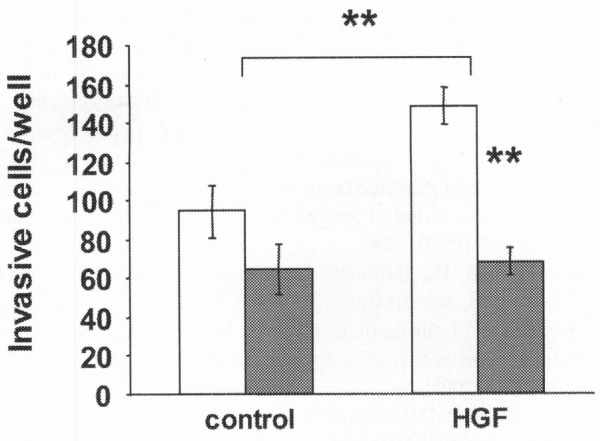

Figure 2. *HGF-induced invasion of MDA-MB-231 cells, as shown using a Matrigel assay. Empty bars: without R59949, solid bars: with R59949. **p<0.01 vs. control.*

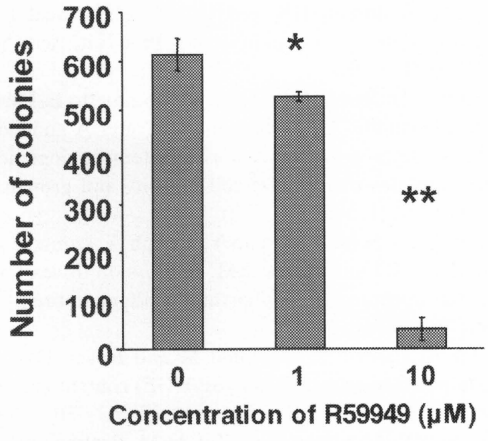

Figure 3. *Soft agar growth of MDA MB 231 breast cancer cells (MTT assay). **p<0.01 and *p<0.05 vs. control.*

invasion of MDA-MB-231 through a matrigel layer, while pretreatment with 1 μM R59949 for 15 min abolished the HGF-induced invasive capability of the cells (Figure 2).

Soft agar growth of the MDA BD-231 breast cancer cell line. The soft agar assay evaluates the ability of cells to form colonies in the absence of adhesion in a semi-solid medium, a feature of neoplastic cells. MDA-MB-231 cells are able to form colonies in soft agar when cultured in 1% FCS. Simultaneous treatment with 1 μM or 10 μM R59949 reduced the number of colonies formed by ~15% and 95% respectively (Figure 3).

Discussion

Treatment of breast cancer with selective ER modulators is an example of a successful therapy targeting estrogen receptor expression (23). However, its efficacy is limited to ER-positive breast tumors, which generally have a better prognosis (1) than ER-negative tumors. Indeed, ER-negative breast tumors are more aggressive (2), although the processes determining local invasion and the formation of metastases, responsible for their aggressiveness, are not completely understood at the molecular level. Revealing the molecular pathways involved in ER-negative hormone-independent breast cancer progression and metastasis may offer suitable targets for the development of new efficient anticancer therapies.

In recent years, diacylglycerol kinases have been intensively investigated either as negative or positive regulators of cell signaling. It has been shown, for instance, that activation of Dgk-α is required for growth factor-induced proliferative and chemotactic signaling (15-18), as well as for negative feedback in TCR signaling (24, 25).

Here we showed that HGF stimulation induced the activation of class I Dgks in MDA-MB-231, an ER-negative human breast cancer cell line considered particularly suitable for pre-clinical studies since it is highly aggressive both *in vitro* and *in vivo*. HGF-induced Dgk activation in turn mediated the passage of MDA-MB-231 cells through a matrigel layer, a peculiarity of aggressive cancer cells which mimics the invasion of extracellular matrices and the ability of these cells to metastasize; the pharmacological inhibition of Dgk activity abolished the effects elicited by HGF. Moreover, we showed that Dgk was involved in the anchorage-independent growth of MDA-MB-231 cells, a typical feature of tumor cells, as the inhibition of Dgk activity drastically reduced the number of colonies formed in soft agar.

These results fully demonstrate the biological relevance of Dgk in signaling pathways leading to cell migration elicited by growth factors, and suggest that class I Dgks could be a suitable target for the development of efficient therapies of ER-negative breast cancer.

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Diacylglycerol kinase-α mediates HGF-induced epithelial cell scatter by regulating Rac activation and membrane ruffling

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ABSTRACT

Diacylglycerol kinases (Dgk) phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), thus turning off and on, respectively, DG-mediated and PA-mediated signalling pathways. We previously showed that HGF, VEGF and ALK activate Dgkα in endothelial and leukaemia cells through a Src-mediated mechanism, and that activation of Dgkα is required for chemotactic, proliferative and angiogenic signalling *in vitro*. Inhere we investigate the downstream events and signalling pathways regulated by Dgkα, leading to cell scatter and migration upon HGF treatment and v-Src expression in epithelial cells. We report that specific inhibition of Dgkα, obtained either pharmacologically by R59949 treatment, or by expression of Dgkα dominant-negative mutant, or by siRNA-mediated downregulation of endogenous Dgkα, impairs *i)* HGF- and v-Src-induced cell scatter and migration, without affecting the loss of intercellular adhesions; *ii)* HGF-induced cell spreading, lamellipodia formation, membrane ruffling and focal adhesions remodelling; *iii)* HGF-induced Rac activation and membrane targeting. In summary, we provide evidence that Dgkα, activated downstream of tyrosine kinase receptors and Src, regulates crucial steps directing Rac activation and Rac-dependent remodelling of actin cytoskeleton and focal contacts in migrating epithelial cells.

INTRODUCTION

Epithelial tissues are characterized by monolayers of highly polarized cells, while *in vitro* epithelial cells grow to form discrete colonies. During embryonic development and tissue repair, as well as through cancer progression, epithelial cells acquire a highly motile and invasive phenotype in a process commonly known as epithelial-mesenchymal transition (EMT) (Thiery, 2002; Thiery *et al.*, 2006). *In vitro*, the scattering of epithelial cells, *i.e.* the dispersal of colonies due to loss of intercellular adhesion and acquisition of cell motility, is triggered by growth factors stimulation and by oncogenes activation, recapitulating the early phases of EMT (Avizienyte and Frame, 2005).

HGF and oncogenic Src induce *in vitro* cell scatter of several epithelial cells, while *in vivo* their inappropriate activation is associated to progression and acquisition of a metastatic phenotype in several epithelial-derived cancer (Irby and Yeatman, 2000; Danilkovitch and Zbar, 2002). Within hours from stimulation of their tyrosine kinase activities, both HGF and v-Src induce scattering of epithelial cell colonies through loss of cadherin-mediated cell-cell adhesions and increase of their motility, due to formation of lamellipodia and remodelling of cortical actin and focal adhesions (Beherens *et al.*, 1993; Lamorte *et al.*, 2002). The signalling pathways by which HGF and v-Src stimulate EMT, cell scattering and invasiveness have been extensively investigated in several epithelial cells (Thiery, 2002). Recruitment of Gab-1, along with activation of PI 3-kinase, PLCγ, Ras and Rac are required (Lamorte *et al.*, 2002 and refs herein). Src plays a crucial role in HGF signalling as its activity is required for HGF-mediated cell motility, anchorage-independent growth and tumorigenesis. Indeed Src mediates HFG-induced tyrosine phosphorylation of catenins, leading to downregulation of cadherin-mediated cell-cell adhesions, and of several focal adhesion proteins required for cell motilitiy and invasiveness, such as FAK, Paxillin and p130Cas (Beherens *et al.*, 1993; Rahimi *et al.*, 1998; Nakaigawa *et al.*, 2000).

Diacylglycerol kinases, which phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), comprise a family of ten distinct enzymes, grouped in five classes each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich atypical C1 domains (Topham and Prescott, 1999; Imai *et al.*, 2005). DG is an established activator of several typical C1 domain-containing proteins, such as PKCs, RasGRPs and chimaerins. Similarly, several signalling proteins have been reported to be regulated by PA, including serine kinases, such as mTor, Raf and atypical PKCs, small GTPase regulating proteins, such as SOS, RhoGDI, Ras- and Rho-GAPs, and signalling lipid metabolizing enzymes, such as PI(4)P 5-kinase and PLC-γ (Topham, 2006; Zhao *et al.*, 2007). However, a common specific PA binding domain has not been identified yet. Thus, by regulating in a reciprocal manner the level of both DG and PA lipid second

messengers, Dgk enzymes may act as terminators of DG-mediated signals as well as activators of PA-mediated ones.

Recent evidences showed that α, ζ and θ Dgk isoforms are regulated by extracellular ligands and play a role in signal transduction (van Blitterswijk and Houssa, 2000; Luo *et al.*, 2003). T cells derived by Dgkα -/- mice feature enhanced DG-mediated RasGRP activity upon TCR activation, leading to over-activation of the Ras pathway and a defect in anergy, while overexpression of Dgkα in T cells impairs TCR signalling (Olenchock *et al.*, 2006). Several evidence in T cells indicate that Dgkα and ζ, by interacting respectively with RasGRP and PKC, up-regulate cell sensitivity to TCR activation by negatively modulating the intensity and the kinetic of DG-mediated signalling (Luo *et al.*, 2003; Sanjuan *et al.*, 2003; Zhong *et al.*, 2003). Conversely, mast cells derived from Dgkζ -/- mice feature a diminished FcεRI-mediated degranulation, correlating with impaired PLCγ activation and calcium response, both likely dependent on PA production (Olenchock *et al.*, 2006).

We have previously shown that in endothelial and leukaemia cells, activation of Dgkα downstream from tyrosine kinase receptors, such as HGF-R, VEGFR-2, and ALK, is required for either chemotactic or proliferative signalling induced by their respective ligands, as well as for cell proliferation upon IL-2 stimulation of T cells (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004; Bacchiocchi *et al.*, 2005). Growth factors stimulate Dgkα through a mechanism requiring complex formation with Src and phosphorylation of Dgkα on Tyr^{335} by Src itself (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2007). The specific signalling pathways regulated by activation of Dgkα still await elucidation.

Herein we investigate the role of Dgkα in HGF-induced cell migration of epithelial cells. We show that Dgkα activation is required for HGF- and v-Src-induced scattering of MDCK cells, and particularly in those mechanisms leading to cell spreading and F-actin cytoskeleton and focal adhesions remodelling. By further investigating the role of Dgkα in HGF early signalling, we show that upon 15 minutes from HGF stimulation, Dgkα activity is necessary for membrane targeting and activation of Rac, and for Rac-regulated formation of membrane ruffles.

These data, by indicating Dgkα as a key signal transducer of motility signals downstream HGF and v-Src, strongly suggest that it may represent a key regulator in the processes of invasion and metastasis.

MATERIALS AND METHODS

Cell culture

MDCK (Madin-Darby canine kidney cells) and MDCK-*ts*-v-Src (Baldanzi *et al.*, 2004) are a kind gift of W. Birchmeier (Berlin). Cells were cultured in high glucose DMEM GlutaMAX™ medium (Gibco), supplemented with 10% fetal bovine serum (Gibco) and antibiotic-antimicotic solution (Sigma), in humidified atmosphere with 5% CO_2. MDCK cells were cultured at 37°C, while MDCK-*ts*-v-Src were normally grown at 40.5°C (inactive v-Src) and shifted to 35°C to achieve v-Src activation.

Reagents

Recombinant human HGF was purchased from Peprotech; R59949 (Diacylglycerol Kinase Inhibitor II) from Sigma. DMSO, vehicle for R59949, was always used in control samples at the same dilution as R59949. Anti-Myc and anti-Rac1 were from Upstate, anti-Paxillin from BD Transduction Laboratories, anti-Paxillin pTyr^{31} and pTyr^{118} and anti-Akt pSer^{473} from Biosource, anti-Akt from Cell Signaling, anti-α-Tubulin from Sigma, anti-Vinculin from Novus Biological, anti-FAK from Calbiochem, Alexa Flour 546/633 Phalloidin from Molecular Probes. Anti-Dgkα was kindly provided by W.J. van Blitterswijk (the Netherlands Cancer Institute, Amsterdam). Secondary HRP-conjugated antibodies were purchased from PerkinElmer Life Sciences; secondary FITC- and TRITC-conjugated antibodies were purchased from DAKO.

Expression vectors, transfections and infections with retroviral vectors

Myc-Dgkα cDNA cloned into pMT2 expression vector has been previously described (Cutrupi *et al.*, 2000). GFP-Dgkα-WT (wild type) was obtained by cloning Dgkα in pcDNA-DEST53 (Invitrogen) using Gateway kit (Invitrogen) according to manufacturer's instructions. Briefly, Dgkα cDNA was inserted in pDONOR 2.11 vector by PCR and BP recombination. LR recombination was performed to transfer Dgkα in pcDNA-DEST53 for N-terminal GFP fusion; detailed information and protocols are available on www.invitrogen.com. $G_{434}D$ point mutation on Dgkα to obtain the kinase-defective dominant negative mutant (GFP-Dgkα-DN) was performed using QuikChange Site-Directed Mutagenesis Kit 22 (Stratagene) as previously described (Cutrupi *et al.*, 2000). PINCOS retroviral vector, PINCOS/Dgkα-DN and PINCOS/Dgkα-WT, expressing both GFP and the inserted gene, have already been described (Cutrupi *et al.*, 2000). Transient transfections were performed using Lipofectamine2000 Reagent (Invitrogen) according to the manufacturer's instructions.

MDCK cells stably expressing PINCOS/empty vector or PINCOS/Dgkα-DN or PINCOS/Dgkα-WT were obtained by infection. Briefly, GP2-293 packaging cell line (Clontech, kindly provided by R. Piva, University of Torino) was transiently co-transfected, by Lipofectamine2000 Reagent (Invitrogen) according to the manufacturer's instructions, with the envelope vector pVSV-G (Clontech) together with PINCOS or PINCOS/Dgkα-DN or PINCOS/Dgkα-WT.. The next day the medium was changed to normal growth medium. Forty-eight hours after infection, the retroviral supernatant was collected, the debris removed by centrifugation at 1500g, and the supernatant was filtered by a 0.45 μm pore filter and added with Polybrene (8 μg/ml). MDCK cells, plated in a six-well plate, were infected by adding 2 ml of retroviral supernatant and 1 ml of growth medium. The day after the first infection cells were re-infected as previously described. Sixteen hours later, cells were placed and maintained in growth medium. Efficiency of infection was about 80%, as measured by FACS analysis and/or observation with fluorescence microscope of GFP expressing cells .

The murine Dgkα, resistant to canine Dgkα siRNAs, was cloned in the lentiviral vector pLenti4V5 (Invitrogen). Lentiviruses were produced following the manufacturer's instructions and used to infect MDCK cells, which were then selected in Zeocin-containing medium to obtain a stably-expressing cell line.

RNA interference

siRNAs against canine Dgkα were chemically synthesized as double-strand RNA (Ambion). Sequences were as follows: C1 sense GCUCAGAAGUGGACAGGAUtt antisense AUUCUGUCCACUUCUGAGCtg; C2 sense CCCAGACAUCCUGAAAACCtt antisense GGUUUUCAGGAUGUCUGGGtc; C3 sense CCUUCCACACCACAAAAACtt antisense GUUUUUGUGGUGUGGAAGGtg. A GAPDH scramble siRNA (Ambion) was used as negative control.

The BLOCK-iT^{TM} Fluorescent Oligo (Invitrogen) is a fluorescein-labelled dsRNA oligomer and was used to obtain indication of the transfection efficiency with siRNAs.

Dgk assay

Dgkα activity was assayed in anti-Myc immunoprecipitates as described (Cutrupi *et al.*, 2000). Briefly, after immunoprecipitation and extensive washing in Lysis Buffer (25 mM HEPES pH 8, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 10% glycerol supplemented with protease inhibitors [Protease Inhibitors Cocktail, Sigma]), Litium Cloride Buffer (500 mM LiCl, 25 mM Tris-HCl pH 8) and TNE (Tris 25 mM pH 8, NaCl 150 mM,

EDTA 1 mM), all supplemented with fresh 1 mM Na_3VO_4, the immunocomplexes were assayed at room temperature for 10 minutes by incubation with 1 mg/ml diolein (Fluka, dried in nitrogen atmosphere, resuspended and sonicated in 1 mM EGTA, 25 mM Hepes pH 8), 5 mM ATP, 10 μCi/sample [γ-^{32}P]ATP (Amersham), 10 mM $MgCl_2$, 1 mM $ZnCl_2$. Lipids were then extracted as described (Graziani *et al.*, 1991), and PA was separated by TLC in chloroform: methanol: water: 32% ammonium hydroxide (60:47:10:3). TLC plates had been previously coated with (potassium oxalate 1.3%, EDTA 5 mM): methanol 3:2. [^{32}P]-PA was identified by co-migration with non-radioactive PA standards (Fluka) stained by incubation in a iodine chamber. Radioactive signals were detected and quantified with GS-250 Molecular Imager and its Phosphor Analyst Software (Biorad). One half of immunoprecipitated lysates was assayed for Dgk activity, while the other half was heat-denatured in Laemmli Buffer, separated in SDS-PAGE, blotted and probed with anti-Myc antibody.

Scatter, chemotaxis and wound healing

For HGF-induced cell scatter, MDCK cells were plated at low density in 24-well plates and allowed to growth in small colonies. Cells were stimulated in serum-free medium with 2 ng/ml HGF for 24 hours, in presence or absence of 1 μM R59949, fixed with 3% paraformaldehyde, 4% sucrose in PBS, and then photographed with phase-contrast optics with a 20x objective (Zeiss). For v-Src induced cell scatter, MDCK-*ts*-v-Src cells were shifted to the permissive temperature of 35°C in 0% FBS medium for 24 hours, in presence or absence of 1 μM R59949.

Chemotaxis assay was performed in a Neuro Probe Standard 48 Well Chemotaxis Chamber according to manufacturer's instructions. Briefly, the bottom chamber was filled with serum-free DMEM containing 50 ng/ml HGF as chemoattractant, in presence or absence of 1μM R59949. 10^5 cells were seeded in the upper chamber and let migrate overnight through a polycarbonate filter coated with 0.1% gelatine. Migrated cells were fixed and stained with Diff-Quick (Dade Behring Inc.) before counting.

In wound healing assay, cells grown to confluency were scratched using a pipette tip. Cells were then allowed to migrate into the wound for 7 hours in serum-free medium containing 2.5 ng/ml HGF, in presence or in absence of 1 μM R59949, and then photographed with phase-contrast optics with a 20x objective (Zeiss). Migration was quantified by calculating the area of wound at time points t_0 (time of wound) and 7h (7 hours after wound). Normalization was obtained by the formula: [area(t_0)-area(7h)]/area(t_0).

Invasion

Invasion assays were performed in serum-free medium in 6.5 mm Transwells with 8 μm-pore size membranes. The Transwell membrane was pre-coated with 10 μg Matrigel (BD Biosciences) in 50 μl of cold serum-free medium and dried overnight at room temperature. 10^5 cells were seeded in the upper chamber of the Transwell apparatus. The lower chamber was filled with DMEM 2% FBS in presence or absence of 100 ng/ml HGF and cells were allowed to migrate for 48 hours. After washing with PBS, the cells on the upper surface of the Transwell membrane were removed using a cotton-tipped swab, while those cells onto the lower face were fixed in glutaraldehyde and stained with crystal-violet. Fixed cells were then photographed and invasion was quantified by optical densitometry**.**

Immunofluorescence

MDCK cells were seeded in small colonies on glass coverslips (Marienfeld) in 24-well cell culture plates. Cells were overnight starved and then stimulated with 10 ng/ml HGF for the indicated times. 1μM R59949 was given as pre-treatment in short-time HGF experiments (15 minutes), while in long-time experiments (from 4 hours onward) was given together with stimulus. After stimulation, cells were washed twice in PBS and fixed by incubation with PBS 3% paraformaldheyde-4% sucrose. Cells were then permeabilized in cold HEPES-Triton Buffer (20 mM HEPES pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100), washed with PBS containing 0.2% BSA and incubated for 15 minutes with PBS containing 2% BSA. 15 μl of primary antibody (1:100 in PBS/2% BSA) was added directly onto each glass coverslip in a humidified chamber for 30 minutes and the excess of antibody was washed away with PBS/0.2% BSA. Cells were then incubated for additional 15 minutes with PBS/2% BSA and FITC-/TRITC-conjugated secondary antibodies and/or Alexa Fluor 546/633 Phalloidin (1:30 and 1:200 in PBS/2% BSA respectively) were added for 30 minutes in the humidified chamber. After washes, each glass coverslip was washed briefly in water and blocked onto a glass microscope slide by Mowiol (20% Mowiol 4-88, 2.5% DABCO in PBS pH 7.4) and let polymerize. Confocal images were acquired with the Leica confocal microscopy TSP2 and LCS Leica confocal software. Basal planes are shown.

Western blotting and cell fractionation.

Cell lysates were prepared after cold PBS washing by scraping on ice in Lysis Buffer (25 mM HEPES pH 8, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 2 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 10% glycerol supplemented with fresh 1 mM Na_3VO_4 and protease inhibitors [Protease

Inhibitors Cocktail, Sigma]). Clarified lysates were denatured by boiling in Laemmli Buffer for direct western blotting.

Detergent-soluble and insoluble fractions were obtained according to Potempa and Ridley (Mol. Biol. Cell, 1998). Briefly, cells were lysed in NP-40 buffer (25 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 1% NP-40, 4 mM EDTA, 25 mM NaF, 10% glycerol supplemented with fresh 1 mM Na_3VO_4 and protease inhibitors) for 30 minutes on a rotating wheel at 4°C. The lysates were centrifuged at 10000 g for 30 minutes, and the supernatant was collected as the NP-40-soluble fraction (S). The pellet was resuspended in 100 µl of 25 mM HEPES pH 7.5, 4 mM EDTA, 25 mM NaF, 1% SDS and 1 mM Na_3VO_4. After addition of 900 µl of the NP-40 buffer, the homogenate was passed 10 times through a 27-gauge needle and left for 30 minutes on a rotating wheel at 4°C. The lysates were then centrifuged at 10000 g for 30 minutes and the supernatant was collected as the NP-40-insoluble fraction (I). Equal sample volumes were loaded for SDS-PAGE.

RacGTP pull-down assay

RacGTP pull-down assays were performed according to Zondag *et al.* (J. Cell. Biol., 2000). Briefly, MDCK cells were seeded in 15 cm-diameter cell culture plates and overnight starved in 0% FBS medium before stimulation with 100 ng/ml HGF for 15 minutes. 1µM R59949, when used, was added with a 30 minute pre-treatment and maintained during the following HGF stimulation. Cells were then washed in ice-cold PBS and lysed with GST-Fish Buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% glycerol, 0.1% Triton X-100 supplemented with fresh 1 mM Na_3VO_4, protease inhibitors and 1 mM DTT) and harvested by scraping. The clarified lysates were incubated for 45 minutes with purified GST-PAK-BD at 4°C, pre-coupled to GSH-Sepharose beads (GE Healthcare). After 3 washes with GST-Fish Buffer, samples were resuspended in Laemmli Buffer, heat-denatured and separated by SDS-PAGE in a 12% polyacrylammide gel. A small amount of each sample was directly denatured in Laemmli Buffer for whole cell lysate proteins analysis.

Statistical analysis

At least triplicates were analysed when quantification was performed. Couples of conditions were compared using Student t-test. Histograms represent means ± standard errors.

RESULTS

Dgkα activation mediates HGF-induced scatter and migration of MDCK cells.

We previously showed that activation of Dgkα in endothelial cells is required for VEGF and HGF-induced chemotaxis (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004). However the role of Dgkα in epithelial cell scattering has never been investigated, as well as the signalling pathways involved.

MDCK cells express endogenous Dgkα and feature a R59949-sensitive Dgk activity associated to anti-phosphotyrosine immunoprecipitates upon HGF stimulation (data not shown). Upon v-Src activation, obtained by shifting MDCK-*ts*-v-Src cells to the permissive temperature, Dgkα is activated in a time-dependent manner reaching a maximum activity after 1h (Figure 1). Activation of Dgkα by v-Src was evaluated by assaying Dgk activity in anti-Myc immunoprecipitates of MDCK-*ts*-v-Src cells transiently transfected with Myc-Dgkα. Similarly, Myc-Dgkα was also activated by HGF in MDCK cells (data not shown), as previously reported in endothelial cells (Cutrupi *et al.*, 2000).

MDCK cells form discrete compact colonies that, upon either HGF stimulation or v-Src activation, undergo scatter, which involves cell spreading, dissolution of inter-cellular adhesions and migration of cells away from one another (Beherens *et al.*, 1993; Weidner *et al.*, 1993; Palacios and D'Souza-Schorey, 2003).

In order to investigate the role of Dgkα in cell scattering and migration, Dgkα activity was inhibited in MDCK cells by R59949, a pharmacological isoform-specific Dgk inhibitor. Cell treatment with 1μM R59949 (Figure 2A), severely impair HGF-induced cell scatter. The specificity of Dgkα inhibition by R59949 cell treatment was verified in a wound healing assay. Indeed, overexpression of Dgk in MDCK cells fully re-establishes HGF-induced cell migration even in presence of 1μM R59949 (Figure 2B).

Moreover, HGF-induced cell scatter was also impaired by stable expression of Dgkα kinase-defective mutant, acting as dominant-negative (Dgkα-DN) (Figure 2C). About 80% of cells were infected with PINCOS/Dgkα-DN, as measured by FACS analysis (data not shown) and as shown in GFP panels; global overexpression of Dgkα-DN is shown by western blot (Figure 2C).

In order to further verify the specificity of Dgkα requirement in HGF-induced cell scatter, the endogenous protein was downregulated by transient transfection of specific siRNAs. Three siRNAs were designed (C1, C2, C3), transiently transfected in MDCK cells, and proved to be effective in knocking down canine Dgkα, as verified by western blot; negative control siRNA does not affect Dgkα expression (Figure 2D). Transfection of MDCK cells, with the same conditions,

with BLOCK-iT Fluorescent Oligo demonstrates that the efficiency of siRNA internalization into MDCK cells is near to 100% (Figure 2D). Similarly to R59949 treatment and expression of Dgkα-DN, C3 siRNA-mediated downregulation of endogenous Dgkα inhibits HGF-induced MDCK cell scatter (Figure 2E). Similar results were obtained with C1 and C2 (data not shown). In order to provide further evidence of the specificity of Dgkα requirement for cell scatter, we generated MDCK cells stably expressing murine Dgkα, whose expression is not affected by any of three siRNAs directed against the canine hortologue (MDCK/Mus-Dgkα). Indeed, transient transfection of C3 (Figure 2E), C1 or C2 (data not shown) in these cells does not affect HGF-induced cell scatter.

We further verified that Dgkα is required for HGF-induced cell migration in a quantitative chemotaxis assay. Indeed 1μM R59949 completely abolishes HGF-induced chemotaxis of MDCK cells toward the HGF-filled lower chamber (Figure 2F), while it does not affect cell basal migration.

A motile phenotype is essential also for the acquired ability of scattering MDCK cells to invade the extracellular matrix, a typical feature of metastatic carcinoma. Thus we verified the role of Dgkα in HGF-induced invasion of MDCK cells through a Matrigel barrier, a common assay to investigate the signalling pathways leading to metastatic progression (Birchmeier *et al.*, 2003). Indeed inhibition of Dgkα by expression of Dgkα-DN, strongly impairs HGF-induced *in vitro* invasiveness of MDCK cells (Figure 2G).

Similarly to HGF-induced cell scatter, inhibition of Dgkα, either by R59949 treatment or downregulation of the endogenous protein by C3 siRNA, strongly impairs MDCK cell scattering induced upon *ts*-v-Src activation (Figure 2H). Similar results were obtained with C1 and C2 siRNAs (data not shown).

Dgkα inhibition uncouples spreading, cytoskeletal remodelling and lamellipodia formation from downregulation of E-cadherin-mediated intercellular adhesions.

In HGF-induced cell scattering, loss of cell-cell contacts is preceded by internalization of E-cadherins at 4-6 hours from HGF stimulation (Beherens *et al.*, 1993; Potempa *et al.*, 1998; Kimura *et al.*, 2006), which occurs concomitantly to colony spreading, so that the area covered by each colony increases two to three fold. At the same time, cells at the colony outer edge undergo dramatic morphological changes, featuring extended lamellipodia, where focal adhesion proteins, such as Paxillin and Focal Adhesion Kinase (FAK), are recruited at new sites of adhesion and at the tips of stress fibres (Weidner *et al.*, 1993; Ridley *et al.*, 1995; Palacios and D'Souza-Schorey, 2003).

We observed that inhibition of Dgkα, either by 1μM R59949 treatment (data not shown) or

by expression of Dgkα-DN, does not affect the HGF-induced internalization and removal of E-cadherins from cell-cell contacts (Figure 3A, panel g), occurring upon 6 hours of cell stimulation. In addition, we performed fractionation of MDCK cell lysates in NP-40-soluble and NP-40-insoluble fractions. Upon 6 hours of treatment, HGF induces a decrease in the amount of E-cadherin in the insoluble fraction, independently from Dgkα-DN expression (Figure 3B).

Conversely, inhibition of Dgkα by 1μM R59949 results in a remarkable reduction of HGF-induced colony spreading upon 4 hours of cell stimulation (Figure 3C). Moreover, staining for F-actin clearly shows that Dgkα inhibition strongly affects HGF-dependent morphological changes such as lamellipodia formation (Figure 3D, panels a,b,c,d). Consistently with inhibition of lamellipodia formation, R59949 treatment severely affects HGF-induced remodeling of focal adhesions spatial organization, as, visualized by staining for FAK (Figure 3D, panels e,f,g,h). Inhibition of Dgkα in unstimulated MDCK cells does not affect their morphology concerning all of the analyzed aspects.

These data strongly suggest that Dgkα is not involved in the mechanisms by which HGF downregulates E-cadherin-mediated intercellular adhesions, and that its inhibition uncouples HGF-induced events leading to loss of intercellular adhesions from the signalling pathways mediating cell spreading, F-actin remodelling, lamellipodia formation and eventually cell migration.

Dgkα is required for HGF-induced membrane ruffles formation and focal adhesions remodelling.

Upon few minutes of HGF stimulation, MDCK cells at the outer edge of colonies undergo intense ruffling. They eject small membrane protrusions, whose formation relies on regulated recruitment of molecular scaffolds to growing focal complexes at new adhesion sites, coupled to the coordinated organization of actin filaments into lamella network and bundled arrays. Eventually membrane ruffles evolve in wider lamellipodia driving and providing direction to cell migration (Small *et al.*, 2002). Thus we verified whether the effects of Dgkα inhibition observed after hours of HGF stimulation derived from impairment of events occurring at earlier time points, such as formation of membrane ruffles and new focal complexes.

We ascertained Dgkα localization in resting or HGF-treated MDCK cell by transiently transfecting a GFP-Dgkα fusion protein. In untreated cells Dgkα displays cytoplasmic localization, but upon 15 minutes of HGF treatment it accumulates at the cell periphery, in correspondence of the protruding plasma membrane (Figure 4A). This observation suggests that Dgkα may play a role in HGF-induced earlier events leading to membrane ruffle formation.

Thus we set to investigate earlier changes in F-actin cytoskeleton organization in response to HGF. Upon 15 minutes of HGF treatment, small membrane ruffles develop on the outer membranes of cells at colony edge (Figure 4B, arrows). The percentage of cells featuring membrane ruffles raises from less than 20% in control cells (vehicle- and R59949-treated cells) to about 50% in HGF-treated cells. In presence of 1μM R59949, the percentage of membrane ruffle displaying cells upon HGF stimulation is reduced to almost control value (Figure 4B). In order to further verify the specificity of Dgkα requirement in HGF-induced ruffle formation, we showed that transient transfection of either C1, C2 or C3 siRNA impairs HGF-induced membrane ruffling and that this inhibition is completely overridden by the expression of the Dgkα murine hortologue, which is not affected by any of three siRNA (Figure 4C). Consistently, HGF fails to induce membrane ruffles in cells expressing Dgkα-DN compared to cells expressing the vector alone (Figure 4D). In conclusion, these data demonstrate that the formation of membrane ruffles occurring upon 15 minutes of HGF treatment depends on stimulation of Dgkα activity.

Membrane ruffles formation implies the recruitment of focal adhesion proteins at new adhesion sites within the ruffle itself. In epithelial cells, Paxillin recruitment to newly-formed focal complexes, where it acts as a scaffold for signalling molecules, is required for HGF-induced signalling leading to cell migration (Lamorte *et al.*, 2003; Ishibe *et al.*, 2004; Chen *et al.*, 2005).

In resting MDCK cells, Paxillin is partially diffuse in the cytoplasm, while in cells at colony edge it is also localized in focal adhesions along the outer plasma membrane (Figure 5A and B, panel a). Upon 15 minutes of HGF stimulation, Paxillin condensates to the newly-formed focal complexes in correspondence of membrane ruffles (Figure 5A, panel c and Figure 5B, panel b). Upon inhibition of Dgkα by either 1μM R59949 (Figure 5A, panel d), or by expression of Dgkα-DN (Figure 5B, panel f), Paxillin accumulates along the outer plasma membrane instead of being recruited in the area of ruffling, while ruffles formation is impaired. Inhibition of Dgkα in unstimulated cells does not significantly affect Paxillin localization either in the cytoplasm or at focal adhesions along the outer plasma membrane.

In order to verify that Paxillin indeed accumulates in structures identifiable as focal complexes, we analyzed its co-localization with Vinculin, a resident protein whose function is to stabilize them (Ziegler *et al.*, 2006). In unstimulated cells Vinculin and Paxillin co-localize at focal complexes along the outer plasma membrane of colony-edge cells and upon HGF stimulation they are both recruited to newly-formed focal complexes in the area of ruffling, in a manner fully dependent on Dgkα activity. In fact, inhibition of Dgkα, while impairing HGF-induced neo-formation of ruffles and focal complexes at membrane ruffles, does not affect Vinculin and Paxillin co-localization (Figure 5C).

Upon growth factor stimulation Src- and FAK-mediated phosphorylation of Paxillin is required to recruit and coordinate multiple signalling complexes, regulating events at the leading edge of migrating cells (reviewed in Brown and Turner, 2004). Phosphorylation of Paxillin on tyrosine 31 and 118 mediates its association with Crk and is required for growth factors-induced Paxillin-mediated migratory signals (Nakamura *et al.*, 2000; Petit *et al.*, 2000). Thus we verified whether inhibition of Dgkα affects HGF-induced phosphorylation of Paxillin Tyr^{31} and Tyr^{118}, identified by anti-phosphotyrosine specific antibodies. Western blot analysis of Paxillin tyrosine phosphorylation reveals that HGF induces Paxillin phosphorylation of both Tyr^{31} and Tyr^{118} in control MDCK cells (Figure 5D). Surprisingly basal phosphorylation of Paxillin in both residues is enhanced in cells expressing Dgkα-DN, and is not further affected by HGF stimulation (Figure 5D).

In summary these data demonstrate that upon minutes of HGF stimulation, activation of Dgkα is required for the formation of membrane ruffles and for the succeeding remodelling of Paxillin- and Vinculin-containing focal complexes.

Dgkα is required for HGF-induced Rac activation and membrane targeting.

The data presented above strongly suggest that activation of Dgkα is involved in the signalling mechanisms leading from HGF-receptor activation to ruffle formation.

Membrane ruffle formation is dependent on the activation of Rac small GTPase, which acts upstream of the recruitment of WAVE and Arp2/3 complexes at new adhesion sites promoting F-actin polymerization (Takenawa *et al.*, 2007). In migrating cells active Rac localization at leading edge is enhanced and allows the coupling with its downstream effectors (Kurokawa and Matsuda, 2005). In MDCK cells HGF activates Rac, whose function is required for HGF-induced cell scatter, spreading and for ruffles and lamellipodia formation (Ridley *et al.*, 1995; Royale *et al.*, 2000).

Activation of endogenous Rac was assayed by GST-PAK pull-down to purify active GTP-bound Rac from lysates of either control or HGF-stimulated MDCK cells. HGF treatment results in activation of endogenous Rac. Inhibition of Dgkα, by either 1μM R59949 or by Dgkα-DN expression, severely impairs HGF-induced Rac activation, without affecting Rac basal state of activation (Figure 6A). Rac activation requires the coordinated activity of its direct upstream regulators, which are recruited in multi-molecular complexes at the cell leading edge. As several Rac GEFs are regulated through their PH domain by D-3 phosphoinositides (Welch *et al.*, 2003), we verified whether inhibition of Dgkα affects the PI 3-kinase pathway, as measured by Akt phosphorylation. Indeed HGF induces Akt phosphorylation in both control and Dgkα-DN expressing MDCK cells (Figure 6B), demonstrating that Dgkα does not mediate Rac activation by regulating PI 3-kinase.

Rac activation is tightly coupled to its targeting to specific cholesterol-enriched membrane microdomains, defined by ligand-activated integrin signalling (Grande-Garcìa *et al.*, 2005). Thus we verified whether inhibition of Dgkα may interfere with HGF-induced targeting of Rac to the plasma membrane. By confocal microscopy, we observed the localization of endogenous Rac in MDCK cells (Figure 7A). In most unstimulated cells, Rac is both cytoplasmic and at intercellular contacts, while only about 20% of colony-edge cells feature Rac at the outer plasma membrane (Figure 7A, panel a). Following 15 minutes of HGF stimulation, the percentage of colony-edge cells featuring Rac at the outer plasma membrane raises to more than 40% (Figure 7A, panel c), while localization of Rac at cell-cell contacts is not affected. Inhibition of Dgkα by 1μM R59949 treatment completely abolishes HGF-induced Rac membrane targeting (Figure 7A, panel d), while it does not significantly affect Rac localization in unstimulated cells (Figure 7A, panel b), nor Rac localization at cell-cell contacts. Similar results were obtained when Dgkα was inhibited upon expression of Dgkα-DN (Figure 7B). Upon HGF stimulation Rac is properly membrane localized in cell infected with the empty vector (Figure 7B, panel b), while it remains predominantly cytoplasmic in Dgkα-DN-expressing cells (Fig. 7B, panel f).

In summary these data demonstrate that Dgkα is required for HGF-induced activation and targeting of Rac to the plasma membrane and for the following formation of membrane ruffles, thus strongly suggesting that Dgkα is involved in the signalling pathways regulating Rac function and targeting upon activation of HGF receptor. Thoroughly, this data demonstrate that Dgkα plays a pivotal role in the migratory signalling downstream HGF, being involved in early molecular events such as Rac activation, membrane ruffles protrusion, and formation and organization of new focal adhesions, and that it consequently regulates the acquisition of a migratory phenotype in epithelial cells.

DISCUSSION

In this study we investigated the role of Dgkα in HGF- and v-Src-induced cell migration. We show that Dgkα specific inhibition, obtained either pharmacologically, or by expression of a kinase-defective dominant-negative mutant, or by siRNA-mediated downregulation of the endogenous protein, impairs both HGF- and/or v-Src-induced cell scatter and migration. This finding is consistent with previous demonstrations from our laboratory that Dgkα is activated by growth factors through a mechanism requiring its tyrosine phosphorylation mediated by Src family tyrosine kinases, and that its function is required for migration of endothelial cells (Cutrupi *et al.*, 2000; Baldanzi *et al.*, 2004; Bacchiocchi *et al.*, 2005; Baldanzi *et al.*, 2007). Moreover these data suggest that Dgkα represents a crucial node in the signalling network downstream Src regulating epithelial cell scattering and switching to a motile mesenchymal phenotype.

Although both HGF stimulation and v-Src activation promote epithelial cell dispersion by coordinating loss of intercellular adhesions and migration of cells away from one another, the two events are regulated through distinct signalling pathways (Palacios *et al.*, 2001). Intriguingly, Dgkα inhibition uncouples the down-regulation of E-cadherin-mediated intercellular adhesions from cell migration, strongly suggesting that Dgkα may regulate specifically those signalling events required for HGF- and v-Src-stimulated epithelial cell motility. Thus we investigated the role of Dgkα in well characterized HGF-induced morphological and molecular events leading to cell migration.

Spreading and lamellipodia protrusion with formation of new focal adhesions at the leading edge are mandatory steps in cell migration (Ridley *et al.* 1995; Small *et al.*, 2002). We show inhere that upon Dgkα inhibition, no cell spreading, lamellipodia extension and remodelling of focal adhesions are observed upon HGF treatment, suggesting that activation of Dgkα is likely to be required for an earlier event. Rapid formation of membrane ruffles, upon minutes from growth factors stimulation, preludes to establishment of extended lamellipodia at the leading edge of migrating cells (Royale *et al.*, 2000). Indeed, upon inhibition of Dgkα, MDCK cells fail to extend membrane ruffles after HGF stimulation. Intriguingly, while recent findings indicate that Dgkα is enriched in the pseudopodia of spontaneously invasive epithelial MSV-MDCK-INV cells (Jia *et al.*, 2005), we show that Dgkα is recruited to membrane ruffles upon HGF treatment. Altogether these data provide the first circumstantial evidence that Dgkα may act in growth factors signalling at the leading edge of migrating cells.

Ruffles formation, cell spreading and lamellipodia protrusion are dependent on Rac small GTPase activation, occurring through its targeting to newly-formed focal complexes (Ridley *et al.*,

1995; Burridge and Wennerberg, 2004; Rossman *et al.*, 2005). Rac targeting and GTP loading are regulated by a complex signalling network involving the recruitment of distinct Rac-regulating proteins to multiple molecular complexes at the leading edge of migrating cells.

An increasing body of evidence suggests that Dgks regulate small GTPases, including Rac, through multiple mechanisms, whose complexity still awaits elucidation. In T cells Dgkα and ζ negatively regulate Ras pathway, by finely tuning the access of RasGRP1, a C1 domain-containing Ras GEF, to its activator DG (Jones *et al.*, 2002; Olenchock *et al.*, 2006, Zha *et al.*, 2006). However, in epithelial cells, neither the overexpression nor the downregulation of Dgkα affects the Ras pathway, as detected by ERK-1/2 phosphorylation (our unpublished results). In addition Dgkγ, but not Dgkα, upon its recruitment to the plasma membrane, negatively regulates PDGF- and EGF-induced Rac activation and membrane ruffling, by enhancing the activity of β2-chimaerin, a Rac GAP containing a C1 and a SH2 domain (Tsushima *et al.*, 2004, Yasuda *et al.*, 2007). These observations provide further support to the previous finding that DG-dependent membrane recruitment of β2-chiamerin determines the extent and the kinetic of EGF-induced Rac activation. (Wang *et al.*, 2006). Conversely, in neurons and skeletal myoblasts Dgkζ acts in a complex with Rac at specific sites of the plasma membrane and control the remodeling of F-actin cytoskeleton leading to neurite extension and membrane ruffle protrusion, possibly by facilitating Rac1 activation and/or localization to the cell surface (Abramovici *et al.*, 2003; Yakubchyk *et al.*, 2005). Furthermore Dgkζ and PI(4)P 5-kinase co-localize with F-actin at lamellipodia protrusions in epithelial cells (Luo *et al.*, 2004), where Dgk-generated PA is required for full activation of PI(4)P 5-kinase activity, consistently with a role of both lipid kinases in positive regulation of Rac function. Interestingly a Dgk and a PI(4)P 5-kinase activities were found to associate in a complex with Rac and RhoGDI (Tolias *et al.*, 1998). RhoGDI forms a complex with Rac, keeping it in a cytosolic inactive GDP-bound form, and upon Rac activation it contributes to Rac targeting to specific sites at the plasma membrane (Moissoglu *et al.*, 2006). As Rac targeting implies the displacement of the interaction between Rac and RhoGDI, the finding that *in vitro* PA and $PI(4,5)P_2$ impair RhoGDI affinity for Rac (Chuang *et al.*, 1993; Ugolev *et al.*, 2006), raises the hypothesis that activation of the RhoGDI-associated Dgk may allow the release of Rac from RhoGDI, and leads to speculate that also Dgkα may regulate Rac activation through this mechanism. Altogether, these data strongly indicate that distinct Dgk isoforms act as regulators of Rac membrane targeting and activation through multiple mechanisms, whose complexity still awaits to be elucidated.

Several Rac GEFs, such as Vav2, DOCK180/Elmo, βPIX and Tiam1, are regulated either directly or indirectly through Src-dependent tyrosine phosphorylation (Lamorte *et al.*, 2002; Servitja *et al.*, 2003; Santy *et al.*, 2005), and/or interaction with $PI(3,4,5)P_3$ (Welch *et al.*, 2003).

Although there is no direct evidence for a role of any Dgk isoforms in the regulation of any Rac GEFs, based on the observations reported inhere, we may discuss several hypothesis, providing a framework for further investigation.

Several data indicate that, upon growth factors and v-Src stimulation, rapid Rac-mediated membrane ruffling occurs through the recruitment of βPIX to Paxillin-containing focal complexes (Cotton *et al.*, 2007). Indeed βPIX mediates rapid ruffles formation upon PDGF, EGF and FGF treatment in different cell types (Lee *et al.*, 2001; Park *et al.*, 2004; Shin *et al.*, 2006), and the interaction between βPIX and Rac is necessary and sufficient for Rac recruitment to membrane ruffles and focal adhesions (ten Klooster *et al.*, 2006). Crk recruitment to tyrosine-phosphorylated Paxillin contributes to βPIX localization to focal complexes (Lamorte *et al.*, 2003). Indeed we show that HGF stimulates phosphorylation of Paxillin on both Tyr^{31} and Tyr^{118}, the two major determinants for Crk association. Surprisingly, the inhibition of Dgkα enhances basal phosphorylation of Paxillin on both residues, but does not affect their phosphorylation upon HGF stimulation, suggesting that Dgkα may affect βPIX function in a complex manner. Moreover βPIX and Dgkα do not associate in a complex, not even upon HGF stimulation (data not shown).

Upon minutes of growth factors stimulation, βPIX recruitment and Rac activation are promoted by rapid GTP/GDP cycling of Arf6, suggesting that Arf6 plays a pivotal role in Rac-mediated membrane ruffling (ten Klooster *et al.*, 2006; Cotton *et al.*, 2007). Furthermore, upon hours of HGF stimulation, ARF6 has been recently shown to regulate Rac targeting at tubule tips of MDCK cells grown in 3D collagen (Tushir and D'Souza-Schorey, 2007). Interestingly, several Arf GAPs are regulated by phospholipids, including PA (Randazzo *et al.*, 2000). Moreover, PLD-induced production of PA downstream of Arf6 is required for Arf6-dependent epithelial cell ruffling and migration (Santy and Casanova, 2001). Thus we may speculate that also Dgkα may contribute to regulate Arf6 function in coordinating Rac activation, focal adhesions remodelling and membrane ruffles formation.

Several Rac and Arf GEFs are regulated by $PI(3,4,5)P_3$, the product of PI 3-kinase. However we can rule out that Dgkα mediates Rac activation by regulating PIP_3 synthesis, as inhibition of Dgkα does not affect HGF-induced activation of Akt, a major PIP_3 target. Conversely, the finding that PIP_3 might contribute to recruit and activate Dgkα (Ciprés *et al.*, 2003) allow to speculate that Dgkα might contribute to couple PIP_3 generation to the activation of one of the PIP_3-dependent Rac GEFs, such as Vav2 and Tiam1. However, the expression of a either wild-type or kinase-defective Dgkα in fibroblasts does not affect PDGF-induced Rac activation and ruffles formation (Tsushima *et al.*, 2004), both mediated by Vav2 (Liu and Burridge, 2000). Moreover, the Rac GEF Tiam1 is

mainly involved in maintaining E-cadherin-mediated epithelial cell-cell adhesions (Mertens *et al.*, 2003), event which we showed to not be regulated by Dgkα, making Tiam1 an unlike target of Dgkα activity.

In conclusion, inhere we clearly demonstrate that activation of Dgkα is required for HGF- and v-Src-induced cell migration. By exploring some significant molecular events affected by Dgkα inhibition, we raise the hypothesis that Dgkα may act in growth factors migratory signalling by mediating Rac targeting and activation, thus revealing a novel signalling pathway linking tyrosine-kinase receptors and Src to small GTPases in the context of cell migration.

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FIGURE LEGENDS

Figure 1. v-Src activates Dgkα. MDCK-*ts*-v-Src maintained at the non-permissive temperature of 40.5°C were transiently transfected with Myc-Dgkα, starved overnight in 0% FBS medium and shifted to the permissive temperature of 35°C for the times indicated. Cell lysates were immunoprecipitated with an anti-Myc antibody. Half of each immunoprecipitate was separated by SDS-PAGE and after blotting was probed with anti-Myc; the other half was assayed for Dgk activity as described in Materials and Methods.

Figure 2. Dgkα is required for HGF-induced cell scatter and migration of MDCK cells (A) MDCK cell colonies were treated, in 0% FBS medium, with HGF 2 ng/ml in presence or absence of 1μM R59949 for 24 hours. Representative fields are shown. (B) Control or MDCK/Dgkα-WT cells were allowed to migrate into the wounded area in 0% FBS medium with 2.5 ng/ml HGF in presence or absence of 1μM R59949 for 7 hours. Quantification was performed as described in Materials and Methods. Means of at least 4 experiments with standard errors are shown. ** = $p<0.005$. The western blot shows the level of Myc-Dgkα-WT expression. (C) MDCK/empty vector or MDCK/Dgkα-DN were treated, in 0% FBS medium, with HGF 2 ng/ml for 24 hours. Representative fields are shown. The western blot shows the level of Myc-Dgkα-DN expression. (D) Lysates of MDCK cells transiently transfected with negative control siRNA or canine Dgkα siRNAs C1, C2 and C3 were separated by SDS-PAGE and after blotting were probed for Dgkα and tubulin. MDCK cell colonies were transfected with Block-IT fluorescent siRNA to evaluate the efficiency of transfection. (E) MDCK and MDCK/Mus-Dkgα cell colonies were transiently transfected with negative control siRNA or canine Dgkα siRNAs and treated, in 0% FBS medium, with HGF 2 ng/ml for 24 hours. Representative fields are shown. (F) MDCK cells were seeded in the upper part of a chemotaxis chamber and induced to migrate in presence of 50 ng/ml of HGF in the bottom part, in presence or absence of 1μM R59949. The histograms represent the number of migrated cells, means of 8 different wells with standard errors. ** = $p<0.005$. A representative experiment is shown. (G) MDCK/empty vector or MDCK/Dgkα-DN cells were seeded in the upper chamber of a Transwell apparatus. Invasion through a Matrigel-covered porous membrane was induced in 48 hours in 2% FBS medium by the presence of 100 ng/ml HGF in the lower chamber. Fixed cells on the Transwells lower face were stained with crystal-violet, photographed and quantified by optical densitometry. Means of three experiments are shown, with standard errors; * = $p \leq 0.05$. (H) MDCK-*ts*-v-Src cell colonies, maintained at the non-permissive temperature of

40.5°C, were transiently transfected with negative control siRNA or canine Dgkα siRNAs, as indicated, placed in 0% FBS medium and shifted to the permissive temperature of 35°C for 24 hours. The same experiment was performed with untransfected cells, in presence or absence of 1μM R59949, as indicated. Representative pictures are shown.

Figure 3. Dgkα is required for HGF-induced cell spreading and lamellipodia formation, but not for down-regulation of E-cadherin-mediated intercellular adhesions. (A) MDCK/empty vector or MDCK/Dgkα-DN cells were treated with HGF 10 ng/ml for 6 hours, fixed and stained for E-cadherin. Representative pictures are shown. Scale bar = 20 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were treated with HGF 10 ng/ml for 6 hours. Cell lysates were fractionated into a NP-40-soluble (S) and a NP-40-insoluble (I) fraction. Equal sample volumes were loaded, separated by SDS-PAGE and probed for E-cadherin. (C) MDCK cell colonies were starved overnight in a 2% FBS medium and treated with HGF 10 ng/ml for 4 hours, in presence or absence of 1μM R59949. Fixed cells were stained for actin filaments with phalloidin. Scale bar = 40 μm. (D) MDCK cells treated as described in (C) were fixed and stained for actin (red, panels a,b,c,d) and FAK (green, panel e,f,g,h). Scale bar = 16 μm. Representative pictures are shown.

Figure 4. Dgkα is required for HGF-induced membrane ruffling of MDCK cells. (A) MDCK cells were transiently transfected with GFP-Dgkα, starved overnight in 0% FBS medium, stimulated with HGF 10 ng/ml for 15 minutes, fixed and stained for actin. Scale bar = 8 μm (B) MDCK cell colonies were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for actin. Scale bar = 16 μm. Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles (arrows). The percentage of cells with membrane ruffles was calculated. Means of three experiments with standard errors are shown. ** = $p<0.005$. (C) MDCK and MDCK/Mus-Dgkα were transiently transfected with negative control siRNA or canine Dgkα siRNAs C1, C2 or C3, starved overnight in 0% FBS medium and treated with HGF 10 ng/ml for 15 minutes. Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles. The percentage of cells with membrane ruffles was calculated. Means of three experiments with standard errors are shown. * = $p<0.05$, ** = $p<0.005$. (D) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for actin (red, panels a,b,c,d). The arrows indicate membrane ruffles in empty vector-infected cells. Scale bar = 16 μm. Representative

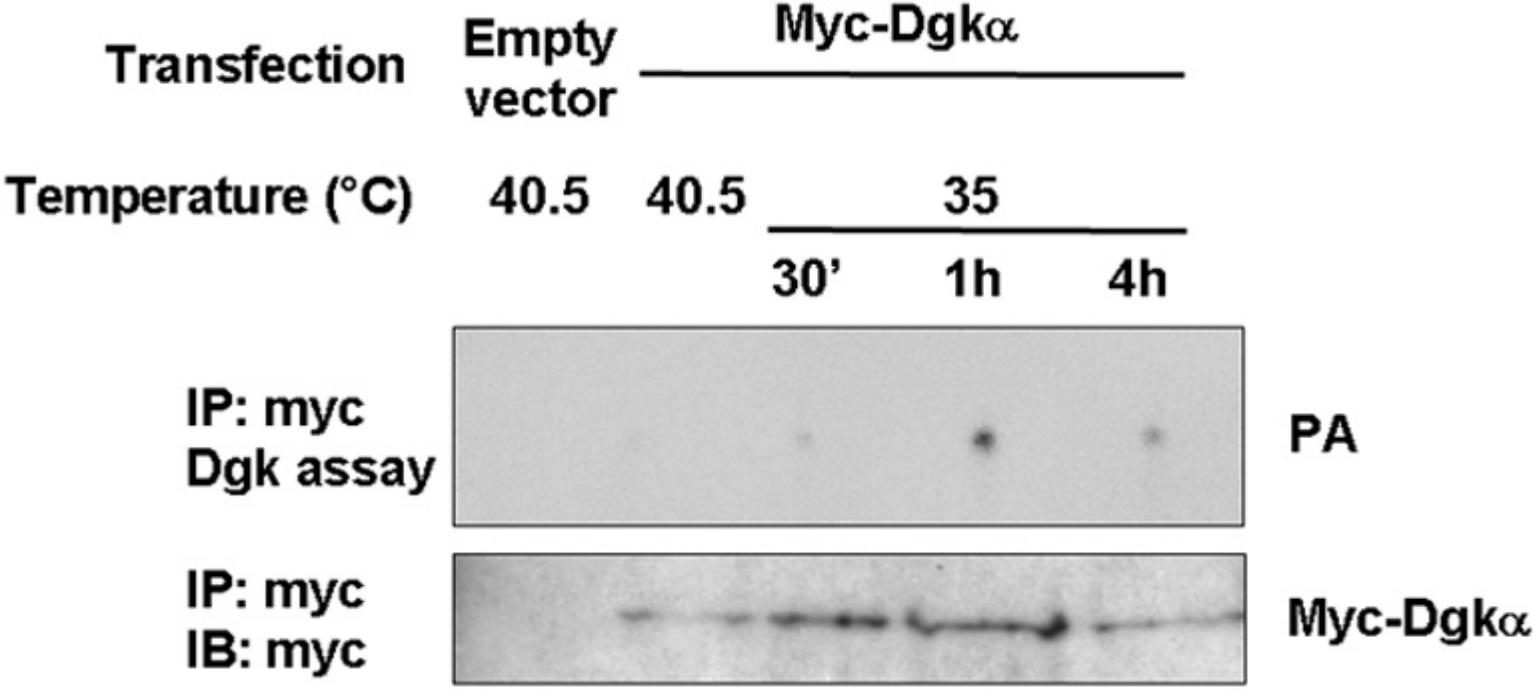
pictures are shown. Confocal acquired images were observed and cells at the edge of colonies were scored for presence of membrane ruffles. Means of three experiments with standard errors are presented. * = $p<0.05$.

Figure 5. Dgkα is required for HGF-induced Paxillin localization to newly-formed focal complexes. (A) MDCK cell colonies were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for Paxillin (green, panels a,b,c,d) and actin (red, panels e,f,g,h). Representative pictures are shown. Scale bar = 16 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for Paxillin (red, panels a,b,e,f). Thick arrows indicate Paxillin localization at focal adhesions in the areas of membrane ruffling, while the slim arrow indicates Paxillin localization at cell periphery in a Dgkα-DN-infected cell, without membrane ruffles. Scale bar = 16 μm. Representative pictures are shown. (C) MDCK cell colonies were treated as described in (A), fixed and stained for Paxillin (green), Vinculin (red) and actin (blue). Representative pictures are shown. Scale bar = 16 μm. (D) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium and treated with HGF 50 ng/ml for 15 minutes. Whole cell lysates were separated by SDS-PAGE and probed with anti-Paxillin $pTyr^{31}$ and $pTyr^{118}$ and anti-Paxillin.

Figure 6. Dgkα is required for HGF-induced Rac activation. (A) MDCK cells were starved overnight in 0% FBS medium, treated with HGF 100 ng/ml for 15 minutes in presence or absence of 1μM R59949 and lysed. GTP-bound active Rac was purified in each sample by pull-down with GST-fused PAK CD domain. MDCK/empty vector and MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with HGF 100 ng/ml for 15 minutes and pull-down assays were performed as described before. (B). MDCK/empty vector and MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with HGF 50 ng/ml for 15 minutes and lysed. Whole cell lysates were separated by SDS-PAGE and probed with anti-Akt $pSer^{473}$ and Akt.

Figure 7. Dgkα is required for HGF-induced Rac localization to the plasma membrane. (A) MDCK cell colonies were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes in presence or absence of 1μM R59949, fixed and stained for Rac (green, panels a,b,c,d) and actin (red). Representative pictures are shown. Scale bar = 16 μm. Confocal acquired images were observed and the percentage of cells at the edge of colonies featuring Rac at the outer plasma

membrane was calculated. Means of three experiments with standard errors are shown. * = $p<0.05$. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with HGF 10 ng/ml for 15 minutes, fixed and immunostained for Rac (red, panels a,b,e,f). Representative pictures are shown. Scale bar = 20 μm. Confocal acquired images were observed and the percentage of edge-of-colony cells featuring Rac at the outer plasma membrane was calculated. Means of three experiments with standard errors are presented. ** = $p<0.005$.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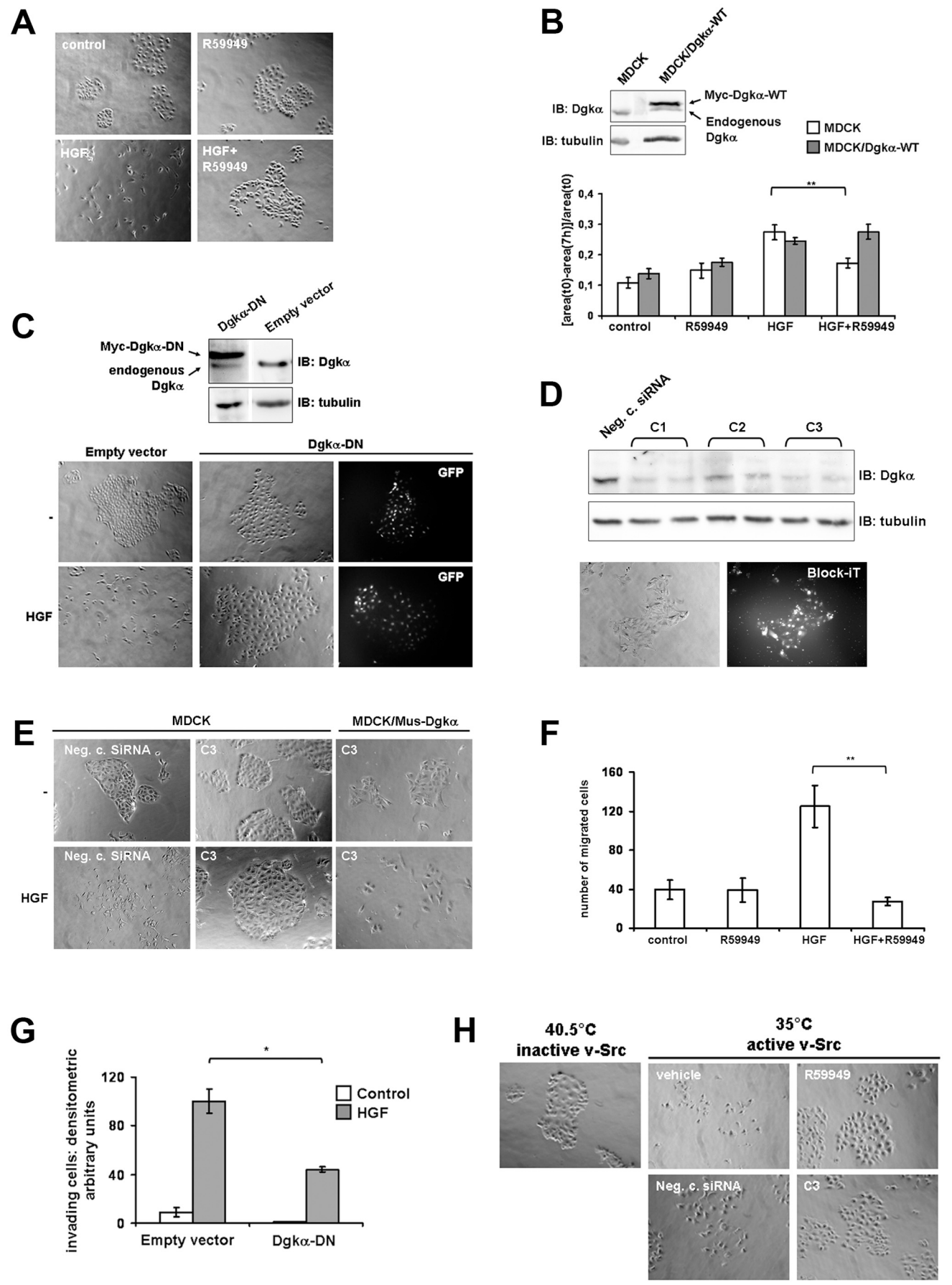
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HGF
HGF+
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IB: tubulin
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Endogenous
Dgkα
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0,4
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HGF
HGF+R59949
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Dgkα-DN
Empty vector
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endogenous
Dgkα
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IB: tubulin
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GFP
GFP
-
HGF
D
Neg. c. siRNA
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IB: Dgkα
IB: tubulin
Block-iT
E
MDCK
MDCK/Mus-Dgkα
Neg. c. SiRNA
C3
C3
Neg. c. SiRNA
C3
C3
-
HGF
F
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120
80
40
0
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R59949
HGF
HGF+R59949
G
invading cells: densitometric
arbitrary units
120
80
40
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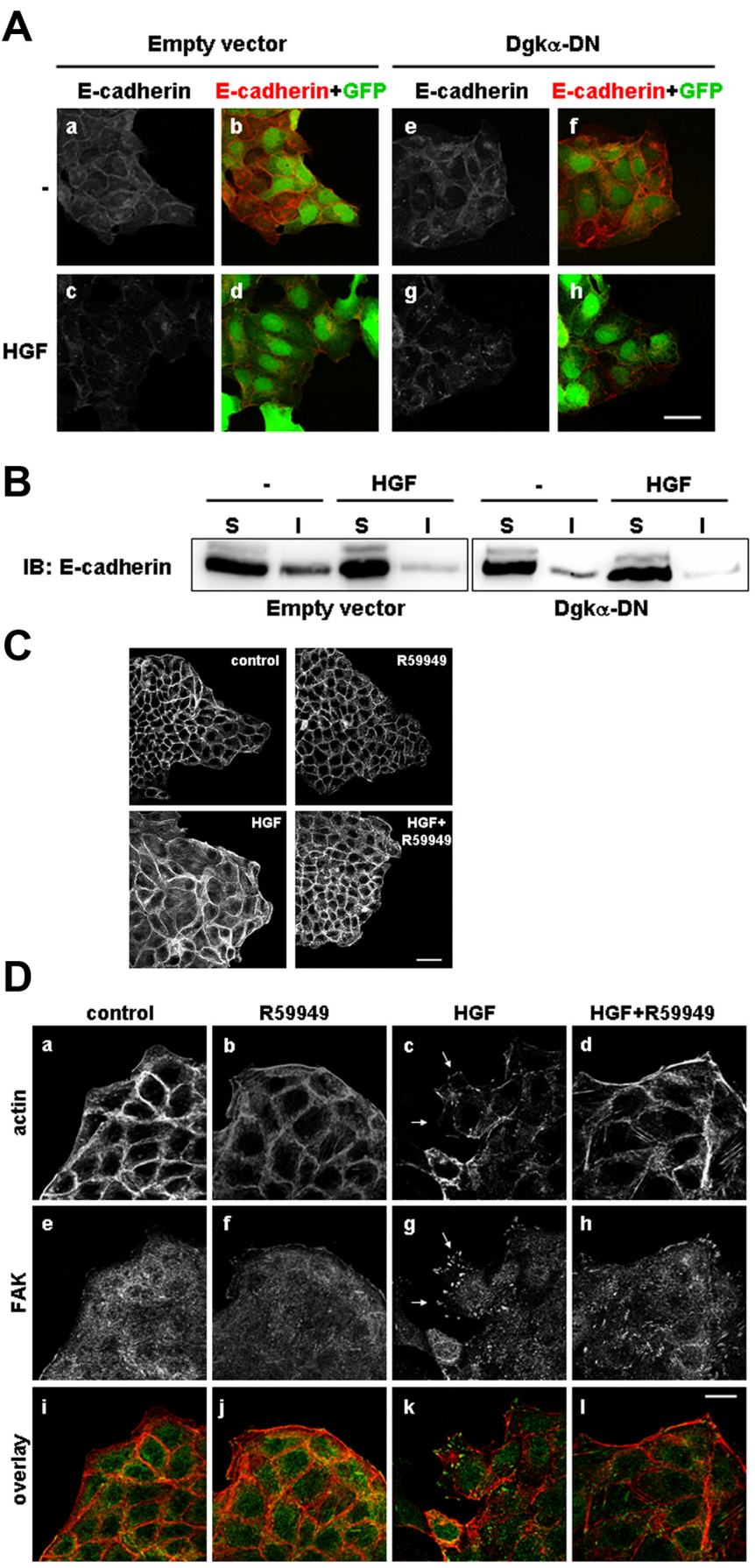
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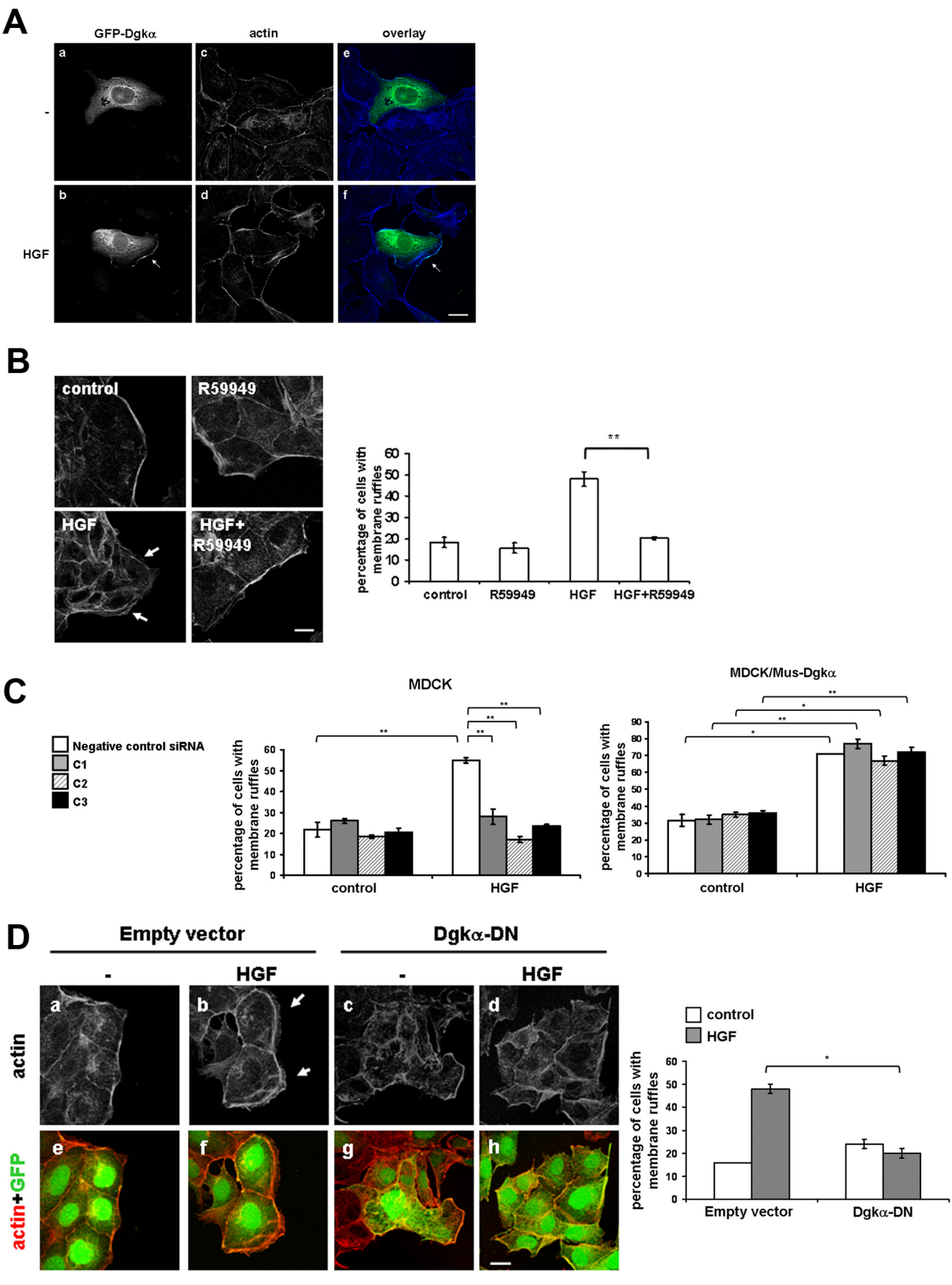
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HGF
HGF+
R59949
percentage of cells with membrane ruffles
control
R59949
HGF
HGF+R59949
C
MDCK
MDCK/Mus-Dgkα
Negative control siRNA
C1
C2
C3
percentage of cells with membrane ruffles
control
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percentage of cells with membrane ruffles
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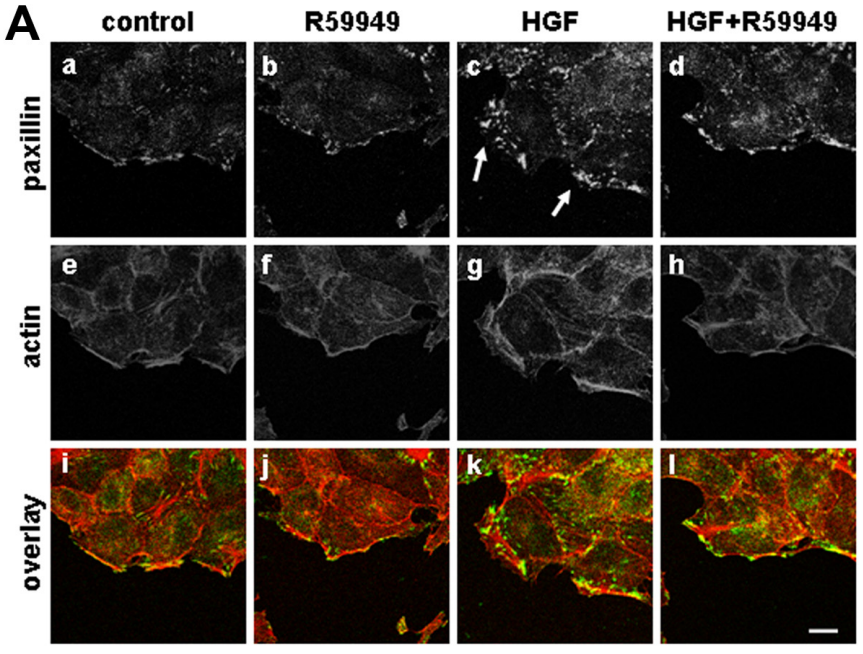
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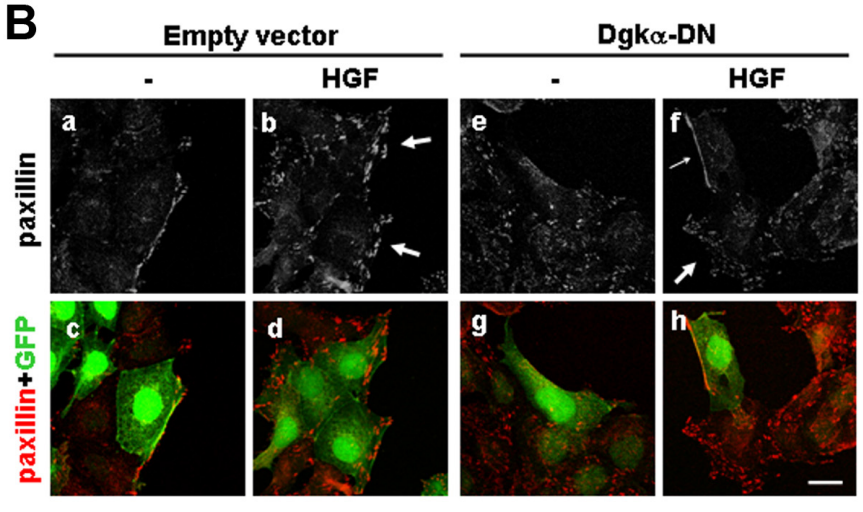
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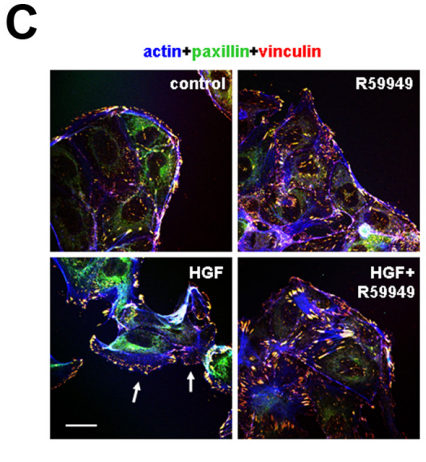
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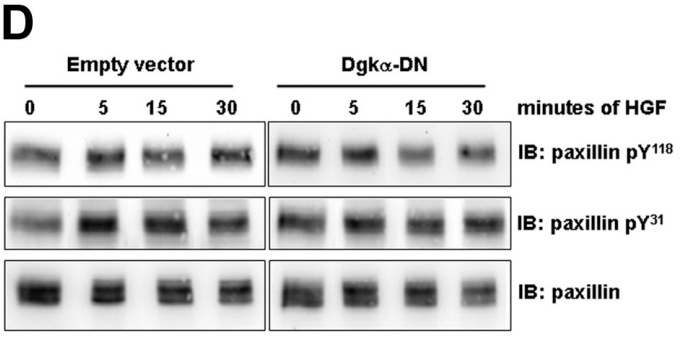
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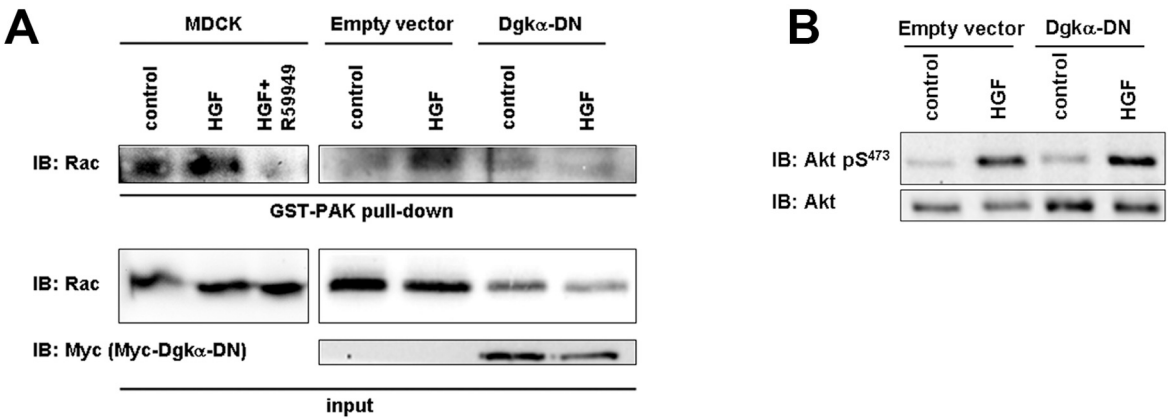
IB: paxillin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IB: Rac
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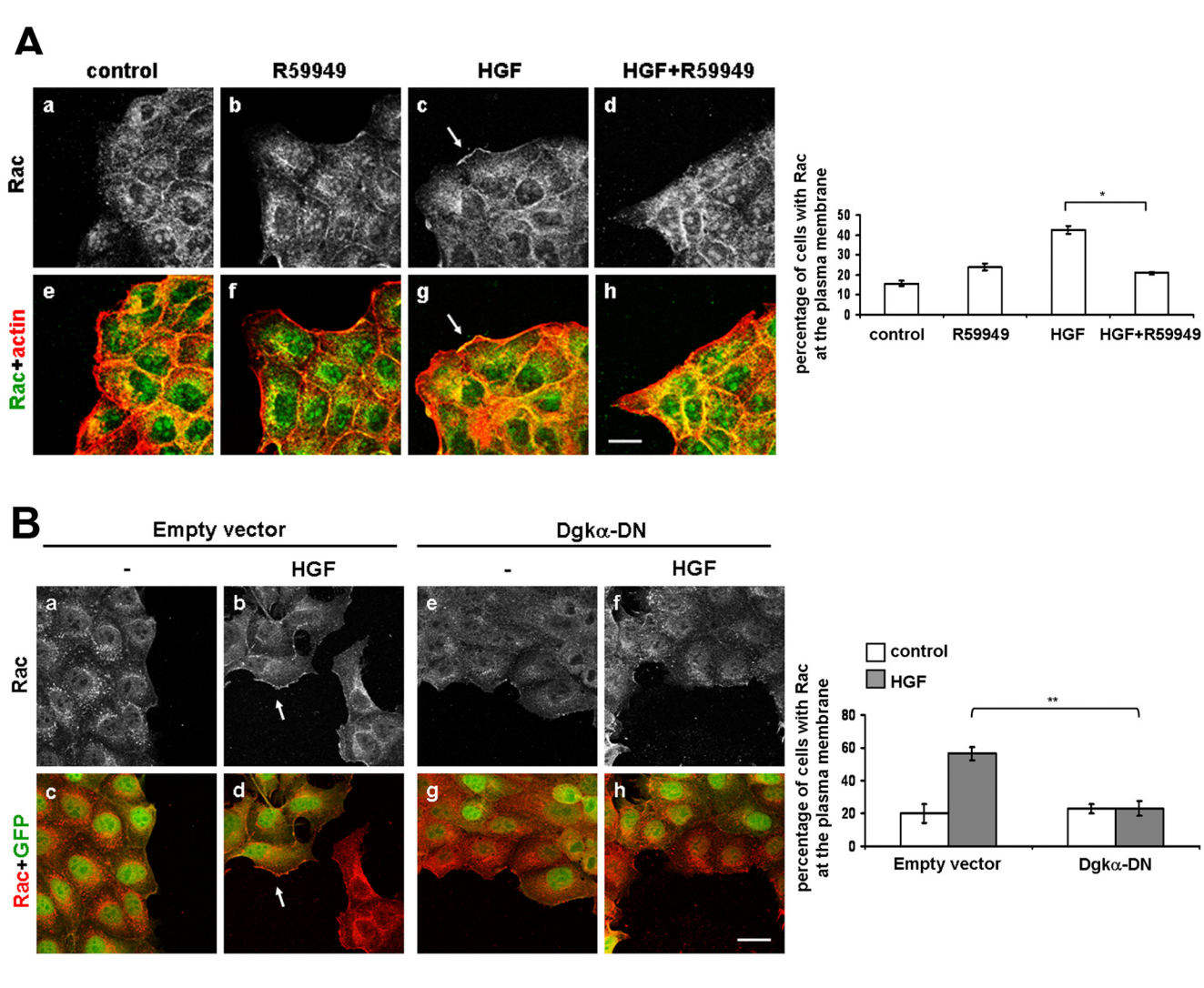
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40
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Empty vector
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80
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percentage of cells with Rac at the plasma membrane
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ORIGINAL 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.

Keywords: ■ | ■ | ■

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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 Luo *et al*., 2003; van Blitterswijk and Houssa, 2000). 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

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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 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

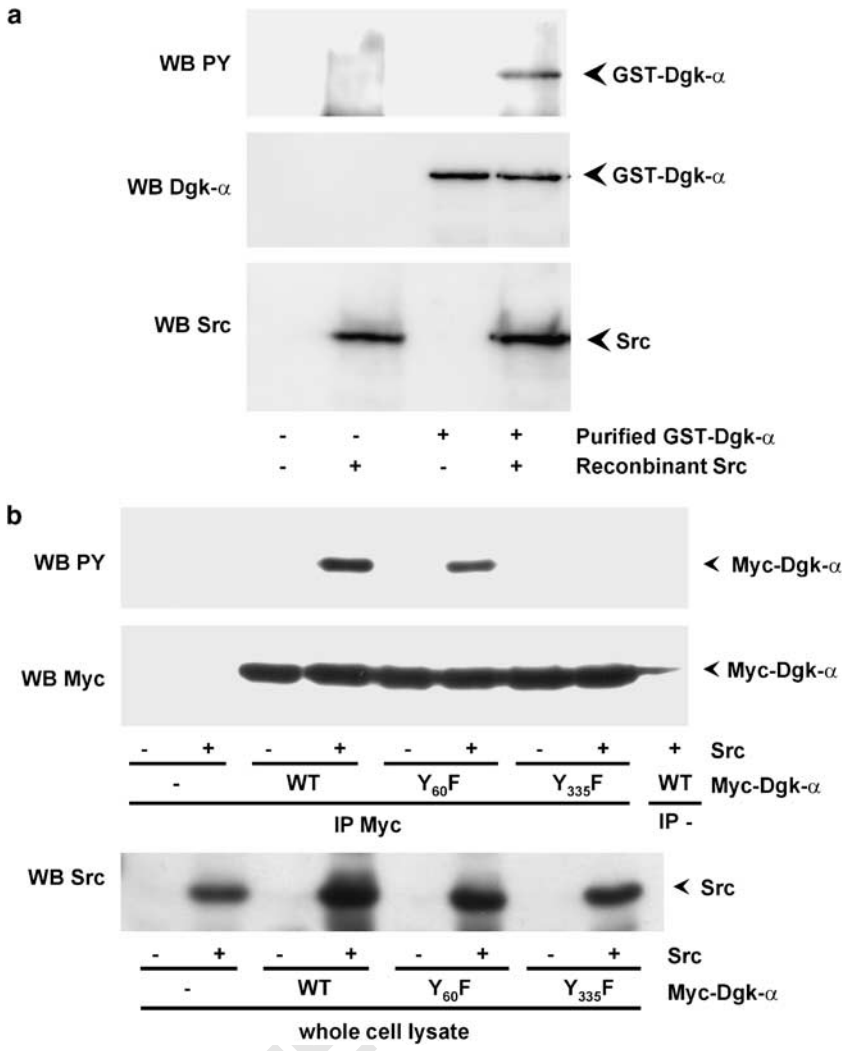

Figure 1 Dgk-α phosphorylation by Src on Y335. (**a**) Partially purified GST-Dgk-α or GST were incubated with or without recombinant Src in kinase buffer for 10 min at 30°C. Samples were split and analysed by western blot with antibodies against phosphotyrosine (upper panel), Dgk-α (middle panel) or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

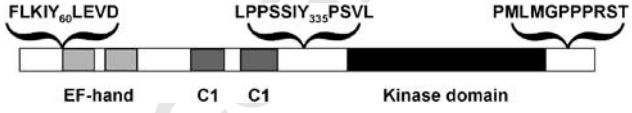

Figure 2 Dgk-α structure. The structure of Dgk-α contains three structurally defined domains: 2 EF-hand motifs, a double atypical C1 domain and a catalytic domain. Y60 lies upstream of the EF-hand motif, Y335 between the second C1 domain and the catalytic domain and the proline-rich region at the C-terminal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

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 interaction of Dgk-α with Src SH3 domain may precede its tyrosine phosphorylation.

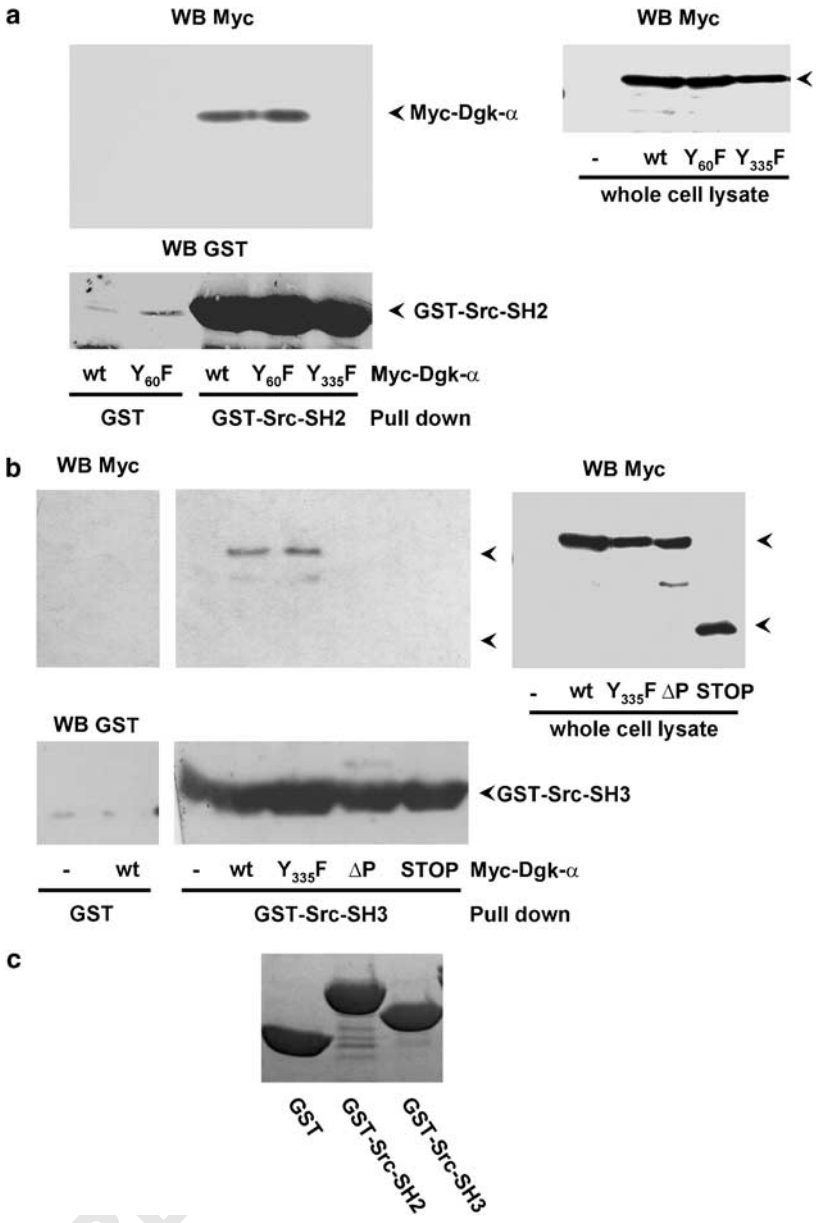

Figure 3 Dgk-α interaction with Src-SH2 and Src-SH3 domains. (**a**) Growing COS-7 cells, transfected with indicated Myc-Dgk-α constructs, were lysed in buffer A. Cell lysates were incubated with agarose-bound purified GST or GST-Src-SH2 for a pull-down assay. Pulled down Myc-Dgk-α (left panel) and Myc-Dgk-α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

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

Table 1

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.

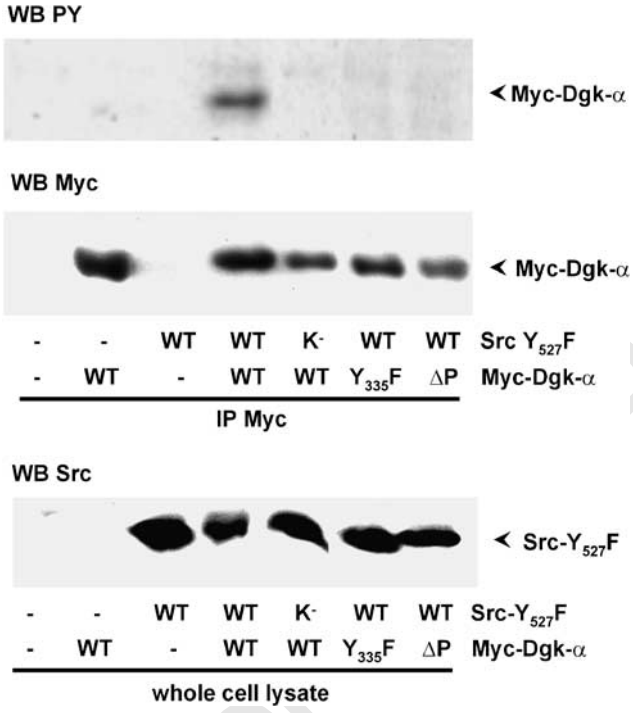

Figure 4 Dgk-α phosphorylation by Src requires Y335- and proline-rich C-terminal sequence. Growing HEK 293T co-transfected with the indicated Myc-Dgk-α and Src-Y527F constructs were lysed in detergent-containing buffer A. Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed by western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

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).

In summary, these results, providing the first evidence *in vivo* that Dgk-α is a target of oncogenic Src,

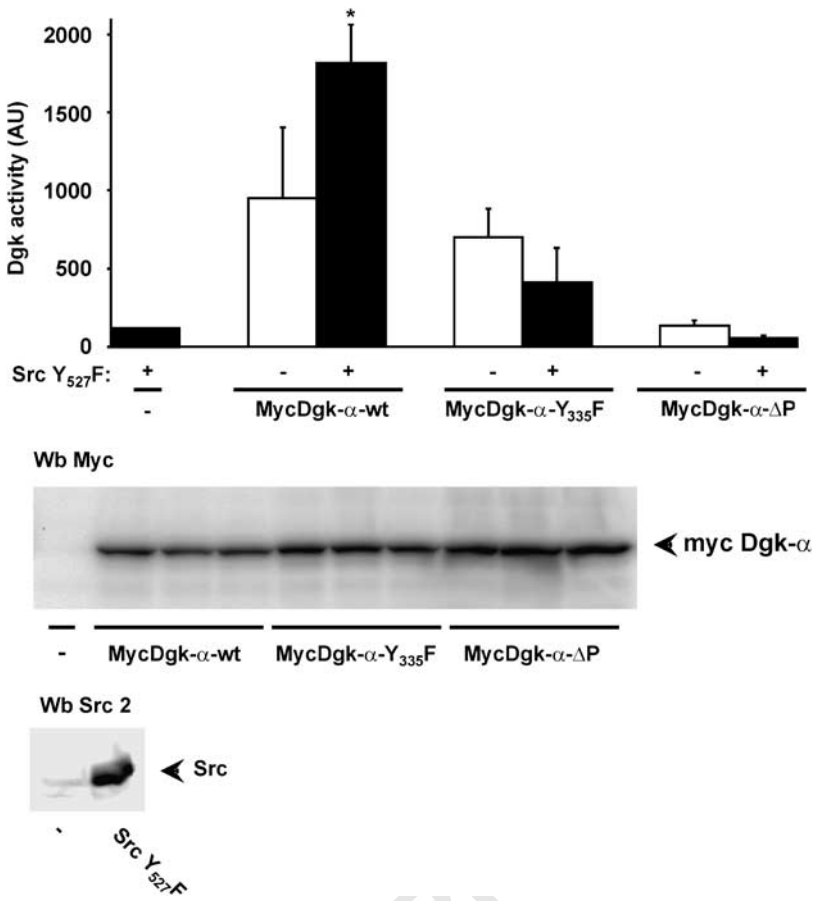

Figure 5 Dgk-α activation by c-Src *in vitro* requires Y335- and proline-rich C-terminal sequence. COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP or Src were homogenized with buffer B in absence of detergent. Cell extracts were mixed as indicated in presence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

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

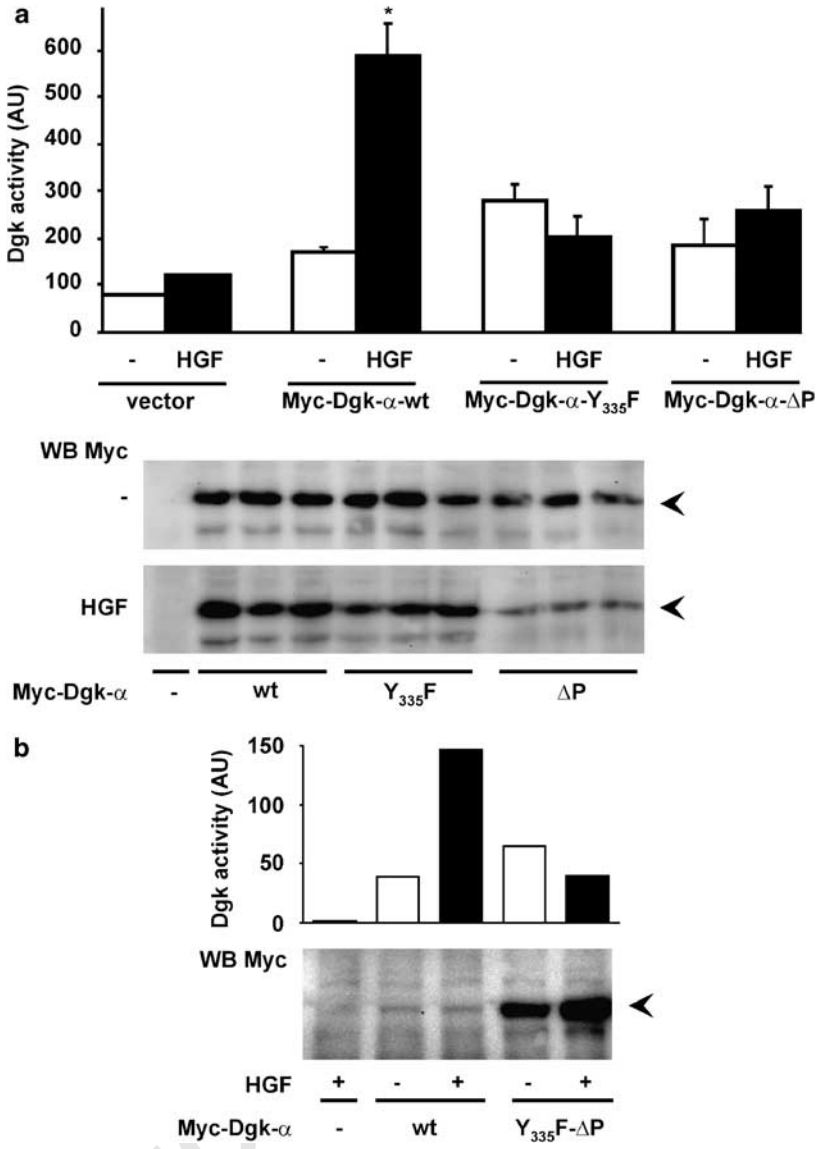

Figure 6 Dgk-α activation by HGF *in vivo* requires Y335- and proline-rich C-terminal sequence. (**a**) COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP were stimulated with HGF (100 μg/ml, 15 min), homogenized with buffer B in absence of detergent and analysed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

both GFP-Dgk-α-Y335F and GFP-Dgk-α-ΔP suggest that the recruitment of Dgk-α to the plasma membrane may occur through vesicular traffic. If this holds true, we should expect that specific inhibition of vesicular traffic between the inner cytosol and the plasma membrane by 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

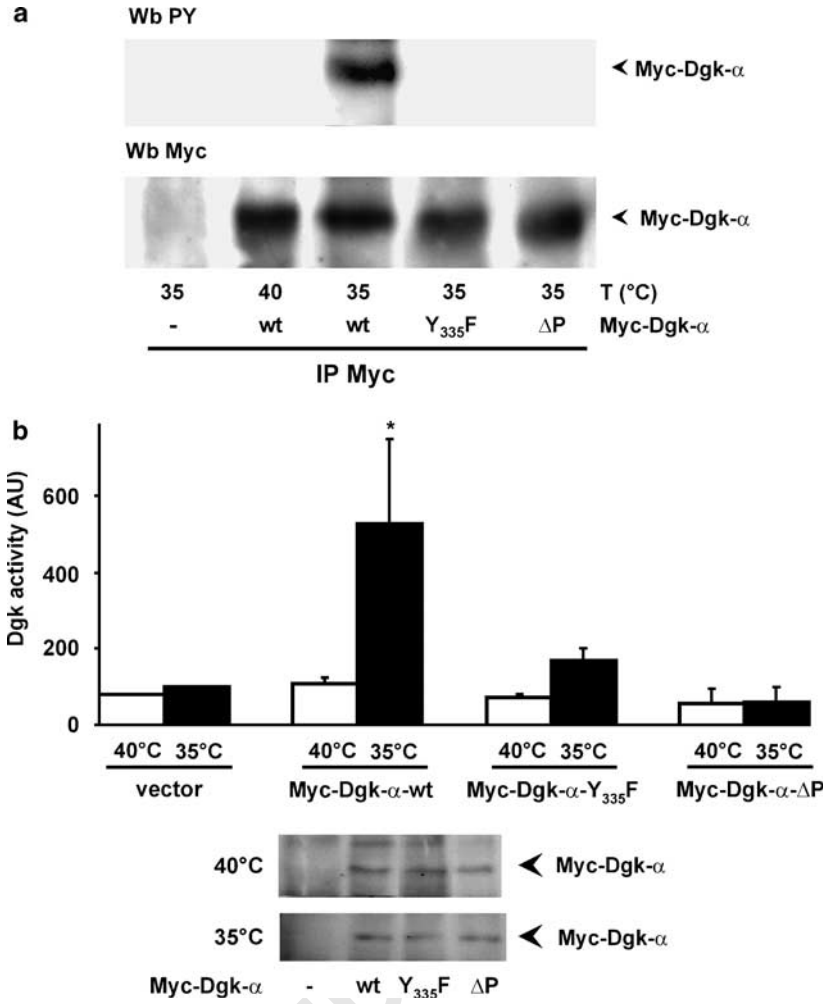

Figure 7 Dgk-α phosphorylation and activation by v-Src requires Y335- and proline-rich C-terminal sequence. Ts-v-Src/MDCK cells transfected with the indicated Myc-Dgk-α constructs, were cultured at nonpermissive temperature (40°C), and, where indicated, shifted at the permissive temperature (35°C) for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

(Cutrupi *et al*., 2000; Baldanzi *et al*., 2004), we investigated whether Y335 contributes to the transduction of HGF pro-migratory signaling. Although HGF 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.

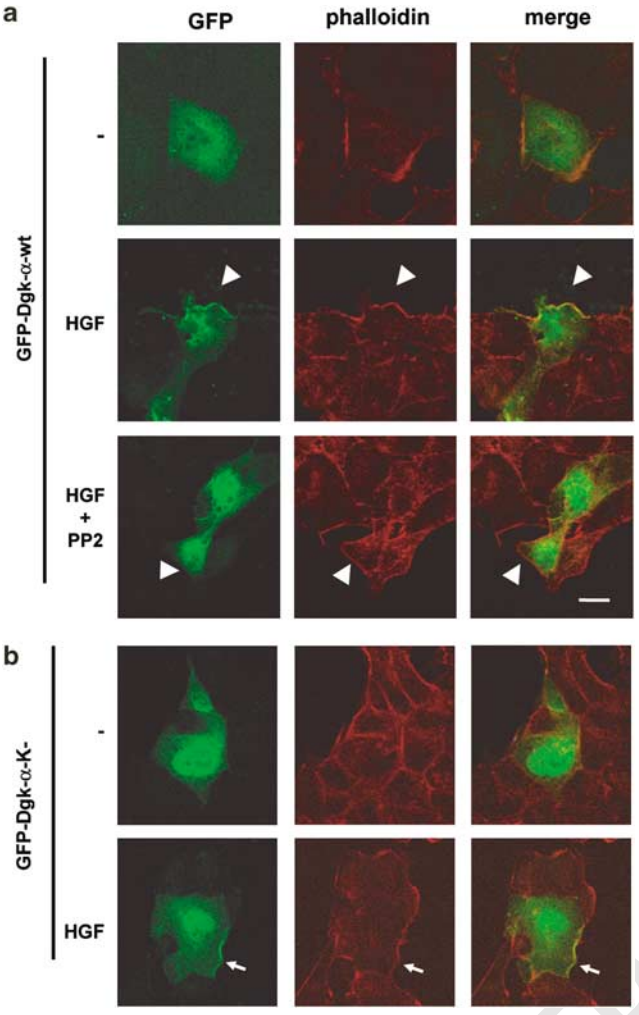

Figure 8 Dgk-α is recruited at cell membrane upon HGF treatment. MDCK cells transfected with GFP-Dgk-α wt or GFP-Dgk-α-K− were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with PP2 (10 μM PP2 for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal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

Discussion

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

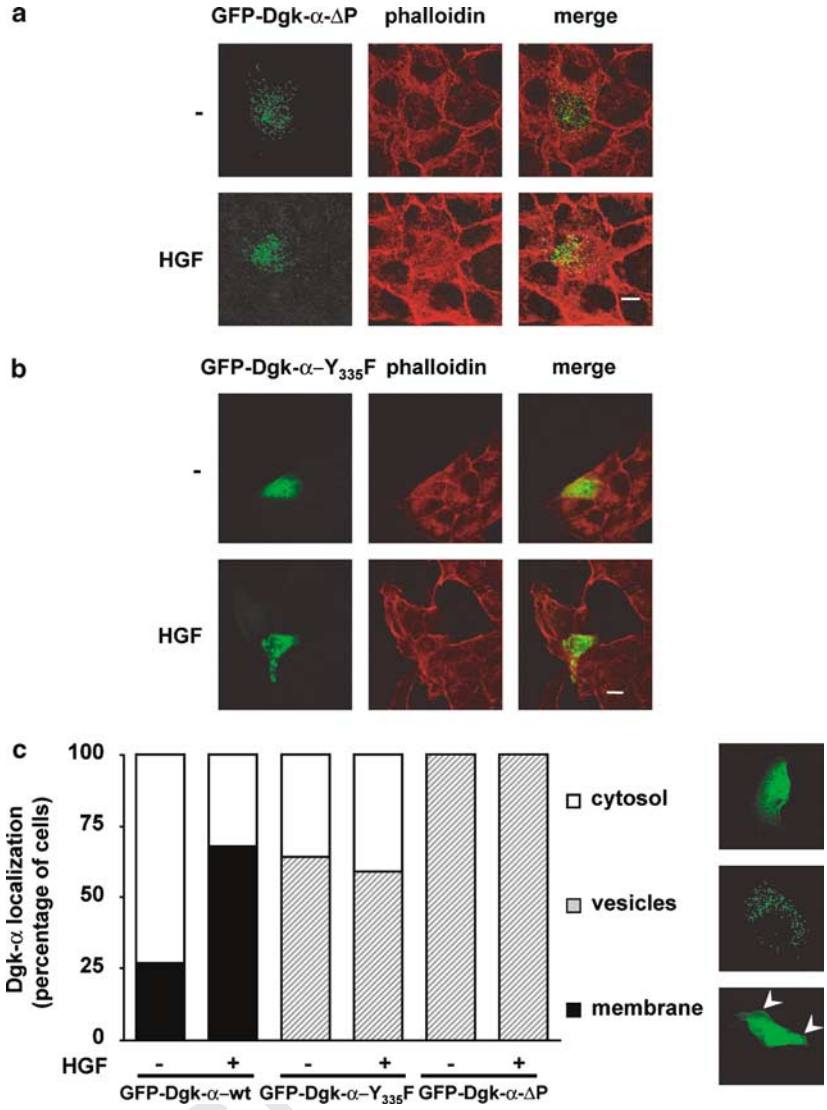

Figure 9 Recruitment of Dgk-α at cell membrane requires Y335- and proline-rich C-terminal sequence. MDCK cells tranfected with either GFP-Dgk-α-wt, GFP-Dgk-α-ΔP (**a**) or GFP-Dgk-α-Y335F (**b**) were stimulated with HGF (50 ng/ml, 15 min). Cells were stained with phalloidin-TRITC and images acquired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

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 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, con-

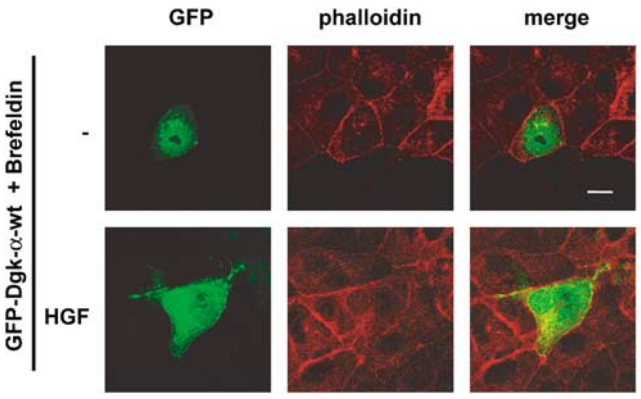

Figure 10 Brefeldin causes accumulation of Dgk-α on cytoplasmic vesicles. MDCK cells transfected with GFP- Dgk-α wt were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with BFA (10 μM BFA for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal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

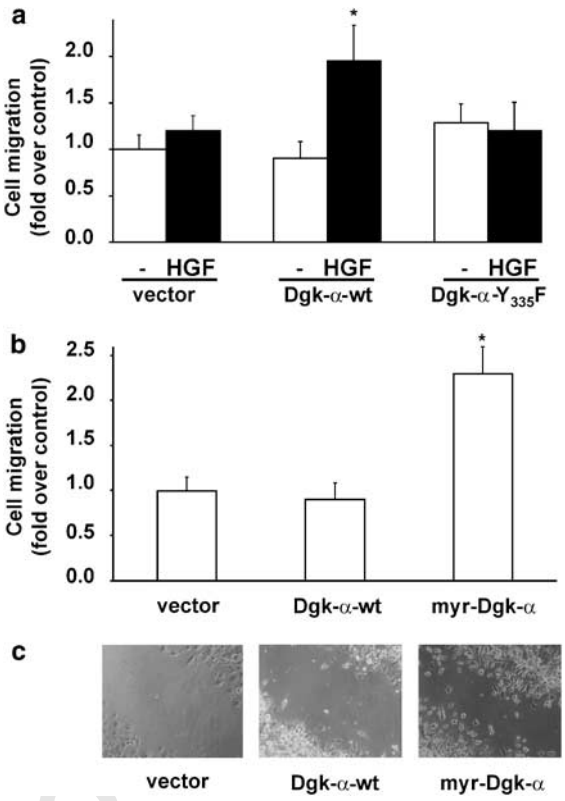

Figure 11 Membrane recruitment of Dgk-α is necessary and sufficient for cell motility. (**a**) COS cells transfected with either Dgk-α wt, or Y335 mutant or empty vector were stimulated to migrate by HGF (100 ng/ml) in a transwell chemotaxis assay. Data are expressed as fold increase over control, values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

versely, 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 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), supplemented with glutamine, 10% fetal calf serum (Gibco) and antibiotic-antimycotic solution (Sigma).

Reagents

Recombinant HGF was from Peprotech, anti-phosphotyrosine 4G10 and anti-Myc 9E10 antibodies were from Upstate Biotechnology. Src-2 anti-Src antibodies were from Santa Cruz. Secondary antibodies anti-mouse and anti-rabbit IgG HRP-labeled were from NEN (PerkinElmer life sciences). Alexa Fluor 456 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) using the Gateway kit (Invitrogen). GST-Dgk-α wt was obtained by cloning Dgk-α wt in pcDEST-27 (Invitrogen) using the Gateway kit (Invitrogen). Detailed information and protocols on the Gateway technology are available on www.invitrogen.com. Point mutations on Dgk-α were obtained using QuikChange Site-Directed Mutagenesis Kit (Stratagene); mutating oligonucleotides were:

Y60F GGGAACATTATCCACTTCGAGGAAGATTTTCAGGAATTGCTG and CAGCAATTCCTGAAAATCTTCCTCGAAGTGGATAATGTTCCC;

Y335F GGCCAGGACACTGGGAAAGATGGAAGATGGAGG and CCTCCATCTTCCATCTTTCCCAGTGTCCTGGCC;

STOP GATGATTTAAATTAGAGCACCTCTGAGGCT and AGCCTCAGAGGTGCTCTAATTTAAATCATC;

K− CGGATTGGTGTGTGGTGACGACGGCACAGTAGGC and GCCTACTGTGCCGTCGTCACCACACACCAAAATCCG.

Dgk-α-ΔP was obtained by insertion of the annealing product of the oligonucleotide CATAACTGCAGTTATGGGCC at the *Apa*I site at position 2168 of Dgk-α wt cDNA. All mutants used have been verified by direct sequencing (MWG biotech or C.R.I.B.I.-BMR). Plasmid encoding Src wt, 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 AGTCCCGGACTGAAACGAACTTGTCCTTCAAGAAA and TTTCTTGAAGGACAAGTTCGTTTCAGTCCGGGACT

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) 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 (Figure 5 and Supplementary material 1) were prepared by collecting the cells with a rubber scraper in buffer B (buffer A without NP-40), homogenizing them with a 23 G syringe (Sigma) and by spinning at 500 *g* for 15 min. Protein concentration was determined by the bicinchoninic acid method (Pierce) 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).

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), 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). 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 ⩾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 ⩾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) 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). The lower chamber was filled with 0,1% FCS medium with or without HGF (50 U/ml) and incubated at 37°C in air with 5% CO2 for 8 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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Ghrelin and Des-Acyl Ghrelin Promote Differentiation and Fusion of C2C12 Skeletal Muscle Cells

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Ghrelin is an acylated peptidyl gastric hormone acting on the pituitary and hypothalamus to stimulate appetite, adiposity, and growth hormone release, through activation of growth hormone secretagogue receptor (GHSR)-1a receptor. Moreover, ghrelin features several activities such as inhibition of apoptosis, regulation of differentiation, and stimulation or inhibition of proliferation of several cell types. Ghrelin acylation is absolutely required for both GHSR-1a binding and its central endocrine activities. However, the unacylated ghrelin form, des-acyl ghrelin, which does not bind GHSR-1a and is devoid of any endocrine activity, is far more abundant than ghrelin in plasma, and it shares with ghrelin some of its cellular activities. Inhere we show that both ghrelin and des-acyl ghrelin stimulate proliferating C2C12 skeletal myoblasts to differentiate and to fuse into multinucleated myotubes in vitro through activation of p38. Consistently, both ghrelin and des-acyl ghrelin inhibit C2C12 proliferation in growth medium. Moreover, the ectopic expression of ghrelin in C2C12 enhances differentiation and fusion of these myoblasts in differentiation medium. Finally, we show that C2C12 cells do not express GHSR-1a, but they do contain a common high-affinity binding site recognized by both acylated and des-acylated ghrelin, suggesting that the described activities on C2C12 are likely mediated by this novel, yet unidentified receptor for both ghrelin forms.

INTRODUCTION

Ghrelin (GHR) is a circulating peptidyl hormone, octanoylated on Ser3, mainly produced by the stomach, which, by acting on the hypothalamus and the pituitary, induces a strong release of growth hormone (GH) and stimulates food intake and adiposity (Kojima *et al*., 1999; Kohno *et al*., 2003; Reimer *et al*., 2003). GHR exerts these activities through binding and activation of growth hormone secretagogue receptor (GHSR)-1a, a G protein-coupled receptor identified previously as the receptor for synthetic growth hormone secretagogues (GHSs) (Howard *et al*., 1996). In addition to its endocrine activities, GHR features several activities in the cardiovascular system in vivo, as it improves cardiac performances after heart damage (Nagaya *et al*., 2001, 2004). Moreover, GHR acts as a vasodilator, enhancing nitric oxide bioactivity in metabolic syndrome patients (Tesauro *et al*., 2005). In vitro, GHR inhibits apoptosis of cardiomyocytes and endothelial cells as well as apoptosis of preadipocytic and preosteoblastic cells, through activation of extracellular signal-regulated kinase (ERK)-1/2 and phosphoinositide 3-kinase/Akt pathways (Baldanzi *et al*., 2002; Kim *et al*., 2004; Kim *et al*., 2005). In addition, GHR induces differentiation of osteoblasts, adipocytes, and neurons by stimulating proliferation of their precursors (Choi *et al*., 2003; Kim *et al*., 2005; Zhang *et al*., 2005), although overexpression of GHR in preadipocytes strongly stimulates their proliferation, impairing rather than promoting adipocytic differentiation (Zhang *et al*., 2004). Conversely, GHR stimulates differentiation of immature Leydig cells by inhibiting their proliferation in vivo (Barreiro *et al*., 2004). GHR is also involved in regulation of cell growth, although it either stimulates or inhibits proliferation in different cell types. Indeed, GHR stimulates proliferation of preosteoblastic cells (Fukushima *et al*., 2005; Maccarinelli *et al*., 2005; Delhanty *et al*., 2006), neuron precursor of the dorsal ganglion (Zhang *et al*., 2005), primary oral keratinocytes (Groschl *et al*., 2005), HEL erythroleukemic cell line (De Vriese *et al*., 2005), zona glomerulosa cells (Andreis *et al*., 2003; Mazzocchi *et al*., 2004), GH3 rat pituitary cell line (Nanzer *et al*., 2004), 3T3-L1 preadipocytes

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Abbreviations used: D-GHR, des-acyl ghrelin; DM, differentiation medium; EGFP, enhanced green fluorescent protein; GHR, ghrelin; GHSR, growth hormone secretagogue receptor; GM, growth medium; MHC, myosin heavy chain.

(Kim *et al*., 2004; Zhang *et al*., 2004), pancreatic adenocarcinoma cells (Duxbury *et al*., 2003), H9C2 cardiomyocyte cell line (Pettersson *et al*., 2002), and several prostate cancer cell lines (Jeffery *et al*., 2002). Conversely, GHR inhibits cell proliferation of cell lines derived from several carcinomas, including prostate (Cassoni *et al*., 2004), thyroid (Volante *et al*., 2003), mammary (Cassoni *et al*., 2001), and lung (Ghè *et al*., 2002), as well as immature Leydig cells (Barreiro *et al*., 2004) and splenic T-cells costimulated by anti-CD3 antibodies (Xia *et al*., 2004).

Des-acyl ghrelin (D-GHR), the unacylated form of GHR, whose concentration in plasma and tissues is higher than that of GHR, does not bind GHSR-1a and is devoid of any central activity on GH release, appetite and adiposity. These observations initially suggested that D-GHR might act as a reservoir of inactive GHR. However, an increasing body of evidence indicates that D-GHR shares with GHR many biological activities and common binding sites on several peripheral tissues and cell types. Indeed, both GHR and D-GHR inhibit apoptosis and recognize common binding sites in H9c2 cardiomyocytes (Baldanzi *et al*., 2002); inhibit proliferation and recognize common binding sites in breast and prostate carcinoma cells (Jeffery *et al*., 2002; Cassoni *et al*., 2001); stimulate proliferation of preosteoblastic as well as GH3 pituitary cells (Fukushima *et al*., 2005; Maccarinelli *et al*., 2005; Nanzer *et al*., 2004; Delhanty *et al*., 2006); stimulate differentiation of osteoblasts in vitro (Delhanty *et al*., 2006); and adipogenesis in vivo (Choi *et al*., 2003), and activate ERK-1/2 and Akt signaling pathways, which mediate their antiapoptotic and proliferative responses.

In most, but not all, of the cells where D-GHR activity was investigated, GHSR-1a is not expressed, strongly suggesting that such pleiotropic activities of both GHR and D-GHR may be mediated by a yet unidentified receptor. In summary, these data indicate that D-GHR shares a subset of biological activities with ghrelin in peripheral tissues through an unidentified receptor distinct from GHSR-1a.

In vivo, GHR treatment has been reported to ameliorate chronic heart failure- and cancer-induced cachexia, whereas its plasma concentration is increased in cachectic patients (Nagaya *et al*., 2001, 2005; Granado *et al*., 2005). However, no studies have addressed whether GHR may act directly on the muscle. Intriguingly, binding sites for hexarelin, a synthetic GHS, have been observed in skeletal muscle (Papotti *et al*., 2000). Based on these observations, we investigated GHR and D-GHR biological activities in skeletal muscle myoblasts.

Skeletal muscle satellite cells are mononucleated myoblasts, which, upon muscle diseases or direct injury, are activated to undergo proliferation and eventually differentiate to form new muscle fibers to allow muscle regeneration. In vivo, differentiation of skeletal muscle involves first the growth factor-sustained expansion of the population of skeletal myoblasts and then cell cycle exit and initiation of terminal differentiation, which involves expression of contractile proteins and formation of multinucleated syncitia by myocytes fusion. The extracellular signals triggering growth arrest and the molecular mechanisms involved in the induction of myoblasts differentiation and fusion still remain to be fully elucidated.

In vitro, muscle differentiation steps can be reproduced with myoblastic satellite-derived cell lines, such as the C2C12 murine myoblast cells used in this study. C2C12 myoblasts proliferate in presence of 10% fetal calf serum (FCS) (growth medium; GM), and undergo differentiation when cultured in 2% horse serum (differentiation medium; DM).

Herein, we provide data demonstrating that both GHR and D-GHR act on skeletal myoblasts by inhibiting cell proliferation and by promoting muscle differentiation and fusion.

MATERIALS AND METHODS

Reagents

Synthetic ghrelin-(1-28), or GHR; Tyr^4-ghrelin-(1-28), or Tyr^4-GHR; truncated ghrelin-(9-28), or GHR-(9-28); des-acyl ghrelin-(1-28), or D-GHR; and motilin were provided by NeoMPS (Strasbourg, France). The anti-myosin heavy chain (MHC; MF-20) and anti-myogenin antibodies were kind gifts of Dr. Mara Brancaccio (University of Torino, Torino, Italy). Anti-phospho-Akt, anti-Akt, anti-phospho-ERK-1/2, anti-ERK-1/2 antibodies, and p38 MAPK assay kit were from Cell Signaling Technology (Beverly, MA). All reagents were from Sigma, unless otherwise indicated.

Cell Cultures

C2C12 myoblasts were grown at low density in a proliferative medium (GM) consisting in DMEM supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml antimycotic. To induce differentiation, cells were allowed to become confluent, and the medium was switched to DM consisting in DMEM supplemented with 2% horse serum, penicillin, streptomycin, and antimycotic as described above.

Western Blot

After the indicated treatments, cells were washed in ice-cold phosphate-buffered saline (PBS) and solubilized with a lysis buffer containing 25 mM HEPES, pH 8, 135 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF 50, 1% NP-40, 10% glycerol, 0.05 mg/ml leupeptin, 0.005 mg/ml pepstatin, 200 μM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4. Lysates were stirred at 4°C for 15 min and centrifuged at 13,000 $\times$ *g* for 15 min at 4°C. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins (20–50 μg protein/lane) were separated by 5–12% SDS-PAGE and transferred to polyvinylidene difluoride filters (Hybond-P; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Membranes were incubated with the primary antibodies, washed with Tris-buffered saline/0.1% Tween, incubated with the appropriate secondary antibody (PerkinElmer Life and Analytical Sciences, Boston, MA), visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences), acquired with VersaDoc 3000 (Bio-Rad), and analyzed with Quantity One software (Bio-Rad). Equal protein loading was further controlled by Ponceau red staining. After anti-phospho-Akt and anti-phospho-ERK-1/2, membranes were stripped with ReBlot Plus (Chemicon International, Temecula, CA) and reblotted with the corresponding total protein antibodies.

Immunofluorescence

Cells were plated on 24-well plates and treated as indicated. At the end of the treatments, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with anti-MHC followed by incubation with the secondary antibody and 4,6-diamidino-2-phenylindole (DAPI), and visualized by fluorescence microscopy (Axiovert 40; Carl Zeiss, Jena, Germany). Each treatment was in triplicate, and each experiment was repeated at least two times. Images were acquired (10 fields/well) and analyzed to determine differentiation and fusion indexes.

Differentiation Index and Fusion Index

To quantify the differentiation and fusion of C2C12 cells after treatments, we calculated the differentiation index as the percentage of MHC-positive cells above total nuclei and the fusion index as the average number of nuclei in MHC-positive cells with at least three nuclei above total number of nuclei, respectively.

Cell Proliferation

C2C12 cells were starved overnight in 0.2% FCS and then maintained for 24 h with or without GHR and D-GHR in GM to evaluate the inhibition of proliferation. At the end of treatments, cells were incubated with 2 μCi/ml [^{3}H]thymidine (GE Healthcare) for 3 h, washed with PBS, treated with 5% trichloroacetic acid (TCA) to precipitate proteins and DNA, and finally lysed by adding 0.5 M NaOH and 0.5% SDS. Positive control for proliferation was GM, whereas negative control was 0.2% FCS. The amount of incorporated [^{3}H]thymidine was evaluated by beta-counter (Tri-Carb 2800TR; Perkin Elmer) analysis. The data presented here are the average of triplicate assays, and similar results were obtained in at least three independent experiments.

p38 Kinase Assay

The ability of GHR and D-GHR to activate p38 was assayed by a specific p38 nonradioactive kinase assay kit from Cell Signaling Technology, according to the protocol provided by the supplier. Briefly, after the indicated treatments, cells were solubilized with a lysis buffer, the phosphorylated p38 was immunoprecipitated, and an in vitro kinase assay was performed using activating transcription factor (ATF)-2 as a substrate. Phosphorylated ATF-2 was finally detected by Western blotting.

Generation of the Ghrelin-expressing Lentiviral Vector (MA1-GHR)

Total RNA from mouse stomach, mechanically triturated in liquid nitrogen, was extracted by TRIzol (Invitrogen), following the manufacturer's instructions. The RNA obtained was retrotranscribed, and the cDNA was used to clone the total ghrelin in the lentiviral vector MA1 (pCCL.sin.PPT.polyA.CTE.eGFP.minhCMV.hPGK.WPRE), a kind gift from Prof. L. Naldini (HSR-Tiget, Milan, Italy), containing a synthetic bidirectional promoter that simultaneously promotes the transcription of two divergent mRNA sequences, one sequence of which encoded for an enhanced green fluorescent protein (EGFP) (Amendola *et al*., 2005). The generated construct has been transfected in myoblasts to verify the ability of this MA1-GHR vector to afford in vitro the expression of the ghrelin gene. Cells were transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Radioimmunoassay (RIA) Analysis

The ability of MA1-GHR vector to afford the expression of the GHR gene in C2C12 myoblasts and the secretion of the hormone in culture medium was assayed by a specific RIA kit from Phoenix Pharmaceuticals (Belmont, CA), according to the protocol provided by the supplier.

GHSR-1a Expression

Total RNA from cultured cells was extracted by Nucleospin RNA II (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions, whereas RNA from mouse brain, mechanically triturated in liquid nitrogen, was extracted by TRIzol (Invitrogen). The RNA obtained was retrotranscribed with SuperScript reverse transcriptase II (Invitrogen). The quality of cDNAs has been assessed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification, and then reverse transcription-polymerase chain reaction (RT-PCR) of GHSR-1a was performed using DNAzyme EXT polymerase (Finnzymes, Espoo, Finland) and the following primers: GHSR-1a exon 1-for 5′-AGTATCGGCCCTGGAACTT-3′, GHSR-1a exon 1-rev 5′-ACGCTCGACACCCATACCAT-3′, GHSR-1a exon 2-for 5′-TGGTGTTTGCTTTCATCCTC-3′, GHSR-1a exon 2-rev 5′-CGGGAACTCTCATCCTTCAGA-3′, GHSR-1a complete-for 5′-AAGGTGGTGGTCACCAAGG-3′, and GHSR-1a complete-rev 5′-CGGTACTTCTTGGACATGATG-3′.

Ghrelin Binding Assay

Tyr^4-GHR was radioiodinated (^{125}I-Tyr^4-GHR; specific activity 2000 Ci/mmol) by using a lactoperoxidase method by GE Healthcare and used as a radioligand in the binding studies. Tyr^4-GHR has been reported to be a reliable probe for labeling GHS-R in tissue or cell membranes and to retain the same GH-releasing potency of the native peptide (Muccioli *et al*., 2001, 2004; Baldanzi *et al*., 2002).

Binding of ^{125}I-Tyr^4-GHR to crude C2C12 myoblast membranes (30,000 $\times$ *g* pellet), and saturation binding analysis were determined as described previously (Muccioli *et al*., 2001, 2004). IC_{50} values of specific radioligand binding were determined by radioligand ghrelin displacement curves with increasing concentrations of unlabeled GHR, D-GHR, GHR-(9-28) fragment, or motilin. The maximal number of binding sites (B_{max}), the dissociation constant (K_d), and the IC_{50} values were calculated with the iterative curve-fitting Prism 4 program (GraphPad Software Inc., San Diego, CA).

Statistical Analysis

Where appropriate, data are presented as the mean ± SEM, and the statistical significance was assessed using Student's *t* test.

RESULTS

Ghrelin and Des-Acyl Ghrelin Promote Differentiation and Fusion of C2C12 Myoblasts in Growth Medium

C2C12 myoblasts, a skeletal muscle satellite-derived cell line, is a common model to investigate cellular and molecular mechanisms of muscle differentiation. Upon culture in 2% horse serum, C2C12 cells exit the cell cycle, differentiate, and fuse into multinucleated skeletal myotubes expressing contractile proteins (Blau *et al*., 1985). The extracellular signals triggering growth arrest and the molecular mechanisms involved in the induction of myoblasts differentiation and fusion still remain to be elucidated.

GHR and D-GHR induce muscle differentiation and fusion of proliferating C2C12 myoblasts in GM (10% FCS), as shown by immunofluorescence microscopy with anti-MHC antibodies. Figure 1A shows typical immunofluorescence images obtained from C2C12 skeletal muscle cells cultured for 72 h in GM in presence or absence of either 10 nM GHR or 10 nM D-GHR. Cells positive for MHC, a marker for terminal differentiation, are red stained, whereas nuclei are blue stained (DAPI). In a representative field of C2C12 cells maintained in GM, only a single MHC-positive cell is visible, but no multinucleated tubes are present, indicating a minimal spontaneous differentiation tendency. In representative fields of C2C12 cells in GM treated with 10 nM GHR or D-GHR, respectively, both single-nucleated MHC-positive cells and multinucleated myotubes are clearly visible.

The extent of differentiation of skeletal muscle cells was measured by differentiation and fusion indexes, reflecting, respectively, MHC expression and multinucleated myotubes formation. Differentiation index is calculated as the number of MHC-positive cells, expressed as percentage of total number of cells counted by DAPI-stained nuclei. Fusion index is calculated as the average number of nuclei contained in MHC-positive cells with at least three nuclei, compared with the total number of nuclei.

Differentiation index of C2C12 myoblasts is significantly increased in a concentration-dependent manner upon 96 h of treatment with rising concentrations of either GHR or D-GHR in GM. Maximal response was observed at 10 nM, whereas minimum significant differentiation was already observed at 1 nM (Figure 1, B and C). Differentiation was already evident and significant upon 48 and 72 h of treatment (Figure 1D).

In addition, the differentiating activity of GHR and D-GHR is not limited to stimulating MHC expression; it also induces myocyte fusion to form multinucleated syncitial myotubes. Fusion index of myocytes cultured in presence of either 10 nM GHR or D-GHR was increased up to 20- to 25-fold after 72 h of treatments, compared with untreated control myoblasts in GM (Figure 1E). Thus, these data clearly show that both GHR and D-GHR activate a complete differentiation program in C2C12 skeletal myoblasts driving both expression of contractile proteins and cellular events leading to the formation of multinucleated myotubes.

Ghrelin and Des-Acyl Ghrelin Induce the Expression of Early and Late Markers of Skeletal Muscle Differentiation in C2C12 Myoblasts

To consolidate the observation that indeed GHR and D-GHR activate a differentiating program in skeletal myoblasts, we have verified the ability of both GHR and D-GHR to induce the expression of myogenin and MHC proteins, as detected by Western blot. While MHC is a late differentiation marker, myogenin is a helix-loop-helix transcription factor whose expression is induced early in differentiation, preceding cell cycle exit (Andres and Walsh, 1996; Zhang *et al*., 1999).

C2C12 cells cultured in GM were treated with 10 nM GHR or D-GHR or switched to DM for either 24 or 72 h. Expression of myogenin and MHC was measured by Western blot of whole cell lysates, and the intensity of the bands was quantified. Figure 2A shows that upon 24-h treatment with both GHR and D-GHR the expression of myogenin is significantly increased compared with control cells in GM, at similar extent of the expression induced by DM. Moreover, upon 72-h treatment, when multinucleated myotubes are formed, the expression of the terminal differentiation marker MHC was significantly induced (Figure 2B). These results

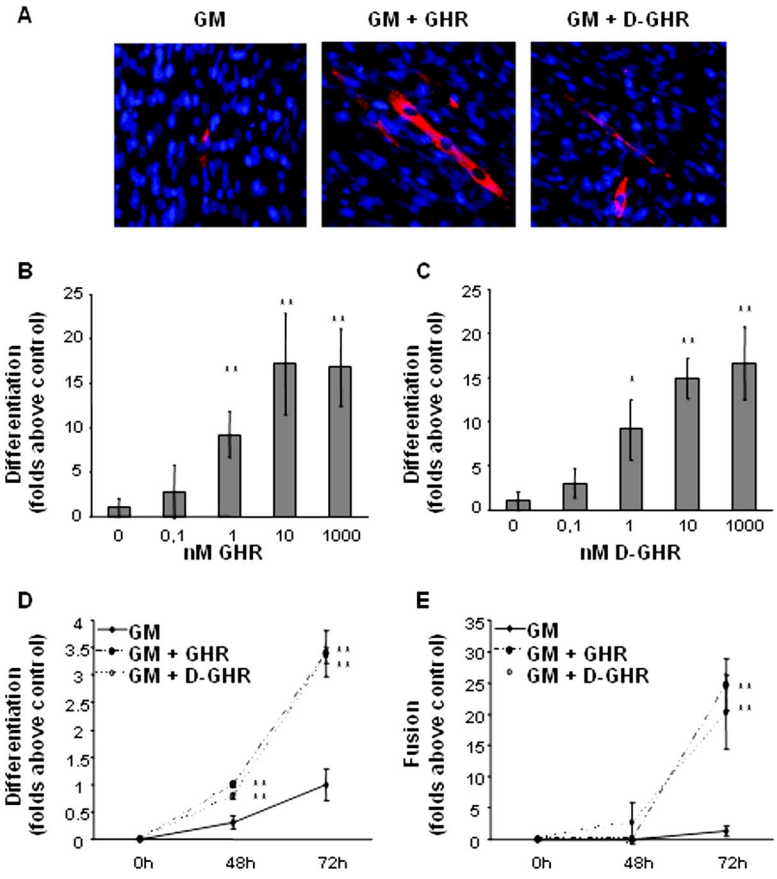

Figure 1. GHR and D-GHR induce differentiation and fusion of C2C12 myoblasts in GM. Cells were treated either with GHR or D-GHR in GM and fixed for staining with anti-MHC antibody and DAPI. (A) Representative immunofluorescences of C1C12 myoblasts treated for 72 h with 10 nM GHR or D-GHR stained with anti-MHC antibody and DAPI are shown. (B and C) Dose–response activity of GHR and D-GHR in inducing differentiation of C2C12 myoblasts after 96 h of treatment. (D and E) Differentiation and fusion indexes after 48 and 72 h of treatment with 10 nM GHR and D-GHR. **$p < 0.01$ versus control.

confirm that GHR and D-GHR are able to promote both early and late steps of skeletal muscle differentiation in GM.

Ghrelin and Des-Acyl Ghrelin Inhibit Proliferation of C2C12 Myoblasts

Proliferating C2C12 myoblasts exit cell cycle upon switching from GM to DM, to initiate terminal differentiation. Because both GHR and D-GHR stimulate differentiation of proliferating myoblasts, we have investigated their ability to inhibit cell proliferation, measured as inhibition of DNA synthesis.

Growing C2C12 myoblasts were starved overnight in 0.2% FCS to synchronize their cell cycles, and then they were maintained for 24 h in GM with or without 10 nM GHR and D-GHR. As positive control of inhibition of proliferation, cells were maintained in 0.2% FCS. Either GHR or D-GHR in GM inhibit DNA synthesis of C2C12 myoblasts of ~25% compared with control cells (Figure 3).

Ghrelin and Des-Acyl Ghrelin Induce Differentiation and Fusion of C2C12 by Activation of p38

Overexpression of a constitutively activated form of MKK6, activating endogenous p38, stimulates muscle differentiation even in the presence of antimyogenic cues (Wu *et al.*, 2000). Because both GHR and D-GHR stimulate the differentiation of proliferating C2C12 myoblasts, we investigated whether GHR and D-GHR induce the activation of p38 (Figure 4A). Indeed, GHR and D-GHR stimulate activation of p38, as measured by its specific kinase activity in vitro on purified ATF-2, suggesting that this pathway may be involved in the differentiative signaling triggered by these factors. To verify such hypothesis, we have investigated whether pharmacological inhibition of p38 impairs GHR

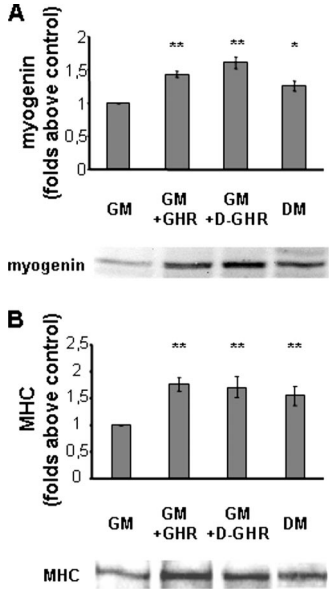

Figure 2. Western blot analysis of myogenic markers expression. C2C12 myoblasts were incubated in GM for 24 or 72 h with 10 nM GHR or D-GHR. The content of myogenin and MHC was measured by Western blot of whole cell lysates and the intensity of the bands quantified. Cells differentiated in DM were considered as positive control. (A) Myogenin expression. (B) MHC expression. Representative Western blots are shown at the bottom of each panel. **$p < 0.01$ and *$p < 0.05$ versus control.

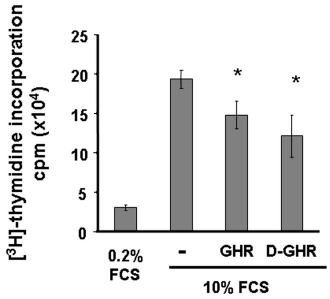

Figure 3. Effect of GHR and D-GHR on basal incorporation of [^{3}H]thymidine into DNA by C2C12 myoblasts. DNA synthesis was estimated by incorporation of [^{3}H]thymidine after a 24-h incubation with or without 10 nM GHR or D-GHR in GM. *$p < 0.05$ versus control.

and D-GHR differentiative activity in C2C12. Indeed, cell pretreatment with 5 μM SB203580 for 15 min significantly inhibited differentiation up to ~40% (Figure 4B), and abolished fusion (Figure 4C), induced by 72-h treatment with 10 mM GHR and D-GHR.

Ghrelin and Des-Acyl Ghrelin Enhance Differentiation and Fusion of C2C12 Myoblasts in Differentiation Medium

To provide further evidence that GHR and D-GHR may participate in the regulation of muscle differentiation, we

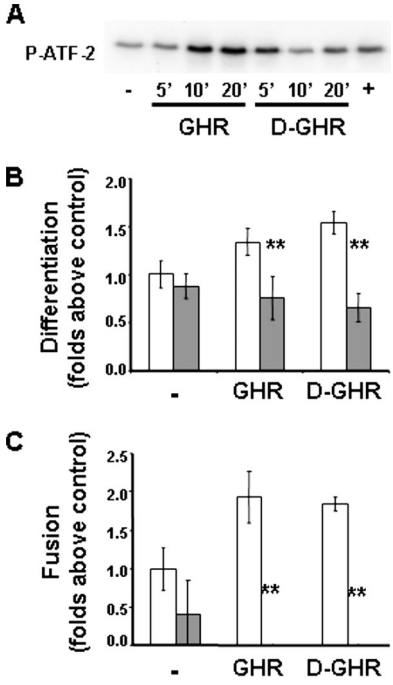

Figure 4. GHR and D-GHR induce differentiation and fusion of C2C12 myoblasts by activation of p38. (A) Kinase activity of p38 on ATF-2 induced by 1 μM GHR and D-GHR in C2C12 myoblasts. Cells were starved in 0.2% FCS overnight and then treated for the indicated times. Treatment with 10 μg/ml lysophosphatidic acid for 5 min was used as positive control (+). (B and C) Inhibition of p38 reduces 10 mM GHR- and D-GHR–induced differentiation and fusion of C2C12. Cells were pretreated with 5 μM SB302580 for 15 min, treated with GHR or D-GHR in GM, and fixed after 72 h for staining with anti-MHC antibody and DAPI to determine the differentiation and fusion indexes. Empty bars, without SB302580; filled bars, with SB302580. **$p < 0.01$ versus control.

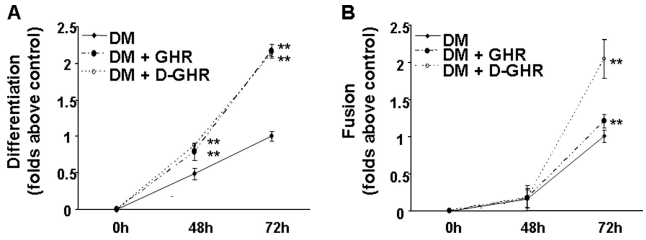

Figure 5. GHR and D-GHR enhance differentiation and fusion of C2C12 myoblasts in differentiation medium. Cells were treated with 10 nM GHR or D-GHR in DM and fixed after 48 and 72 h for staining with anti-MHC antibody and DAPI in order to determine the differentiation index. ** $p < 0.01$ versus control.

investigated whether they affect DM-induced differentiation and fusion of C2C12 myoblasts. Indeed, after 72-h treatment with either GHR or D-GHR in DM, both differentiation index (Figure 5A) and fusion index (Figure 5B) increased up to twofold, compared with nontreated cells in DM.

Furthermore, we artificially generated a ghrelin autocrine loop by ectopic expression of ghrelin gene in C2C12 myoblasts. The murine ghrelin gene was subcloned in a lentiviral vector under the control of a bidirectional promoter, which simultaneously promotes the expression of EGFP (MA1-GHR). Lentiviral vector expressing EGFP alone was used as control. After transfection with MA1-GHR vector, ghrelin secretion, as assayed in culture medium by RIA, was twofold compared with control cells (data not shown).

C2C12 cells transiently expressing either EGFP alone or EGFP and GHR were induced to differentiate in DM. After 72 h from transfection, differentiation index of ghrelin-overexpressing cells is increased by ~45% compared with EGFP-expressing cells. Similarly, fusion index is also increased by ~80% compared with control cells. Untransfected cells feature differentiation and fusion indexes similar to those of cells expressing EGFP alone, indicating that the viral construct does not affect differentiation and fusion by itself (Figure 6).

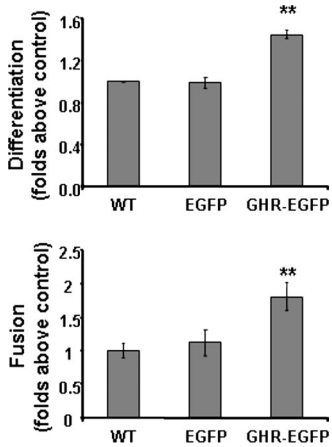

Figure 6. Increase of differentiation and fusion of C2C12 myoblasts overexpressing murine GHR. Cells transfected with a lentiviral vector expressing both GHR and EGFP under control of a bidirectional promoter, a lentiviral vector expressing EGFP only, and C2C12 wild-type cells were induced to differentiate switching the GM to DM. Cells were fixed after 72 h in DM and stained with anti-MHC antibody and DAPI to determine differentiation index (top) and fusion index (bottom). **$p < 0.01$ versus control.

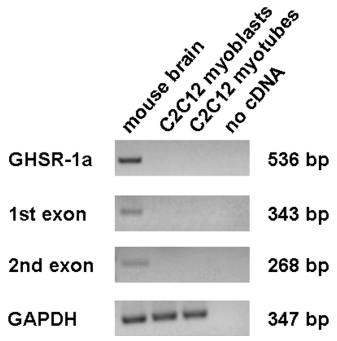

Figure 7. Expression of GHSR-1a, by RT-PCR, in C2C12 myoblasts and myotubes. Mouse brain was used as positive control for amplification of complete receptor, first exon, and second exon. Qualitative controls for samples were performed using mouse GAPDH.

C2C12 Skeletal Muscle Cells Do Not Express GHSR-1a, but They Contain High-Affinity Binding Sites That Are Recognized by Both Ghrelin and Des-Acyl Ghrelin

We previously showed that GHSR-1a, the only known GHR receptor, is not expressed in cardiomyocytes and endothelial cells where GHR and D-GHR inhibit cell death. Thus, we have assayed by RT-PCR the expression of GHSR-1a in C2C12 myoblasts and in differentiated myotubes, by using cDNA from whole mouse brain as positive control. PCR reactions were performed using primers amplifying either the first exon, common to both GHSR-1a and GHSR-1b, or the second exon, specific of GHSR-1a. In addition, to avoid false positives due to genomic contamination, we also used intron-spanning primers, to amplify the complete GHSR-1a. No expression was detected in both undifferentiated and differentiated C2C12 myocytes (Figure 7), suggesting that GHR and D-GHR activities in C2C12 skeletal muscle cells are not mediated by GHSR-1a.

To further investigate the identity of the receptor mediating the effects of GHR and D-GHR on C2C12 skeletal muscle cells, we performed binding studies of radiolabeled GHR (^{125}I-Tyr^4-GHR) to membranes of C2C12 myoblasts. Binding experiments with increasing concentrations of ^{125}I-Tyr^4-GHR (0.035–6 nM) revealed the existence of saturable and high-affinity binding sites in C2C12 cells (Figure 8A) with an apparent K_d and a B_{max} value (mean ± SEM of 3 independent experiments) of 0.39 ± 0.04 nM and 26.3 ± 2.0 fmol/mg protein, respectively. Binding of radiolabeled GHR was displaced in a dose-dependent manner and with equal efficacy by unlabeled GHR and D-GHR. The IC_{50} values calculated from competition binding curves, expressed as nanomolar concentrations (mean ± SEM of three independent experiments), were 1.20 ± 0.09 for GHR and 1.32 ± 0.08 for D-GHR. In contrast, motilin or GHR-(9–28), a synthetic truncated GHR derivative, was able, at the maximal concentration tested, to displace only 14–37% of the specifically bound radiolabeled GHR (Figure 8B). Accordingly, neither GHR-(9-28) nor motilin were able to induce C2C12 myoblasts differentiation (Figure 8C). These findings provide evidence that GHR and D-GHR may act directly as factors modulating cell proliferation and differentiation on C2C12 myoblasts through binding to a specific receptor that is distinct from GHSR-1a.

DISCUSSION

Skeletal muscle satellite cells are quiescent mononucleated myoblasts, located between the sarcolemma and the basal membrane of terminally differentiated adult muscle fibers. On muscle diseases or direct injury, quiescent satellite cells

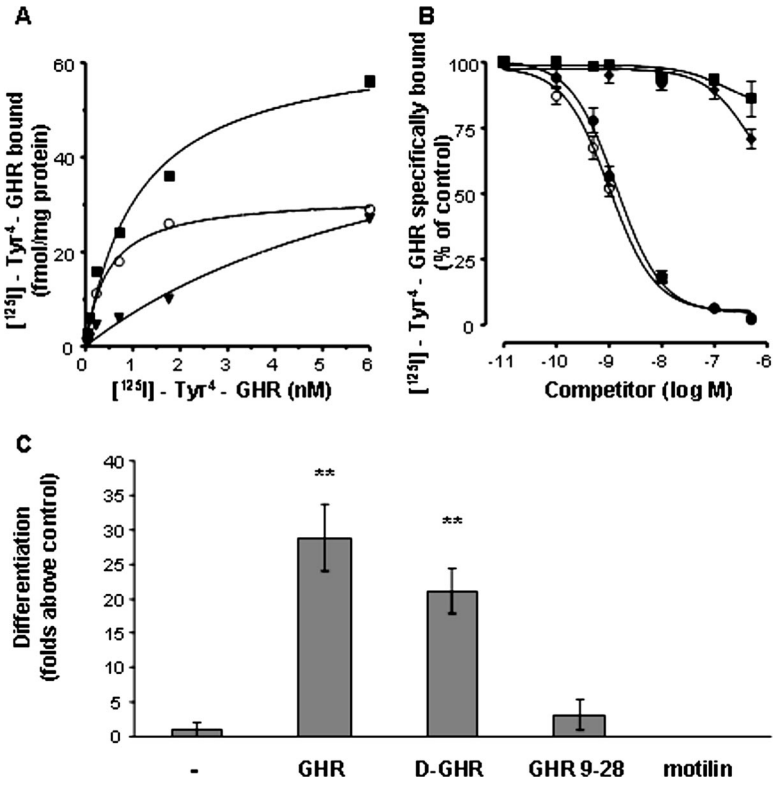

Figure 8. C2C12 myoblasts contain a GHR receptor distinct from GHSR-1a. (A) Specific binding (○) was determined by incubation of crude membranes with increasing concentrations of radiolabeled GHR (^{125}I-Tyr^4-GHR) in the absence (total binding, ■) or in the presence (nonspecific binding, ▼) of 1 μM unlabeled GHR. Data are the average of triplicate determinants. Similar results were obtained in at least two other independent experiments. (B) Displacement curves of radiolabeled GHR by unlabeled GHR (○), D-GHR (●), GHR-(9-28) fragment (♦), and motilin (■). Values are the mean ± SEM of three independent experiments. (C) Cells were fixed after 72 h treatment with 10 mM GHR, D-GHR, GHR-(9-28) fragment, and motilin in GM and stained with anti-MHC antibody and DAPI to determine the differentiation index. **$p < 0.01$ versus control.

are activated to undergo proliferation and eventually differentiate to allow muscle regeneration.

Skeletal muscle regeneration involves, sequentially, satellite cell proliferation, commitment to terminal differentiation, cell fusion into multinucleated syncitia, and muscle fiber formation.

Such mechanisms leading to muscle regeneration are poorly understood; they seem to recapitulate the embryonic program of differentiation, although the extracellular factors regulating such processes may be different.

Satellite cell differentiation into skeletal muscle can be subdivided into temporally separable events, coordinated by the expression of proteins of the muscle regulatory factors family, such as myogenin, and of cyclin-dependent kinase inhibitor of the p21 family (Andres and Walsh, 1996), resulting in cell cycle exit and commitment to terminal differentiation. Later on, expression of muscle contractile proteins, such as MHCs and myosin light chains (MLCs), are hallmarks of phenotypic differentiation. Finally, fusion of myocytes into multinucleated myotubes is the terminal step of muscle differentiation.

The growing interest in skeletal muscle regeneration is associated to the opening of new therapeutic strategies for several muscular degenerative pathologies such as dystrophies, muscular atrophy, and cachexia associated to aging, cancer, chronic heart failure, and acquired immunodeficiency syndrome as well as the treatments of skeletal muscle injury after trauma.

Although GHR is a circulating hormone mainly secreted by the stomach, it is also synthesized in a number of tissues, suggesting both endocrine and paracrine effects (Gnanapavan *et al*., 2002).

The evidence that 1) GHR up-regulation is specifically associated to either congestive heart failure (CHF)- or cancer-induced cachexia (Nagaya *et al*., 2001, Shimizu *et al*., 2003) and that its administration strongly prevents CHF-associated cachexia (Nagaya *et al*., 2004); 2) GHR, D-GHR, and GHSs inhibit apoptosis of cardiac myocytes (Filigheddu *et al*., 2001; Baldanzi *et al*., 2002); and 3) skeletal muscle features high binding sites for synthetic GHSs (Papotti *et al*., 2000), lead us to speculate that GHR and D-GHR may act directly also on skeletal muscle. Indeed, we observed that both GHR and D-GHR stimulate tyrosine phosphorylation of several proteins and activate ERK-1/2 and Akt (data not shown), indicating that both factors could exert a biological activity on these cells.

Here, we show that nanomolar concentrations of both GHR and D-GHR induce the differentiation of proliferating skeletal myoblasts in a concentration-dependent manner and promote their fusion into multinucleated syncitia in vitro. The cellular and molecular mechanisms by which GHR and D-GHR elicit these responses are not known. Cell cycle withdrawal is a prerequisite for myogenic terminal differentiation (Walsh and Perlman, 1997). Indeed, the ability of GHR and D-GHR to reduce DNA synthesis of proliferating C2C12 myoblasts is highly consistent with their prodifferentiative activity. However, inhibition of cell proliferation is not sufficient to elicit muscle differentiation. For example, myostatin inhibits both proliferation and differentiation of C2C12 myoblasts, through down-regulation of MyoD and myogenin expression (Joulia *et al*., 2003). Conversely, GHR and D-GHR, beyond inhibiting cell proliferation, induce the expression of myogenin, which is required for the complete program of differentiation of skeletal myoblasts to proceed (Zhang *et al*., 1999). To our knowledge this is the first evidence for an extracellular factor able to induce muscle differentiation of proliferating skeletal myoblasts in GM.

In proliferating C2C12 myoblasts, activation of p38 pathway obtained by overexpression of constitutively active MKK6 is sufficient to induce myogenin expression, cell cycle exit, and skeletal muscle terminal differentiation (Wu *et al*., 2000). Thus, we investigated whether GHR and D-GHR prodifferentiative activity is mediated by p38. Consistently, inhibition of p38 by cell treatment with SB203580 resulted in the partial albeit significant inhibition of GHR and D-GHR-induced differentiative activity. In addition, we also showed that both GHR and D-GHR activate p38. Altogether, these data demonstrate that GHR and D-GHR act as antiproliferative and prodifferentiative factors by stimulating the p38 pathway.

The lack of expression of GHSR-1a in either C2C12 myoblasts and skeletal muscle tissue (Gnanapavan *et al*., 2002) as well as the activity exerted by D-GHR suggest that GHR- and D-GHR–differentiating activities are mediated by a yet unidentified receptor, common to both acylated and unacylated peptide and distinct from GHSR-1a. Indeed, here we showed that C2C12 cells feature high-affinity common binding sites for both GHR and D-GHR. Such binding sites are specific, because they do not recognize either N-terminal truncated ghrelin or motilin, which are unable to induce differentiation. These studies also demonstrate that the N-terminal portion of the GHR peptide is required for binding and induction of C2C12 muscular differentiation. Together, these data provide further evidence for novel GHR receptor subtypes, which do not discriminate between the acylated and unacylated peptide. Although evidence for common GHR and D-GHR receptors have been reported in several cells, including a cardiomyocyte-derived cell line (Baldanzi *et al*., 2002), this is the first evidence for their expression in skeletal muscle.

We also verified whether the ghrelin gene is up-regulated in C2C12 myoblasts induced to differentiate in DM. However, no difference of ghrelin expression was detected by real-time RT-PCR between proliferating and differentiating cells (data not shown), suggesting that GHR gene product is not involved in DM-induced skeletal muscle differentiation in vitro.

By showing that GHR and D-GHR stimulate terminal differentiation of skeletal myoblasts in vitro, we may raise the hypothesis that the function of GHR gene may be involved in skeletal muscle differentiation in vivo. However, the lack of a consistent phenotype in GHR knockout mice, suggests that GHR function is not required for myogenesis during development. Consistently, we have not detected any GHR expression in somites or related structures during embryonic development by in situ hybridization (data not shown). However, although not essential for embryo development, GHR might be involved in the complex process of myogenesis in the adulthood, i.e., in regenerative processes of skeletal muscle. This hypothesis is consistent with the data showing that FGF6 is not required for muscle development, but is required in the adult for damage-induced muscle regeneration (Floss *et al*., 1997).

Upon muscular injury, skeletal myoblasts are activated to terminally differentiate through an autocrine/paracrine loop. We may speculate that GHR would contribute to skeletal muscle plasticity, promoting the differentiation and fusion of myoblasts in the damaged muscles. If this hypothesis would be proved, the activation of the receptor mediating GHR and D-GHR differentiative activity as well as the overexpression of the hormone may provide novel therapeutic strategies for the reduction or retardation of several skeletal

muscle pathologies, including dystrophies, atrophies, and cachexia.

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DES-ACYL GHRELIN PROTECTS FROM SKELETAL MUSCLE ATROPHY

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ABSTRACT

Skeletal muscle atrophy is a debilitating process associated to several diseases, fasting, or disuse, resulting in a massive loss of muscle mass and functionality. Des-acyl ghrelin is a peptide produced by the ghrelin gene which undergoes acylation to generate ghrelin.Ghrelin stimulates GH release and positive energy balance through binding to its receptor GHSR-1a. Des-acyl ghrelin, which does not bind GHSR-1a, nevertheless shares with ghrelin the ability to stimulate skeletal myoblasts differentiation and to inhibit apoptosis.

Here we show that des-acyl ghrelin and ghrelin inhibit skeletal muscle atrophy *in vitro*, by activating the Akt/mTOR pathway. *In vivo* up-regulation of circulating D-GHR, obtained by either pharmacological treatment or in transgenic mice, inhibits skeletal muscle atrophy induced by either fasting or denervation, through a mechanism which does not involve GHSR-1a-mediated activation of GH/IGF-1 axis.

Moreover, by showing that both des-acyl ghrelin and ghrelin activate Akt and mTOR in the skeletal muscle of GHSR-1a deficient mice, we provide the first genetic evidence that ghrelin and des-acyl ghrelin activate anti-atrophic signaling independently of GHSR-1a. Altogether these results, besides unveiling a novel direct anti-atrophic activity of des-acyl ghrelin, offer also new therapeutic strategies, alternative to IGF-1, for the treatment of skeletal muscle atrophy and cachexia.

Skeletal muscle atrophy consists in a massive loss of muscle structural proteins, which leads to muscle weight decrease and causes a progressive loss of muscle function. Skeletal muscle atrophy is induced by muscle denervation and disuse, and it is also the key component of cachexia, a catabolic, debilitating response to several physio-pathological conditions, such as fasting, cancer, diabetes mellitus, AIDS, renal failure. Cachectic patients not only sustain a decreased quality of life, but also face a worse prognosis of the underlying disease, making cachexia an important target for treatment (Tisdale, 2002).

Ghrelin (GHR) is a circulating peptidyl hormone, acylated on Ser3, mainly produced by the stomach, which, by acting on the hypothalamus and the pituitary, induces a strong release of growth hormone and stimulates food intake and adiposity (Kojima et al., 1999; Kohno et al., 2003; Reimer et al., 2003). GHR exerts these activities through binding and activation of GHSR-1a, a G protein-coupled receptor previously identified as the receptor for synthetic growth hormone secretagogues (GHSs) (Howard et al., 1996). In addition to its endocrine activities, GHR features several activities in the cardiovascular system *in vivo*, such as the improvement of cardiac performances following heart damage (Nagaya et al., 2001; Nagaya et al., 2004). *In vitro*, GHR inhibits apoptosis of several cell types, including cardiomyocytes and endothelial cells through activation of PI3K/Akt and ERK-1/2 pathways (Baldanzi et al., 2002).

Des-acyl ghrelin (D-GHR), the un-acylated form of GHR, whose plasma concentration is higher than GHR, does not bind GHSR-1a and is devoid of any activity on GH release. For these reasons D-GHR has been considered for many years just a reservoir of inactive GHR.

However, an increasing body of evidence indicates that D-GHR shares with GHR many biological activities and common binding sites on several peripheral tissues and cell types (Baldanzi 2002; Jeffery 2002; Sato 2006; Filigheddu 2007; Granata et al, 2007; Soares, 2008). Indeed, both GHR and D-GHR recognize common binding sites in H9c2

cardiomyocytes and in C2C12 skeletal myoblasts, where they stimulate, respectively, anti-apoptotic and differentiative signaling (Baldanzi et al., 2002; Filigheddu et al. 2007).

In vivo, GHR treatment ameliorates cachexia induced by several pathological conditions (Granado et al., 2005; Nagaya et al., 2001; Nagaya et al., 2005). Although GHR may inhibit cachexia by stimulating food intake, positive energy balance and release of GH and IGF-1, the mechanisms underlying the anti-cachectic activity of GHR have not been fully elucidated. Since we have previously shown that both GHR and D-GHR stimulate terminal differentiation of skeletal myoblasts and activate PI3K/Akt, a major anti-atrophic signaling pathway (Filigheddu et al., 2007; Baldanzi et al., 2002), we investigated whether GHR and D-GHR could protect skeletal muscle from atrophy both *in vitro* and *in vivo*. Inhere we provide the first evidence that both D-GHR and GHR protect skeletal muscle from experimentally induced atrophy and that they activate *in vivo* an anti-atrophic signaling pathway which does not involve GHSR-1a and the activation of GH/IGF-1 axis.

RESULTS

D-GHR and GHR prevent dexamethasone-induced atrophy in C2C12-derived myotubes via the PI3K/Akt/mTOR pathway.

C2C12 represent a valuable model to study *in vitro* skeletal muscle atrophy induced by the synthetic glucocorticoid dexamethasone (DEXA). C2C12 myoblasts were differentiated in myotubes for 5 days and then treated with DEXA 1 μM for 24 h (Sandri M et al. 2004 Cell) in presence or absence of 10 nM D-GHR or GHR (Filigheddu N et al. 2007 Mol Biol Cell). Treatment with DEXA resulted in a reduction of myotube diameter and in the induction of the muscle-specific ubiquitin ligase Atrogin-1 (also known as MAFbx), which is rapidly induced in several model of muscle atrophy (Bodine SC 2001 Science). Both D-GHR and GHR impair DEXA-induced reduction of myotubes diameter (FIG. 1a) and induction of Atrogin-1 expression (FIG. 1b). IGF-1 10 ng/ml was used as positive control of atrophy protection.

Since atrophy can be prevented both *in vitro* and *in vivo* by the activation of the PI3K/Akt/mTOR pathway (Bodine SC 2001 Nature Cell Biol; Latres E 2005 JBC) and D-GHR and GHR are able to activate Akt in several cell lines, including H9C2 cardiomyocytes (Baldanzi et al. 2002 J Cell Biol), we investigated whether D-GHR and GHR could activate Akt in this model. Indeed, we observed that in C2C12-derived myotubes D-GHR and GHR induce phosphorylation of Akt and mTOR (FIG. 1c); moreover, incubation of atrophying myotubes with 100 nM wortmannin or 20 ng/ml rapamycin, inhibitors of PI3K and mTOR, respectively, fully reverted the protective effect of D-GHR and GHR, as seen as myotube diameter (FIG. 1d).

Transgenic mice overexpressing D-GHR are protected form starvation-induced atrophy

In order to extend the above findings *in vivo*, we used our strain of transgenic mice with cardiac-specific ghrelin gene expression (αMHC/GHRL). In these transgenic mice,

overexpression of the ghrelin gene in the heart results in a 50-fold increase in circulating D-GHR levels without affecting acylated GHR circulating levels, as already observed in other transgenic mice overexpressing the ghrelin gene (Zhang et al. Endocrinology 2008; Kasuga et al, Gut 2005). Moreover, consistently with the inability of D-GHR to activate GHSR-1a and promote GH release and adiposity, transgenic mice do not feature any change in circulating IGF-1 concentration, tibial and nose-to-anus length and body mass index as compared to their wild type littermates (Table 1). In addition, fasting causes the decrease of IGF-1 and the increase of GHR circulating concentrations at the same extent in both wild type and transgenic mice. Thus, these data strongly indicate that the upregulation of circulating D-GHR in transgenic mice, does not activate GHSR-1a in the pituitary and hypothalamus, does not stimulate the GH/IGF-1 axis and does not affect endogenous GHR regulation.

To investigate whether D-GHR might be a protective factor in muscle wasting, we experimentally induced skeletal muscle atrophy in mice by food deprivation. Upon 48h fasting, the weight of gastrocnemius was decreased of about 14% in wild type animals as compared to fed animals, and of about 9% in transgenic animals. These data indicate that increased circulating concentration of D-GHR results in 30% protection from fasting-induced loss in gastrocnemius mass (FIG. 2a). Accordingly, the mean fiber size, as seen as cross-sectional area (CSA), was reduced by 29% in wild type mice and by 19% in transgenic mice compared to fed animals, indicating a 34% protection (Fig.2 B). Furthermore, we investigated whether upregulation of D-GHR circulating concentration, inhibits the induction of Atrogin-1. Upon 48 h food deprivation, Atrogin-1 expression in gastrocnemia of wild type animals dramatically increased up to 30 folds, while in D-GHR transgenic mice Atrogin-1 induction was significantly reduced of one third (FIG 2c).

Noteworthy, neither weight nor muscle fiber CSA of the gastrocnemius of fed transgenic mice are increased compared to wild type animals, indicating that D-GHR does not induce

hypertrophy on skeletal muscle *per se* (Table 1), nor affects fibers size distribution (data not shown).

D-GHR inhibits denervation-induced muscle atrophy

In order to verify whether D-GHR anti-atrophic activity described above depends on a direct activity on the skeletal muscle and is not due to an activity on energy balance, we assessed the ability of D-GHR to protect from denervation-induced muscle atrophy. Sciatic nerve resection was performed both in αMHC/GHR mice and in wild type littermates, to induce atrophy of the gastrocnemius. Upon 7 days from denervation, the gastrocnemia of wild type animals showed a 21% decrease in weight and a reduction of mean fiber area of 34%. Conversely, in transgenic animals denervation-induced loss of muscle weight was only 13% and loss of CSA was 19%, resulting in about 40% protection (FIG. 2d and e). In addition, sciatic denervation caused an induction of Atrogin-1 in the gastrocnemius which was reduced by 40% in transgenic animals (FIG. 2f).

D-GHR treatment induces phosphorylation of Akt/mTOR in muscle and inhibits starvation- and denervation-induced atrophy

In order to determine the potential clinical relevance of the above findings, we assessed the ability of D-GHR to protect from muscle wasting upon subcutaneous administration at pharmacological doses, which have been previously used for *in vivo* studies (Nagaya N et al. Circulation 2001; Tack J et al Gut 2005).

We observed that within 30-60 minutes from subcutaneous injection, D-GHR induces Akt and mTOR phosphorylation in mouse gastrocnemius (FIG. 3a), indicating that, *in vivo*, D-GHR activates Akt and mTOR signaling pathways as it does in C2C12 myotubes. Consistently with the findings in transgenic mice, D-GHR treatment protected wild type mice from experimentally-induced skeletal muscle atrophy. Atrophy was induced by either food

deprivation or sciatic nerve resection as described above. During the experimental periods, the animals received injections of either saline vehicle or 100 μg/Kg D-GHR twice a day. Either upon 48 hours fasting or 7 days from sciatic nerve resection, gastrocnemia were collected, weighted and processed for further analysis. D-GHR treatment reduced by 50% the fasting-induced loss in gastrocnemius weight compared to saline-treated animals (FIG. 3b), and exerted a significant protection from fasting-induced loss of fiber CSA (FIG. 3c). In addition, D-GHR treatment of denervated mice resulted in a 25% protection from gastrocnemius muscle weight loss, and in a 30% protection from fiber CSA decrease (FIG. 3d and e).

D-GHR induces phosphorylation of Akt/mTOR in muscle of GHSR-1a$^{-/-}$ mice

The findings reported above, along with previous evidence indicating that cells derived from skeletal and cardiac myoblasts feature common binding sites for D-GHR and GHR and do not express GHSR-1a (Filigheddu et al.; Baldanzi et al.), strongly suggest that D-GHR stimulates anti-atrophic signaling in skeletal muscle through activation of a receptor distinct from GHSR-1a. In order to verify this hypothesis, we injected 100 μg/Kg of either D-GHR or GHR in GHSR-1a$^{-/-}$ mice (Sun Y et al, 2004). We observed that, within 30-60 minutes from injection, both D-GHR and GHR induce phosphorylation of Akt and mTOR, providing the first genetic evidence that the observed effects elicited by D-GHR on skeletal muscle are indeed imputable to a receptor different from GHSR-1a.

DISCUSSION

Several studies have provided evidence that acylated GHR protects from cachexia associated to a wide range of pathological conditions (Nagaya, 2004; Nagaya 2005; Nagaya 2006; Balasubramaniam, 2009). Anti-cachectic activity of GHR has been suggested to depend on binding to GHSR-1a, which stimulates positive energy balance and GH/IGF1 axis (Dixit VD et al. J Clin Invest 2004; Xia Q et al. Regul Pept 2004). This would be consistent with the well know ability of IGF-1 to protect skeletal muscle from atrophy by activating Akt and mTOR pathways. (Stitt, 2004; Latres, 2005). In this study we demonstrate that D-GHR exerts a strong anti-atrophic activity on skeletal muscle without involvement of GHRS-1a receptor. *In vitro*, we induced atrophy by treating C2C12-derived myotubes with dexamethasone that induces characteristic features of the atrophic condition, such as reduction of myotube diameter and induction of muscle-specific E3 ubiquitin-ligases (Sandri M et al. Cell 2004; Stitt TN et al. Mol Cell 2004).

Our demonstration that *in vitro* both D-GHR and GHR inhibit dexamethasone-induced atrophy of myotubes through activation of Akt and mTOR, clearly shows that they share the ability to activate a common anti-atrophic signaling pathway in skeletal muscle differentiated cells, consistently with similar findings previously obtained in cardiomyocytes and skeletal myoblasts (Baldanzi 2002; Filigheddu 2007).

The *in vivo* direct anti-atrophic activity of GHR and D-GHR on skeletal muscle can be verified only by increasing the circulating concentration of D-GHR, because an increase of circulating GHR would counteract skeletal muscle atrophy by also activating the GH/IGF-1 axis, confounding the mechanicistic interpretation of the results. Upregulation of circulating D-GHR was obtained either upon D-GHR pharmacological treatment of mice or in the αMHC/GHRL transgenic murine model. The transgenic mice, which have been obtained by specific over-expression of ghrelin gene in the myocardium, features a 50 fold increase of circulating D-GHR without any change of circulating GHR as compared to their wild type

littermates. The lack of increase in acylated GHR concentration may likely depend by the negligible myocardial expression of GOAT, the only enzyme responsible for acylation of D-GHR to GHR (Gutierrez et al. PNAS 2008; Yang et al. Cell 2008). These findings are in accordance to other ghrelin transgenic models featuring high D-GHR circulating levels, in which circulating acylated GHR concentration was not affected (Asakawa et al. Gut, 2005; Zhang W et al Endocrinology 2008).

Inhere we showed that upregulation of circulating D-GHR, obtained both upon pharmacological treatment or in transgenic mice, impairs experimentally-induced atrophy of skeletal muscle. This finding provides further support to the tenet that D-GHR and GHR activate a direct anti-atrophic signaling in the skeletal muscle, independently from GHR activity on GH and IGF-1 release. The possibility that anti-atrophic activity of D-GHR *in vivo* may depend on its conversion to acylated GHR is ruled out by the recent finding that acylation of D-GHR to GHR occurs only intracellularly, through the action of GOAT (Gutierrez et al. PNAS 2008; Yang et al. Cell 2008).

Moreover, consistently with the inability of D-GHR to bind and activate GHSR-1a, the receptor mediating GHR-induced GH release and positive energy balance, αMHC/GHRL transgenic mice do not feature any activation of GH/IGF-1 axis, as proved by the unchanged tibial and whole body length and by the unaffected levels of circulating IGF-1. In addition, hepatic and muscular IGF-1 mRNA, measured by real-time RT-PCR, was not increased neither in transgenic mice nor upon D-GHR treatment as compared to their respective controls (data not shown). Finally, the observation that muscle fiber CSA of αMHC/GHRL transgenic mice is not increased as compared to wild type animals, suggests that D-GHR does not induce hypertrophy *per se.* Altogether these data strongly indicate that *in vivo* anti-.atrophic activity of D-GHR is not mediated by increase of IGF-1.

The finding that D-GHR not only impairs fasting-induced, but also denervation-induced skeletal muscle atrophy, suggests that D-GHR may activate a general anti-atrophic

signaling *in vivo*, rather than just regulating energy balance. Indeed, we observed that D-GHR injection rapidly induces phosphorylation of Akt and mTOR in skeletal muscle, as it does *in vitro.*

Since *in vitro* both D-GHR and GHR activate a common anti-atrophic signaling pathway, it is expected that in vivo even GHR treatment would activate Akt and mTOR, thereby impairing skeletal muscle atrophy. Indeed, in skeletal muscle of GHSR-1a deficient mice, both GHR and D-GHR rapidly phosphorylate Akt and mTOR, ruling out a possible confounding effect of GHSR-1a-mediated upregulation of IGF-1. Furthermore, this finding provides the ultimate evidence that D-GHR and GHR anti-atrophic signaling in skeletal muscle is not mediated by GHSR-1a, the only known GHR receptor. Accordingly, C2C12 myotubes do not express GHSR-1a, but feature high affinity GHR and D-GHR common binding sites (Filigheddu et al. 2007). So far, no alternative receptor for GHR and D-GHR has been identified, but a growing body of evidence indicates that a novel receptor, distinct from GHSR-1a, mediates GHR and D-GHR anti-apoptotic and pro-differentiative activities in several cell types (Granata, 2007; Fukushima, 2005; Delhanty et al., 2006; Nanzer et al., 2004 Kim SW et al., 2005; Choi et al., 2003; Zhang et al., 2005). Although the identity of the putative novel GHR/D-GHR receptor is yet unknown, our findings have important biological and therapeutic implications, since they provide the proof of concept that that D-GHR, even upon pharmacological treatment, by activating a direct anti-atrophic signaling within the skeletal muscle, has a strong and specific potential for the prevention or treatment of atrophy without inducing release of GH nor of IGF-1.

MATERIALS AND METHODS

Reagents

Synthetic ghrelin-(1-28) or GHR, and des-acyl ghrelin-(1-28) or D-GHR were purchased from PolyPeptide Laboratories. Anti-phospho-Akt, anti-Akt, anti-phospho-mTOR, and anti-mTOR antibodies were from Cell Signaling Technology. All reagents were from Sigma, unless otherwise stated.

Cell cultures and myotubes analysis

C2C12 myoblasts were grown in DMEM supplemented with 20% fetal calf serum (Gibco, Invitrogen), penicillin (100 u/ml), streptomycin (100 μg/ml) and antimycotic (0.25 μg/ml). In order to induce differentiation into myotubes, C2C12 myoblasts were allowed to become confluent and the medium was switched to DMEM supplemented with 2% horse serum (Gibco, Invitrogen), penicillin, streptomycin and antimycotic as above.

For measurement of myotube diameters, myotubes were fixed and stained with Diff-Quik (Baxter) and diameter was quantified by measuring a total of >100 myotube diameters from five random fields in 3 replicates at 40x magnification using Image-Pro Plus software (MediaCybernetics) as described (Sandri et al., 2004).

Western Blot

Following the indicated treatments, cells were washed in ice-cold phosphate buffered saline and solubilized with a lysis buffer containing 25 mM Hepes (pH 8), 135 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM $ZnCl_2$, 50 mM NaF, 1% Nonidet P 40, 10% glycerol, Protease Inhibitors Cocktail, 1 mM Na_3VO_4. Lysates were stirred at 4°C for 15 min and clarified at 13,000*g* for 15 min at 4 °C.

Muscles were homogenized at 4 °C in RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1mM EGTA, 50 mM NaF, 160 mM NaCl, 20 mM Tris-HCl pH 7.4) containing 1 mM DTT, Protease Inhibitors Cocktail, and 1 mM Na_3VO_4. Homogenates were then stirred at 4 °C for 15 min and clarified at 13,000*g* for 15 min at 4 °C. Protein concentration was determined by Quant-iT Protein Assay Kit (Invitrogen). Proteins (20-50 μg protein/lane) were separated by 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen), and transferred to polyvinylidene difluoride filters (Hybond-P, GE Healthcare). Membranes were incubated with the indicated antibodies, visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and analyzed with Quantity One software (Bio-Rad). Following anti-phospho-Akt and anti-phospho-mTOR, membranes were stripped with Re-Blot Plus (Chemicon, Millipore) and re-blotted with the corresponding total protein antibodies.

Transgenic animal generation and treatment

All experiments were conducted on young adult male FVB1 wild type, FVB1 αMHC/GHRL transgenic, C57BL/6J wild type, and C57BL/6J GHSR-1a$^{-/-}$ mice (Sun Y et al. PNAS 2004), matched for age and weight, in compliance with the guidelines of Institutional Animal Care and Use Committee at Piemonte Orientale University.

Transgenic FVB1 animals were obtained by cloning the murine ghrelin gene (GHRL) under control of the cardiac promoter sequences of the βMHC 3' UTR and the first three exons of the αMHC isoform (De Acetis et al, 2005). Transgene integration and expression were confirmed by PCR and real time RT-PCR, respectively (data not shown). Phenotypical characterization and experiments were carried out on hemizygote animals. GHR, D-GHR, and IGF-1 plasmatic levels were measured by EIA kits (SPI-BIO for D-GHR and GHR, RnDsystems for IGF-1), according to the manufacturers' instructions.

Starvation-induced atrophy was achieved by 48 h food removal (Sandri et al 2006), while denervation-induced muscle atrophy was obtained 7 days upon resection of the sciatic nerve (Hishiya et al 2006). At the end of the experimental period, mice were sacrificed and muscles were collected and processed for RNA extraction, or embedded in resin, sectioned perpendicularly to their main axis, and stained for histology.

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RNA extraction and analysis

Total RNA from cultured cells was extracted by Nucleospin RNA II (Macherey-Nagel), while RNA from mouse muscles, mechanically triturated in liquid nitrogen, was extracted by TRIreagent (Applied Biosystems). The RNA obtained was retrotranscribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and real-time PCR was performed with the ABI7200 Sequence Detection System (Applied Biosystems) using the following assays: Mm00499518_m1 (Atrogin-1/MAFbx), Mm00439560_m1 (IGF-1), Mm00446953_m1 (Gusb), and Mm00506384_m1 (cyclophilin F).

Muscle fiber size assessment

Muscle fiber cross sectional area (CSA) was assessed, in three muscles from each group, using the stereological method described in Geuna et al. 2001. Data are expressed as fiber size distribution and as the percentage of CSA reduction in comparison to controls.

Statistical analysis

Data are presented as the mean ± SEM, and the statistical significance was assessed, where appropriate, using Student's T test. The variation among groups was compared by means on nonparametric Wilcoxon and Mann-Whitney U tests. Statistical significance was assumed for $p<0.05$. All statistical analysis was performed with SPSS for Windows version 13.0 (SPSS; Chicago, IL).

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TABLE 1

	wild type	**transgenic**
D-GHR (pg/ml)	445.4 ± 155	25000.5 ± 360 [#]
GHR (pg/ml)	41.7 ± 1.6 (fed) 75.7 ± 8.8 (starved)	39.3 ± 1.5 (fed) 68.2 ± 9.5 (starved)
IGF-1 (ng/ml)	748.5 ± 56 (fed) 398 ± 93 (starved)	765.5 ± 120 (fed) 328 ± 37 (starved)
tibial length (mm)	19.65 ± 0.11	19.62 ± 0.22
nose-to anus length (mm)	91.59 ± 0.51	90.61 ± 0.95
body mass index (g/cm^2)	3.32 ± 0.12 (fed) 2.93 ± 0.06 (starved)	3.33 ± 0.08 (fed) 2.92 ± 0.09 (starved)
Gastrocnemius weight (mg)	134.86 ± 4.6	137.2 ± 5.62

LEGEND TO TABLE AND FIGURES

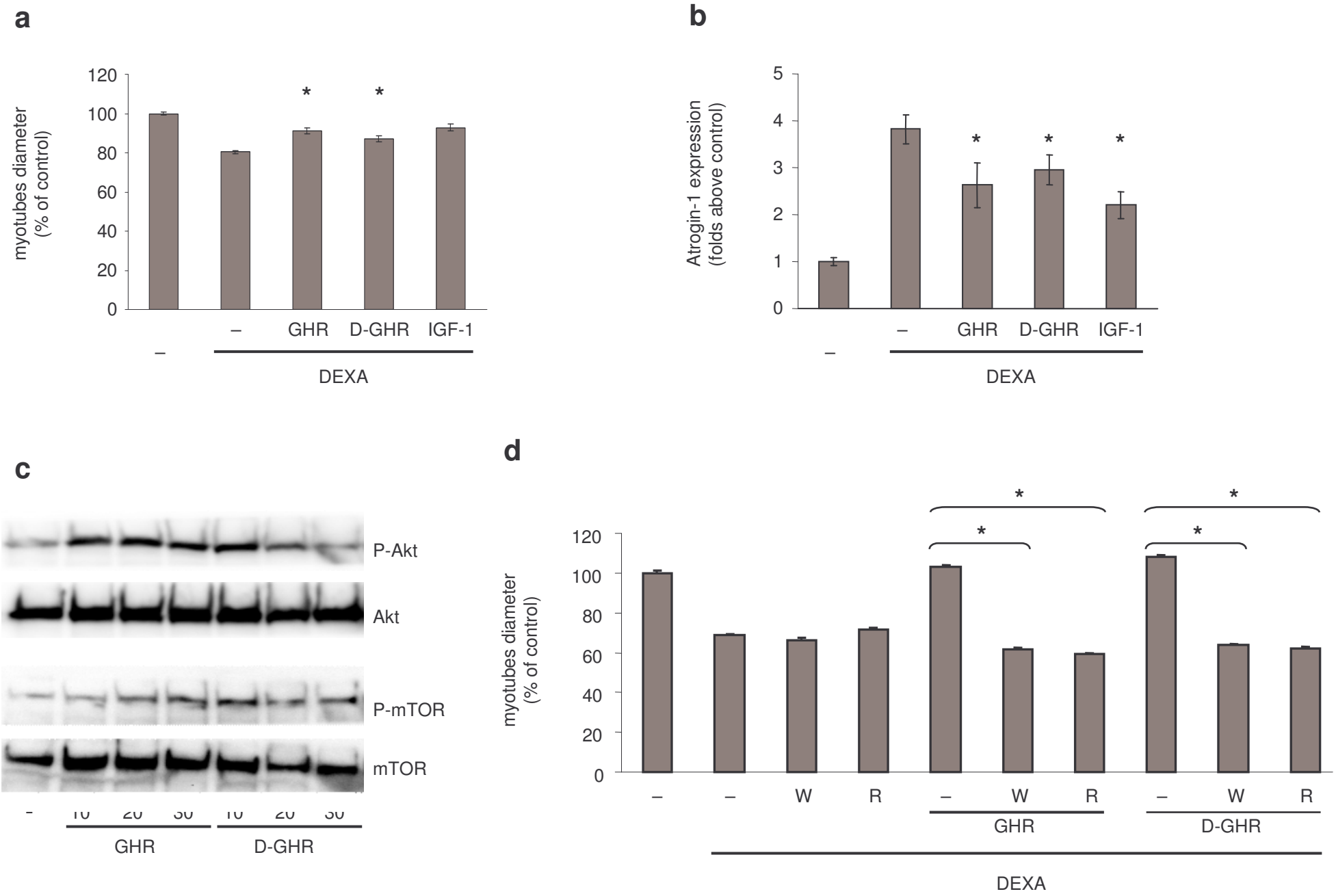
TABLE 1. **Phenotypical characterization of αMHC/GHRL mice.**

Young male mice (fed n = 7 for each group; starved n = 4 for each group) were sacrificed and measurements of the indicated parameters were carried out as described in Materials and Methods. # $p < 0.01$ *vs* wild type animals.

FIGURE 1. **GHR and D-GHR protect C2C12 myotubes from DEXA-induced atrophy by activation of PI3K/Akt/mTOR pathway.** C2C12 myotubes were treated for 24 h with 1 μM DEXA in presence or absence of 10 nM GHR or D-GHR and either (**a**) fixed and stained for myotube diameter measurement, or (**b**) processed for Atrogin-1 expression analysis. IGF-1, at 10 ng/ml concentration, was used as positive control of protection from atrophy. * $p<0.01$ *vs* DEXA-treated cells. (**c**) C2C12 myotubes were treated for the indicated times with 1 μM D-GHR or GHR, lysed, and phosphorylation of Akt and mTOR detected by western blotting. (**d**) C2C12 myotubes were treated for 24 h with 1 μM DEXA, in presence or absence of either 10 nM GHR or D-GHR, 100 nM wortmannin or 20 ng/ml rapamycin. Myotubes were then fixed and stained for thickness measurement. * $p < 0.01$ *vs* inhibitors-free treatments.

FIGURE 2. **αMHC/GHRL transgenic mice are protected from starvation- and denervation-induced skeletal muscle atrophy**. (**a-c**) Atrophy was induced by 48h food deprivation and was evaluated by (**a**) mean percent of weight loss, (**b**) CSA reduction and (**c**) Atrogin-1 induction. * $p<0.01$ *vs* control. (fed wild type and transgenic mice n = 7, starved wild type n = 5, starved transgenic n = 6). (**d-f**) Atrophy was induced by sciatic nerve resection and evaluated as (**d**) mean percent of weight loss versus weight of the muscle on the unperturbed side, (**e**) CSA reduction, and (**f**) Atrogin-1 induction. * $p<0.05$ *vs* control. (wild type n = 6, transgenic n = 4).

Figure 3. D-GHR treatment activates anti-atrophic signaling in both wild type and GHSR-1a$^{-/-}$ mice. (**a**) Wild type mice were injected s.c. with 100 μg/kg D-GHR and at the indicated times gastrocnemia were removed, lysed, and phosphorylation of Akt and mTOR detected by western blotting. (**b-e**) Mice (n = 6 for each group) were treated with 100 μg/kg D-GHR or saline and skeletal muscle atrophy was induced either by (**b,c**) 48 h food deprivation or by (**d,e**) denervation. Atrophy of gastrocnemius was evaluated by (**b,d**) mean percent of weight loss and (**c-e**) CSA reduction. * $p<0.01$ *vs* control. (**f**) GHSR-1a$^{-/-}$ mice were injected s.c. with 100 μg/kg GHR or D-GHR and at the indicated times gastrocnemia were removed, lysed, and phosphorylation of Akt and mTOR detected by western blotting.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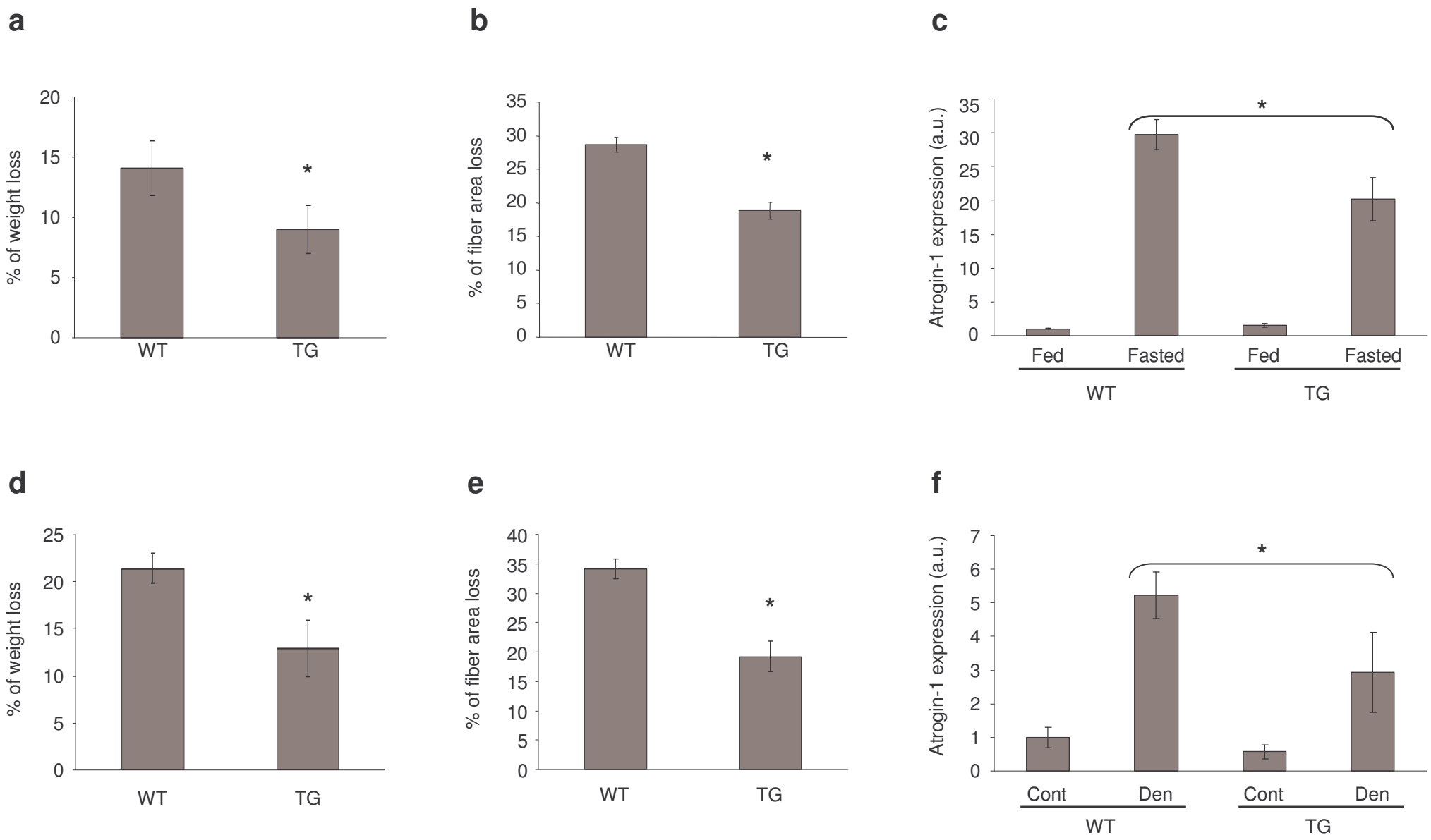
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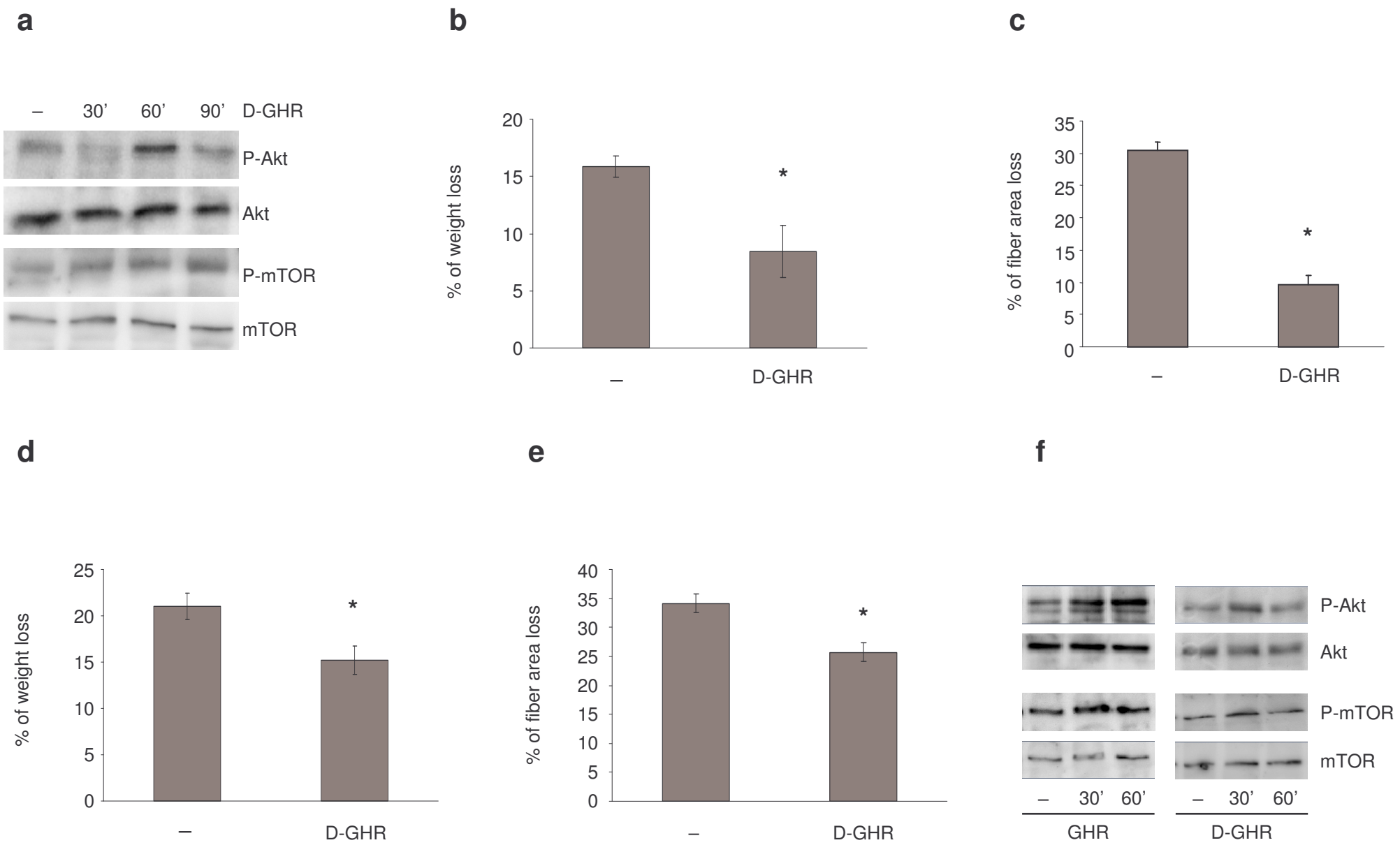
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Hepatic expression of hemochromatosis genes in two mouse strains after phlebotomy and iron overload

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Background and Objectives. Iron homeostasis is tightly regulated in mammals according to the needs of erythropoiesis and the iron stores present. This regulation is disrupted in hereditary hemochromatosis (HH), a genetic disorder characterized by increased intestinal iron absorption, leading to iron overload. The genes coding for HFE, transferrin receptor 2 (TFR2), ferroportin (SLC40A1 or FPN1), hepcidin (HEPC) and hemojuvelin (HJV or RGMC) are responsible for different types of genetic iron overload. All these genes are highly expressed in the liver and their protein products are likely components of a single hepcidin-related pathway. In order to gain insights into the molecular relationship among the HH proteins we evaluated the hepatic expression of HH genes in conditions of iron restriction or overload.

Design and Methods. Data were obtained after phlebotomy, to activate the *erythroid regulators* and following parenteral iron dextran loading, to activate the *store* regulators, in two mice strains (C57BL/6 and DBA/2). HH genes and proteins expression were analyzed by quantitative real time polymerase chain reaction and by Western blotting, respectively.

Results. *Hepc* RNA was reduced after phlebotomy and increased in iron overload. A statistically significant reduction of hepatic *Fpn1* RNA expression was observed after phlebotomy; this effect was more evident in the DBA/2 strain. *Fpn1* increased in C57BL/6 mice, but not in the DBA/2 ones in parenteral iron loading. *Fpn1* protein did not change substantially in either condition. *Hfe*, *Rgmc* and *Tfr2* expression was not influenced by phlebotomy. In parenteral iron overload, *Tfr2* gene and protein expression decreased concomitant to the increase in *Hepc*, while *Hfe* RNA remained constant.

Interpretations and Conclusions. Our results indicate that regulation of hepatic *Fpn1* differs from that reported for duodenal *Fpn1*. Furthermore, taken the differences in gene expression in dietary overload (increased *Hfe* but not *Tfr2*), distinct roles are suggested for *Hfe* and *Tfr2* in *Hepc* activation.

Key words: iron, hemochromatosis, mouse, hepcidin, ferroportin.
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Iron is an essential element for growth and functionality of all mammalian cells, thus its balance is maintained through a tight regulation of intestinal absorption, in order to avoid excessive uptake and iron-mediated cell toxicity. This regulation is titrated on the basis of iron needs for erythropoiesis and on the consistency of iron stores present, according to the proposed model of *erythroid* and *store* regulators.[1,2] Advances in understanding iron homeostasis derive from molecular genetic studies of hereditary hemochromatosis (HH), a genetic disorder characterized by inappropriately high intestinal iron absorption. Identification of the genes involved in HH has revealed new proteins regulating iron homeostasis. Disruption of HFE produces classic, adult-onset HH[3], whereas mutations of hepcidin (HEPC)[4] and of hemojuvelin (HJV or RGMC)[5] lead to the juvenile form, characterized by early onset of severe iron loading. Mutations of transferrin receptor 2 (TFR2) cause type 3 HH, with an early presentation but an intermediate clinical phenotype.[6] The inactivation of each of these four genes results in the same pattern of hepatocyte iron accumulation with reticuloendothelial sparing,[7] although preferential iron deposition in the heart and pituitary gland is observed in the juvenile disease.[8] SLC40A1 gene, encoding ferroportin1 (Fpn1)/IREG1/MTP1, is mutated in type 4 HH,[9,10] which is also known as *ferroportin disease*, and has a dominant inheritance and preferential iron storage in macrophages in most patients.[11]

Among the HH proteins, hepcidin has a key control role and is considered the final effector of the pathway that regulates iron homeostasis. In mice models, its overexpression causes severe iron deficiency,[12] whereas its ablation results in iron overload.[13] The central position of the liver in iron homeostasis is inferred by the observation that all HH genes are highly expressed in hepatocytes.[5,13-15] However, the interrelationships among the different proteins and their relationship with the

storage and *erythroid* regulators remain poorly understood. The evidence is that HFE modulates hepcidin, since the levels of hepcidin are inappropriate to the degree of iron loading in both *Hfe*-deficient animals [16,17] and HFE C282Y homozygous patients.[18] Furthermore, hepcidin is able to correct the *Hfe* defect in transgenic mice.[19] Hepcidin is absent or present at only low levels in the urine of juvenile patients with HJV mutations,[5] suggesting that HJV is hepcidin-related. Low hepcidin levels are also found in patients with TFR2 mutations,[20] implying that TFR2 is another modulator of hepcidin. Furthermore, hepcidin is regulated by hypoxia/anemia both in human hepatoma cells and in animal models[21,22] and is strikingly increased in inflammation.[23]

Recent data have shown that in cultured cells hepcidin interacts directly with ferroportin 1, causing its internalization and lysosomal degradation.[24] This is expected to occur physiologically in duodenal cells to impair iron absorption and in macrophages to restrict iron release in the presence of high hepcidin levels. Indeed in *Hepc*-deficient mice a high level of expression of *Fpn1* was observed in duodenum, spleen and liver macrophages.[25]

The purpose of this study was to evaluate the variations of hepatic expression of the HH genes and especially the *Hepc/Fpn1* relationships, after phlebotomy and parenteral iron overload in two mouse strains characterized by different *Hepc* expression.

Design and Methods

Animal care

Mice purchased from Harlan S.r.l, were housed in the barrier facility at the Department of Clinical and Biological Sciences, University of Turin, Italy and maintained on a standard diet.

All procedures in the mice were carried out in compliance with the guidelines of Institutional Animal Care and Use Committee at our University. All experiments were performed on animals of ten weeks of age. Because of significant differences in iron loading between sexes, only females were used.[26] The studies were carried out in C57BL/6 and in DBA/2 mice, since these strains are characterized by a distinct response to iron variation, the former being more resistant and the latter more susceptible to iron loading.[27]

Treatment protocols

Induction of anemia through phlebotomy

To induce acute anemia, 0.5 mL of blood were extracted by a single retro-orbital puncture from the previously anesthetized mice (Avertin, 2,2,2,-tribromoethanol; Sigma-Aldrich, St Louis, MO, USA). Animals were sacrificed at 6, 9, 12, 15, 48, 72 hours and 7 days after treatment and tissues were collected for RNA and protein extraction. Five animals were used for each time point and nine served as controls.

Induction of parenteral iron overload

Secondary iron overload was induced by two intraperitoneal administrations of 20 mg (1 g/kg body weight) iron dextran (Sigma Chemicals, St Louis, MO, USA) separated by 15 days. Animals were sacrificed two weeks after the second injection. Each animal's liver was dissected and snap-frozen immediately for RNA and protein analysis. Five animals were treated and five served as controls.

Measurements of hematologic parameters

Blood obtained by phlebotomy was collected into tubes containing EDTA (Sarstedt s-monovette, Aktiengesellschaft & Co., Numbrecht, Germany). Blood cell counts and erythrocyte parameters were determined using an EPICS Coulter Profile II (Coulter Electronics, Hialeah, FL, USA) automatic analyzer.

Histology and Perls' staining

For histological studies and iron staining, tissues were fixed in aqueous formaldehyde solution (buffered 4% vol/vol) and embedded in paraffin. For histological assessment of non-heme iron deposition, slides of liver sections were stained with Perls' Prussian blue. Hematoxylin-eosin (H&E) counterstaining was performed to mark the nucleus and cytoplasm by standard procedures.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

For reverse transcription, starting from 1 μg of total RNA, random hexamers at a concentration of 25 μM and 100 U of the reverse transcriptase (Applera, Milan, Italy) were added to the reaction mixture.

Levels of gene expression were measured by quantitative real time PCR (qRT-PCR). The PCR reactions and fluorescence measurements were performed using an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The amount of HH gene transcripts was evaluated using a qRT-PCR assay based on specific sets of primers, designed to be complementary to the different exon sequences to avoid co-amplification of genomic DNA, and probes (Assays-on-Demand, Gene Expression Products) supplied by Applied Biosystems (Foster City, CA, USA). Hepc primers used for qRT-PCR amplify both *Hepc 1* and *Hepc 2*. For the PCR reaction, 5 μL of cDNA were added to 15 μL of PCR reaction mix containing 10 μL of TaqMan Universal PCR Master Mix (Applera), 1 μL of assay in a final volume of 20 μL. The PCR procedure was started with a step of 2 min at 50°C to activate the UNG enzyme, followed by 10 min at 95°C to inactivate the UNG

enzyme and to provide a *hot start* activating the AmpliTaq polymerase. Next, 50 cycles of denaturation (95°C for 15 sec), followed by annealing and extension (60°C for 60 sec) were performed. All analyses were carried out in triplicate; results showing a discrepancy greater than one cycle threshold in one of the wells were excluded.

Northern blot

RNA preparation and Northern-blot analysis were performed with standard methods. Serum amyloid A3 probe was kindly provided by Fiorella Altruda, University of Turin, Italy. The blot was rehybridized with a GAPDH probe in order to normalize the amounts of RNA.

Data analysis

The values obtained were normalized using β-glucuronidase (GUS) as a control gene. The results were analyzed using the ΔΔCt method, as the efficiencies of amplification of both the target and reference genes were determined and found to be approximately equal. Briefly, the threshold cycle (C_T) indicates the cycle number at which the amount of the amplicon reaches the fixed threshold (usually 50%). ΔC_T is the difference between threshold cycle for the gene of interest and the reference gene ($C_{Ttarget} - C_{Treference}$). $\Delta\Delta C_T$ is the difference between ΔC_T of the sample and ΔC_T of a RNA calibrator. A pool of normal mice liver RNA was used as calibrator. The final result is expressed as $2^{-(\Delta\Delta CT)}$.

The statistical significance of the differences of mRNA expression between controls and treated mice was evaluated using one way ANOVA analysis followed by Dunnet's post-hoc test for phlebotomy experiments and Student's t test (unpaired, two tailed) for the iron overload experiments.

Western blot

Western blotting was perfomed with anti-human Tfr2 antibody (14E8),[28] which has demonstrated cross-reactivity with mouse proteins. Rabbit anti-mouse polyclonal anti-Fpn1 antibodies were kindly provided by David Haile, University of Texas, USA.[29] Hepatic tissue was collected, homogenized and lysed. Ten micrograms of proteins were run in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted according to standard protocols. All Western blot analyses were performed on both C57BL/6 and DBA/2 mice tissues.

Results

Hepc expression in basal conditions differed in the two strains of animals studied and also in individual animals. As previously reported,[26] *Hepc* levels, as assessed by RT PCR, were higher in DBA/2 mice than in the C57BL/6 strain, and were predominantly accounted for by *Hepc2,* whereas C57BL/6 expressed more *Hepc1* (*data not shown*). The assay used for qRT-PCR experiments cannot discriminate *Hepc1* and *Hepc2*. However, since it has been demonstrated that *Hepc2* has no effect on iron metabolism,[30] we assumed that the differences observed are due to inter-strain variations in *Hepc1*.

Phlebotomy protocol

Induction of anemia was demonstrated at 6 hours after phlebotomy by measuring hemoglobin levels (Figure 1). Hemoglobin concentration then remained stable and returned to normal levels 7 days after treatment in both strains. *Hepc* expression was up-regulated at 6 hours, decreased at 12-15 hours and recovered at 48-72 hours in both strains. *Hepc* expression was significantly lower ($p<0.05$) within 12 hours after phlebotomy (9 hours in DBA/2) in the animals with induced anemia than in the non-treated animals in both strains (Figure 1A). Northern blot of the same samples showed that serum amyloid A3, a marker of inflammation,[31] increased at 6-15 hours (*data not shown*).

Fpn1 transcripts decreased significantly 6 hours after phlebotomy ($p<0.05$), before the *Hepc* decrease in both strains (Figure 1B). *Fpn1* levels returned to normal 9 hours after phlebotomy in C57BL/6 mice while they paralleled *Hepc* levels in DBA/2 animals. At the protein level, *Fpn1* was slightly decreased in C57BL/6 mice at 6-12 hours after phlebotomy, then remained stable; DBA/2 *Fpn1* did not show significant variations (Figure 1C).

Hfe, *Rgmc* and *Tfr2* transcript levels did not change significantly after phlebotomy in either strain (*data not shown*).

Parenteral iron loading

The protocol of parenteral iron dextran administration we used dramatically increased reticuloendothelial and also parenchymal iron. We did not measure liver iron concentration but, as shown by Perls' staining (Figure 2), iron deposition in C57BL/6 and DBA/2 liver was massive. *Hepc* levels were significantly up-regulated in iron-loaded mice. *Hepc* expression increased by about 7-fold relative to basal levels in C57BL/6 mice ($p<0.001$) and 3-fold in DBA/2 mice ($p<0.001$) (Figure 3A).

Tfr2 transcript levels were downregulated in iron-loaded animals. The reduction was approximately 2-fold in both the C57BL/6 and DBA/2 strains with a statistically significant difference as compared to controls ($p<0.001$ and $p<0.01$, respectively) (Figure 3B). Tfr2 protein levels were reduced accordingly in C57BL/6 mice (Figure 3D upper panel). *Fpn1* tran-

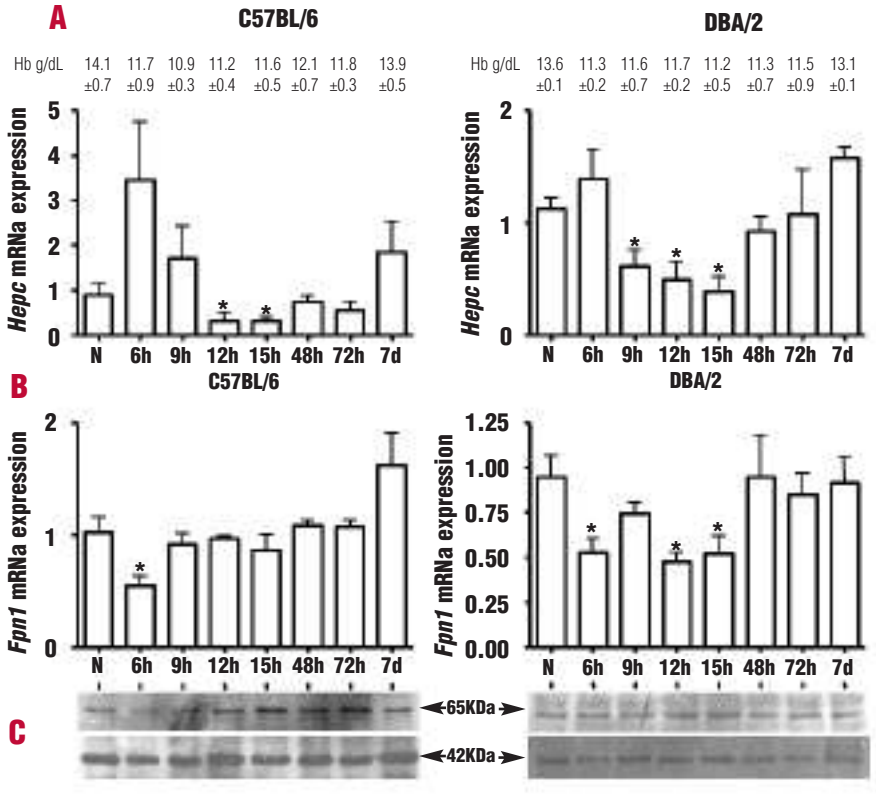

Figure 1. *Hepc* and hepatic *Fpn1* variations in phlebotomized mice. A. Hepatic expression of *Hepc in* C57BL/6 (left) and DBA/2 (right) phlebotomized mice. Hemoglobin values (Hb) are reported. The time course of the experiment (h/d= hours/days after phlebotomy) is illustrated. Five animals were used for each point and nine served as controls (N). The statistical analysis was performed using the ANOVA test, as described in the *Design and Methods*. *Hepc* expression data were normalized to *Gus* cDNA. The asterisk indicates a statistically significant difference ($p<0.05$) vs controls. Results are expressed as mean ± SEM (standard error mean). B. Results of real time PCR (qRT-PCR) of hepatic *Fpn1* mRNA. h/d= hours/days after phlebotomy. N = control mice. Results are expressed as mean + SEM. Statistical analysis was performed as above. *Fpn1* expression data were normalized to Gus (β-glucuronidase) cDNA. The asterisk indicates a statistically significant difference ($p<0.05$) vs controls. C. Western blot of liver Fpn1 (upper lanes) compared with β-actin (lower lanes) of phlebotomized C57BL/6 and DBA/2 mice. The time course of the experiment is reported above the lanes. Molecular sizes in kilodalton (kDa) are shown. N: normal mice.

script levels increased significantly in C57BL/6 mice ($p<0.001$), but did not change after iron loading in DBA/2 animals (Figure 3C). The levels of *Fpn1* protein remained rather stable in both strains (Figure 3D lower panel and *data not shown*). No significant variations were observed for *Hfe* and *Rgmc* transcripts (*data not shown*).

Discussion

The liver is central to the regulation of iron homeostasis in mammals, as demonstrated by the high hepatic expression of HH genes, especially *Hepc*. The evidence is that both *erythroid* and *storage* needs are mediated by *Hepc*,[24] but whether and how the other genes respond to the regulators remains unknown. We have studied hepatic expression of murine HH genes in conditions that modulate iron requirements and *Hepc* production, activating either the *storage* (*Hepc* increase) or the erythroid (*Hepc* decrease) regulators.

A dramatic decrease of *Hepc* in parallel with anemia had been previously observed after multiple blood withdrawal (total 1.5 mL in 24 hours) in C57BL/6 mice.[21] In this study after a single withdrawal of 0.5 mL of blood we observed *Hepc* up-regulation at 6 hours, but thereafter a striking reduction occurred with normalization within 72 hours. The increase at 6 hours is inflammation-related as demonstrated by the simultaneous increase of the inflammatory serum amyloid A3 protein RNA.[31]

The reduction in *Hepc* occurred before any possible change of marrow erythroblast activity and is likely hypoxia-related, consistent with hypoxia being a major determinant of *Hepc* suppression.[21] As shown in Figure 1, *Hepc* levels returned to normal before correction of anemia, suggesting that hypoxia was rapidly compensated. We did not observe significant variation of expression of any of the other genes

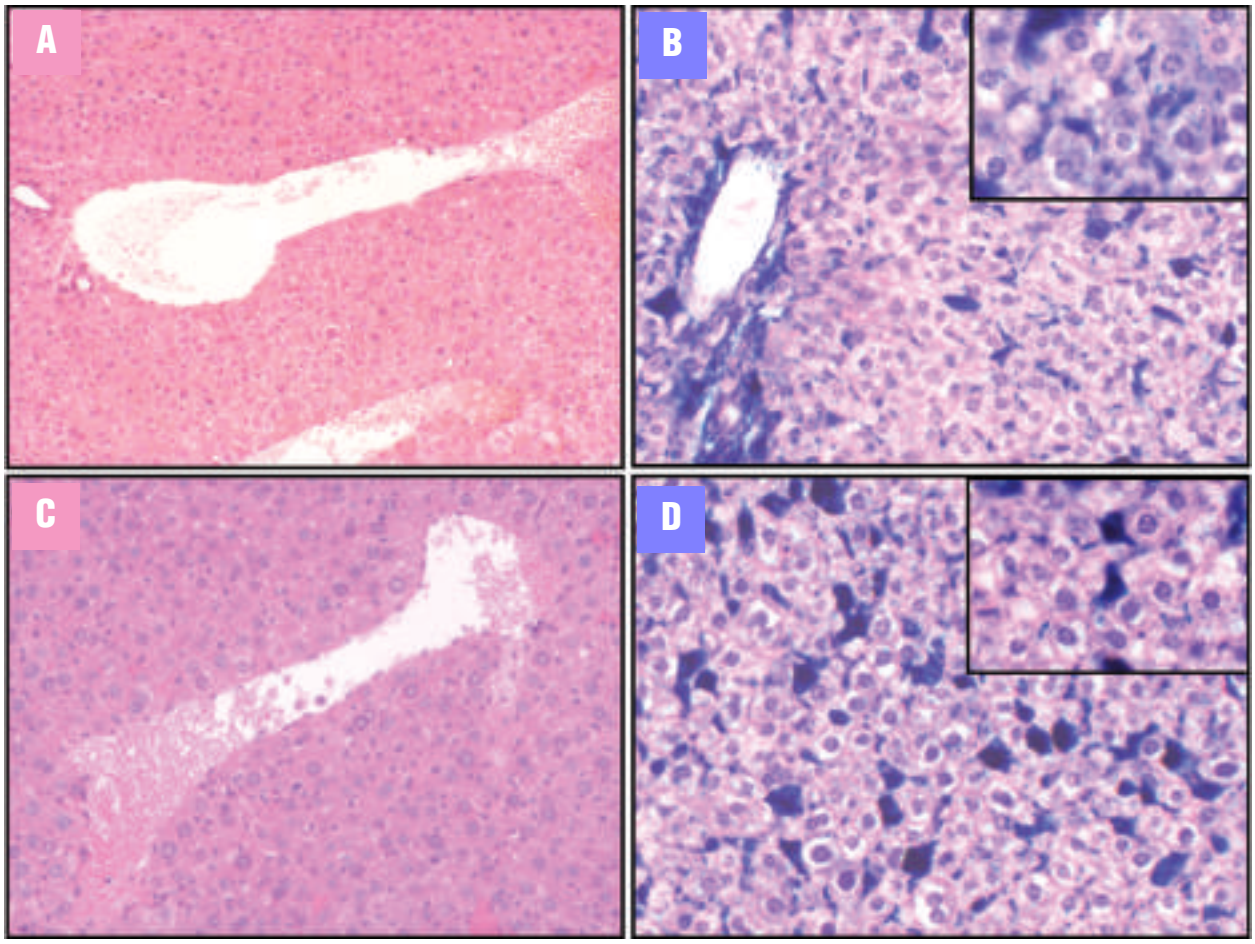

Figure 2. Histology and Perls' staining of mice liver in basal conditions and after iron overload. Panels A and C show the histology of C57BL/6 and DBA/2 control mouse liver respectively (×100), with normal lobular architecture. Panels B and D show heavy iron deposition in Küppfer cells and hepatocytes (Perls' staining, ×100) of the iron dextran treated C57BL/6 and DBA/2 mice, respectively. The inset is a higher-power view (×200).

(*Tfr2, Hfe, Rgmc*) in parallel with the decrease in *Hepc*, indicating that these genes are not transcriptionally co-regulated with *Hepc*. The only exception concerned *Fpn1*. After phlebotomy *Fpn1* RNA was significantly but transiently reduced in both strains, likely as a result of inflammation, as reported in rat liver in response to lipopolysaccharide.[32] From 9 hours liver *Fpn1* RNA parallels *Hepc* RNA variations in DBA/2, while its level overlaps that of controls in C57BL/6. Fpn1 protein levels remained stable, except for a slight reduction in C57BL/6 mice in the first hours after phlebotomy. As expected, a remarkable increase in *Hepc* was observed in mice treated with intraperitoneal iron injections, with this increase being more striking in C57BL/6 mice than in the DBA/2 animals. Interestingly, increased *Fpn1* RNA expression was recorded only in the C57BL/6 strain, but Fpn1 protein levels, although with individual variations, did not change significantly.

Decreased/stable hepatic *Fpn1* after phlebotomy and stable levels in iron overload suggest a distinct regulation of liver *Fpn1*, as compared with duodenal *Fpn1*, whose levels are inversely related to *Hepc* levels.[33] *Fpn1* RNA has a functional 5' UTR IRE motif[29,34] and may undergo post-transcriptional regulation. *In situ* hybridization studies have demonstrated that *Fpn1* mRNA in rat liver shows a decreasing gradient of intensity from periportal to central hepatocytes, indicating a correlation of *Fpn1* expression with iron deposition.[14] The discrepancy between hepatic and duodenal *Fpn1* expression might also be related to different rates of protein degradation. Iron-driven post-transcriptional regulation could operate in the liver and be overtaken by the *Hepc* effect in duodenum and macrophages. A distinct *Fpn1* regulation could reflect a different iron storage function of liver and macrophages. Unfortunately, in our study we cannot separate the contribution of Küppfer cells and that of hepatocytes. Preserved Fpn1 activity would permit iron export from hepatic stores when intestinal iron absorption and macrophage release are inhibited by high *Hepc* levels. After phlebotomy when

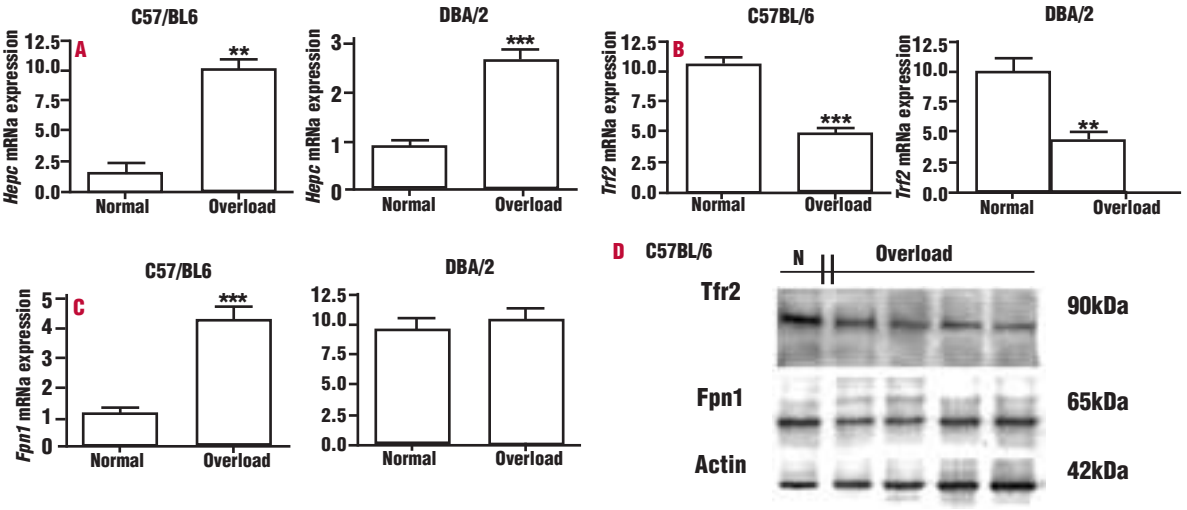

Figure 3. Expression of HH genes and proteins in iron-loaded animals. A-C. Results of qRT-PCR experiments of hepatic mRNA of C57BL/6 (left panels) and DBA/2 (right panels) mice after treatment with iron dextran. Five animals were treated and five served as controls. Statistical evaluation was performed using the Student's t test. The asterisks indicate statistically significant differences vs controls (**p<0.01; ***p<0.001). D. Western blot of liver proteins (Tfr2, Fpn1 compared with beta actin) of C57BL/6 iron-overloaded mice. Band sizes in kilodalton (kDa) are shown on the right. N: normal mice.

Hepc is low, lack of increase of Fpn1 protein could preserve hepatocyte iron, if iron storage were limited. The finding that in *Hepc*-deficient, *Usf2* -/- mice, which are characterized by high liver iron, Fpn1 protein is increased but prevalently in Küppfer cells might be in keeping with our hypothesis.[25] In parenteral iron overload a consistent reduction of *Tfr2* transcript and protein was observed in both strains, irrespective of the degree of *Hepc* activation, whereas *Hfe* expression did not change. A modest reduction of *Tfr2* expression after secondary iron overload in normal mice was observed by analyzing liver RNA on an iron chip.[17] In dietary iron overload *Tfr2* expression is unchanged, whereas that of *Hfe* is increased[35,36] (*and data not shown*), suggesting that transcription of the two genes responds to different signals. Since Tfr2 protein is stabilized *in vitro* by exposure to diferric transferrin, TFR2 has been proposed to be a sensor of transferrin saturation.[37,38] TFR2 is an hepcidin activator *in vivo*, since patients with TFR2 mutations, have low or undetectable urinary levels of hepcidin,[19] but its function is likely distinct from that of HFE. TFR2 could activate hepcidin production, to decrease iron absorption following increase of transferrin saturation. The downregulation of *Tfr2* we observed in chronic iron overload, may suggest that other mechanisms maintain the persistent *Hepc* activation.

All authors meet the criteria for being contributing authors. AR and CC were responsible for the study design, AB, PV, PP and AR did the experimental procedures on animals, FD, EGr, SC and EGo performed the molecular analyses of the samples. All the authors participated in the data interpretation, the drafting of the paper and its final version revision with their suggestions and criticisms. The authors declare that they have no potential conflict of interest.

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