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A FASCINATING JOURNEY FROM THE EFFECTS OF GHRELIN ON SKELETAL MUSCLE CELL LINES TO THE STUDY OF SATELLITE CELL

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PREFACE

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

Part I. When I entered the Molecular Medicine PhD programme under the scientific supervision of Professor A. Graziani, my first objective was to complete the undergoing research on the effects of ghrelin on skeletal muscle cells. The results obtained during the years following this line have been recently published on Molecular Biology of the Cell (Filigheddu et al, 2007) and represent the first proof of a direct effect of both ghrelin and des-acyl ghrelin on myoblasts.

While I was carrying on the investigation about the effects of ghrelin on skeletal muscle cells, I got involved in the study of the skeletal muscle pathology, regeneration and therapeutical approaches: in particular, I have worked on an *in vitro* model of muscle atrophy and I was responsible for the first steps of the production and analysis of two *in vivo* models of ghrelin transgenic over-expression. Both studies gave encouraging preliminary results, which I briefly present inhere, but still need deep investigation in order to be conclusive.

In approaching the muscle pathology and regeneration, my interest grew towards satellite cells, the resident stem cells of the skeletal muscle. In order to follow my interest and increase my competency in working in the muscle field, I moved to London during the last year of my PhD, in the laboratory of Dr. P. Zammit, where I have been learning the up to date techniques to work with satellite cells. While in Dr. Zammit's laboratory, I have been involved in a number of different research projects aimed to elucidate the satellite cell physiology. Inhere, I introduce two studies I participated to, which resulted in publications during the last year, clarifying the biochemical pathway leading to Myf5 activation and the role of β-catenin in satellite cell fate choice, respectively (Perez-Ruiz et al, 2007; Perez Ruiz et al, 2008).

Part II. Although my main research focus in Prof. Graziani's laboratory was on ghrelin and skeletal muscle, during my PhD I also gave my contribution to the investigation of diacylglycerol kinase α (Dgkα) that my colleagues were conducing. Inhere, I present a brief overview on Dgkα, its regulation and on cell migration in order to introduce two papers we recently published: in the first paper, we analyzed in detail the molecular determinants responsible for Dgkα activation by Src in response to HGF stimulation (Baldanzi et al, Oncogene 2008); in the second one, we showed the importance of Dgkα activity in HGF-induced epithelial cell migration and we provided first insights of the downstream pathway involved (Chianale et al, 2007).

PART I

1. GHRELIN

1.1. Discovery and structure

During the late '90s a number of synthetic peptides stimulating the release of the Growth Hormone (GH), both *in vitro* and *in vivo,* have been developed and analyzed (reviewed in Aimaretti et al, 2004). The so called GH secretagogues (GHSs) were shown to be acting through binding an orphan G protein-coupled receptor, named Growth Hormone Secretagogue Receptor 1 (GHSR-1) after this finding (Howard et al, 1996).

Two isoforms of the GHSR-1 obtained by alternative splicing are known: the GHSR-1a, characterized by a seven transmembrane loops structure, typical of G protein-coupled receptors, responsible for binding to the GHSs and stimulating the release of GH; and the GHSR-1b, a truncated form with only five transmembrane loops, considered an inactive isoform to date. The GHSR-1a is mainly expressed in the hypothalamus and the pituitary, while the GHSR-1b is ubiquitously expressed at low levels.

GHSR-1a natural ligand was finally identified by evaluating the effects of cellular extracts from various tissues on its activation. The peptide was named ghrelin (Kojima et al, 1999). Ghrelin is a 28 aminoacid peptide, mainly produced by the stomach; both its nucleotidic and aminoacidic sequences are highly conserved in all species from which it has been cloned so far (Palyha et al, 2000). 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 ubiquitous, suggesting the possibility of local paracrine and/or autocrine activities on cells throughout the body.

The mature ghrelin peptide is obtained by serial proteolitical cleavages from a pre-pro peptide of 117 aminoacids and is then released outside the cell and in the blood stream, to act as hormone.

Recently, it has been found that from the same pre-pro peptide another 28 aminoacids peptide is obtained, named obestatin (Zhang et al, 2005). Obestatin is 23 aminoacids downstream of ghrelin in the pre-pro peptide sequence and it was initially reported to bind to GPR39, an orphan G protein-coupled receptor. However, this finding was not confirmed by further investigation from other groups (Holst et al, 2006).

Ghrelin exists in two different isoforms: the first one has a post-translational modification, namely 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) (Figure 1). Importantly, the octanoylation on Ser3 is a necessary requirement to bind to GHSR-1a and to induce GH release (Kojima et al, 1999).

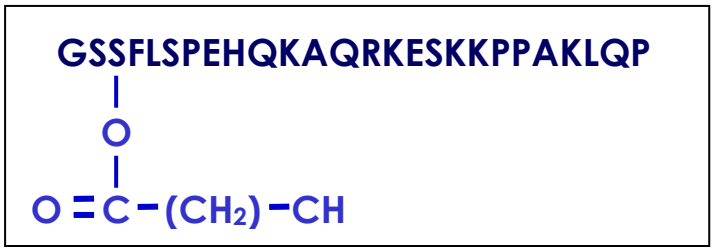

Fig.1 Ghrelin primary structure and octanoylation. Des-acyl ghrelin is identical, but lacks the acylic group on Ser3.

Interestingly, the des-acyl GHR circulating in the plasma 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, to be activated by acylation upon requirement. Very soon though, it became clear that not only GHR has many more activities than initially thought, but also that des-acyl GHR has activities on its own, sometimes, but not always, overlapping with GHR's ones.

1.2. Ghrelin as a gut-brain hormone

During the last years a number of studies have analyzed the role of ghrelin as a gut-brain hormonal signalling molecule, able to influence behaviour and contributing to regulate energy homeostasis together with a number of other molecules, such as peptide YY, pancreatic polypeptide, glucagon-like peptide 1, axyntomodulin, cholecistokinin, leptin and others (reviewed in Murphy and Bloom, 2006; and in Wren and Bloom, 2007).

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).

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).

Although in the last years ghrelin has been considered one of the most promising anti-obesity targets, by the analysis of single gene or congenic knock-out mice for ghrelin (*ghrelin -/-*) and GHSR (*Ghsr -/-*) it has been recently shown that a more complex regulation of energy homeostasis should be taken in account (Sun et al, 2008). Indeed, *Ghsr-/-* mice are refractory to the stimulatory effects of ghrelin on GH release and appetite, confirming that the GHSR is a physiologically relevant ghrelin receptor (Sun et al, 2004). Despite this, *ghrelin*-/- mice and *Ghsr*-/- mice have normal growth rates and normal appetite under conditions of standard laboratory housing (Sun et al, 2003; Sun et al, 2004; Wortley et al, 2004).

By analyzing adult congenic mutant mice (*ghrelin -/-*; *Ghsr -/-*) fed with a high fat diet, no differences in body weight and energy expenditure were evident between wild-type littermates and null genotypes; however, under caloric restriction, the weight loss of *ghrelin-/-* and *Ghsr-/-* mice was identical to wild-type littermates, but blood glucose levels were significantly lower (Sun et al, 2008).

These results suggest that the primary metabolic function of ghrelin in adult mice is to modulate glucose sensing and insulin sensitivity, rather than directly regulate energy intake and energy expenditure.

In fact, the finding that the leptin deficient *ob/ob* mouse bred onto the *ghrelin*-/- background has the same hyperphagic and obese phenotype as the *ob/ob* mouse, indicates that ghrelin unopposed by leptin does not play a dominant role in orexigenic regulation and fat deposition (Sun et al, 2006). Therefore, the leading hypothesis arising from the last researches is that the ghrelin/GHSR signalling pathway is a modulator rather than a dominant regulator of energy homeostasis.

1.3. Activities on the cardiovascular system

The vast majority of studies on the GH-independent activities of GHR and des-acyl GHR focused on the cardiovascular system.

Before the cloning of ghrelin it was already clear that a number of GHSs, such as hexarelin, exerted a protective effect on cells of this system, seen as apoptosis inhibition on cardiomyocytes *in vitro* (Filigheddu et al, 2001) and cardio-protection *in vivo* (Locatelli et al, 1999).

Starting from these results, in the laboratory of Prof. Andrea Graziani the effects of both GHR and des-acyl GHR on endothelial and cardiac cells have been evaluated. Results obtained with cell lines and primary cell cultures revealed that both GHR and des-acyl GHR are able to inhibit the apoptosis induced by various stimuli (serum deprivation, Fas-L administration, doxorubicin) in a rat cardiac cell line (H9C2), primary mouse cardiomyocytes and endothelial cells (PAE, a cell line derived from pig aorta) (Baldanzi et al. JCB, 2002). Moreover, the existence of a second ghrelin receptor, able to bind both the acylated and the non-acylated isoform was postulated, since a protective effect was exerted by both peptides and a high affinity binding site was found on the cells used in this study for both GHR and des-acyl GHR, while the GHSR-1a was absent. To date, even though a number of other studies strengthened this hypothesis, an alternative receptor binding both GHR and des-acyl GHR has not been identified yet.

Biochemically, the anti-apoptotic activity of GHR on cardiomyocytes and endothelial cells is coupled with the stimulation of the phosphorylation and subsequent activation of ERK 1/2 and Akt (Baldanzi et al, 2002).

The cardio-protective activity of the ghrelin peptides observed *in vitro* has been confirmed by a variety of *in vivo* evidences, both in rats and humans. It has been shown that GHR is able to protect from cellular damage induced by heart failure in rats (Nagaya et al, 2001a) and from ischemia-reperfusion injury induced on a model of rat isolated heart (Chang et al, 2004). Recent results suggest that ghrelin administration in rats would attenuate left ventricular remodelling following myocardial infarction via the suppression of cardiac sympathetic activity (Soeki et al, 2007). Moreover, cardiac parameters, such as left ventricular function, and exercise capacity are improved following treatment with ghrelin in human patients with chronic heart failure (Nagaya and Kangawa, 2003; Nagaya et al, 2004).

An important hemodynamic effect induced by ghrelin is vasodilatation, beneficially observed in patients with chronic heart failure (Nagaya et al, 2001b) and metabolic syndrome (Tesauro et al, 2005). Interestingly, both GHR and des-acyl GHR show similar vasodilator potency and efficacy in reversing endothelin 1-induced constriction in human artery (Kleinz et al, 2006).

1.4. Other activities

As previously mentioned, although the X/A-like cells in the gastric fundus mucosa are mainly responsible for the production of ghrelin, the peptide is ubiquitously expressed. As consequence, likely it can also elicit paracrine or autocrine actions on various cell systems beside its endocrinological activities.

Since ghrelin discovery, its effects on various tissues and cell lines have been evaluated, especially in terms of influence on proliferation and differentiation. A number of tumoral cell lines have been analyzed as well.

Ghrelin has been shown to promote proliferation in H9C2 rat cardiomyocyte cell line (Pettersson et al, 2002), several human prostate cancer cell lines (Jeffery et al, 2002), human pancreatic adenocarcinoma cells (Duxbury et al, 2003), rat and human zona glomerulosa cells (Andreis et al,

2003; Mazzocchi et al, 2004), rat pituitary somatotroph cell line (Nanzer et al, 2004), mouse 3T3-L1 preadipocytes (Zhang et al, 2004; Kim et al, 2004), rat osteoblasts (Fukushima et al, 2005; Maccarinelli et al, 2005), primary oral human keratinocytes (Groschl et al, 2005) and HEL erythroleukemic human cell line (De Vriese et al, 2005).

On the other hand, and sometimes in disagreement with the results listed above, it has been demonstrated that ghrelin inhibits cell growth of human breast carcinoma and derived cell lines (Cassoni et al, 2001), human lung carcinoma cell line (Ghé et al, 2002), human foetal thyroid and follicular carcinoma cell lines (Volante et al, 2003), prostatic carcinoma cell lines (Cassoni et al, 2004), rat immature Leydig cells (Barreiro et al, 2004) and mouse splenic T cells when these are co-stimulated by anti-CD3 (Xia et al, 2004).

The ability of ghrelin to influence differentiation has been mainly evaluated on adipose tissue cells, since one of ghrelin main action *in vivo* is the stimulation of adiposity and GHSR-1a is abundantly expressed in adipose tissue.

Indeed, different groups reported different results on 3T3-L1 preadipocytes: in fact, Sasaki and colleagues showed a pro-differentiative effect of ghrelin on such cells (Choi et al, 2003), while Mulholland and colleagues observed adipogenesis inhibition (Zhang et al, 2004).

Ghrelin has also been shown to affect bone formation not only by promoting proliferation, but through a direct pro-differentiative activity on osteoblastic cells (Fukushima et al, 2005; Maccarinelli et al, 2005).

2. SKELETAL MUSCLE DIFFERENTIATION

2.1. Skeletal muscle structure and formation

The myofibre is the functional, contractile unit of the adult skeletal muscle; it is a syncytial cell composed of a high number of post-mitotic myonuclei, derived from the massive fusion of myoblasts during development and muscle formation. An individual skeletal muscle may be made up of hundreds, or even thousands, muscle fibres bundled together and wrapped in a connective tissue covering.

Skeletal muscles vary considerably in size, shape, and arrangement of fibres. 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).

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.

Skeletal muscles of the trunk in vertebrates are derived from the somites, segmental blocks of paraxial mesoderm which form on either side of the neural tube (reviewed in Buckingham et al, 2003). Somitogenesis begins around E8 in the mouse and proceeds for several days. Briefly, the sclerotome and the dermomyotome quickly originate from the somites. While the sclerotome would give rise to the cartilage of vertebral column and ribs, the dermomyotome is the source of muscle precursors and other cells. At this point, the formation of the myotome follows, and the mature muscle is obtained from it.

In the adult, the skeletal muscle is a slow renewing tissue. In normal conditions, the number of myofibres per muscle does not undergo significative variations during the adult life, while the protein content may dramatically increase (hyperplasia) or decrease (atrophy).

2.2. Satellite cells and skeletal muscle differentiation

As already mentioned, in the adult the skeletal muscle is a slow renewing tissue and the myonuclei in the myofibres are post-mitotic. Thus, a certain degree of physiological turnover has to be carried out and specific events can require a massive regeneration, i.e. a muscle injury.

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

A number of other cellular types have been proposed to contribute to skeletal muscle regeneration (reviewed in Cossu and Biressi, 2005), such as mesoangioblasts (Sampaolesi et al, 2006), pericytes (Dellavalle et al, 2007) or bone marrow-derived stem cells (LaBarge and Blau, 2002). Nevertheless, satellite cells are still considered the main, physiological source of new myofibres during routine turnover or following a muscle injury.

Satellite cells derive the name from their peculiar position on the myofibre, on the periphery, right under the basal lamina (Mauro, 1961) (Figure 2).

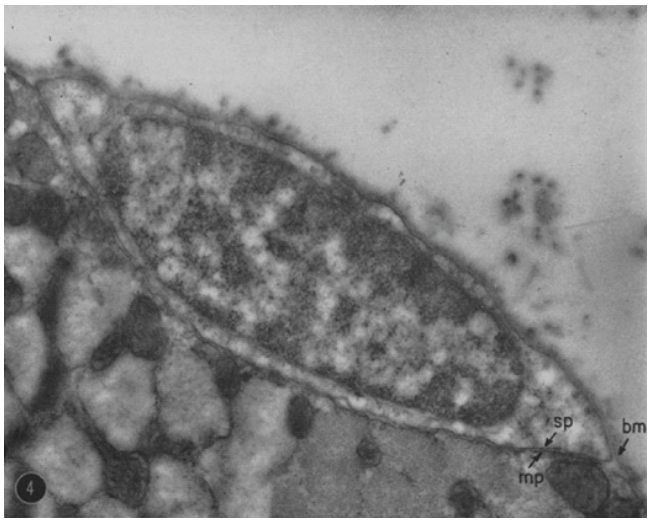

Fig. 2 The first electronic micrography of a rat satellite cell. In 1961, Mauro firstly identified the satellite cell underneath the basal lamina of the muscle fibre and proposed its function as reserve cell. A new field in the skeletal muscle research was born (from Mauro, 1961).

Unlike the myonuclei, satellite cells are in a reversible quiescent state. Upon specific triggering signals, satellite cells 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 (Whalen et al, 1990; Zammit et al, 2002).

Although the specific signals able to trigger the satellite cells response have not been identified 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).

Each step from activation to the fusion of hundreds of daughter cells is characterized by the expression of specific markers. Apart from their anatomical localization and functional characteristics, quiescent satellite cells can be identified by the expression of a peculiar set of molecular markers (reviewed in Kuang and Rudnicki, 2007): among them M-cadherin (Irintchev et al, 1994), MNF -myocyte nuclear factor- (Garry et al, 1997), which is also expressed by a small, but significant, percentage of myonuclei, the transcription factor Pax7 (Seale et al, 2000), CD34 and Myf5 (Beauchamp et al, 2000), the last two defining the vast majority of satellite cells present on a single fibre. In particular, the transcription factor Pax7 is widely used to define satellite cells because of its specificity for this pool of cells (while other markers are also expressed by other cell types in different systems). In fact, Pax7 is essential to specify the satellite cell pool, as Pax7-/- mice develop normally but lack satellite cells and fail to effectively regenerate following a muscular damage (Seale et al, 2000). Moreover, a good monoclonal antibody against Pax7 is commercially available.

Following a triggering signal, the activation of satellite cells is marked by the re-entering in the cell cycle from a G0 reversible state. At this stage, Pax7 is

still expressed and the expression of the transcription factor MyoD is induced very quickly, within 24 hours from activation, while a first division is occurring. However, MyoD expression is necessary but not sufficient to undergo terminal differentiation.

Activated, proliferating satellite cells, expressing both Pax7 and MyoD are at a crossroad: they can suppress the expression of Pax7 and maintain MyoD, therefore committing to irreversible differentiation; otherwise, they can maintain Pax7 and down-regulate MyoD. In this second case, they would return to quiescence and be available for future events requiring their activation (Zammit et al, 2004) (Figure 3).

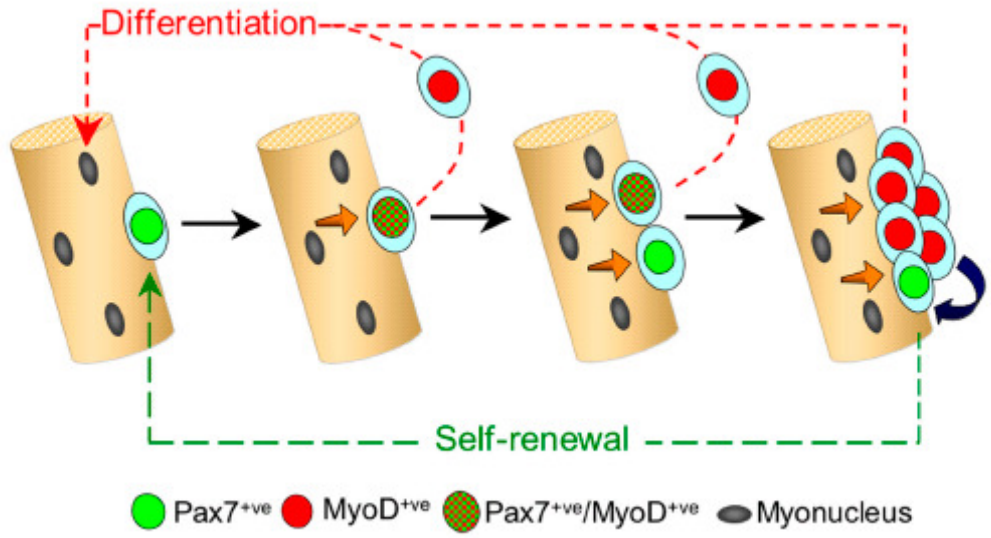

Fig. 3 The proposed model for satellite cell self-renewal. Each satellite cell has the property of giving rise to a highly proliferating and eventually differentiating progeny, as well as to maintain a "stem" population with identical properties. Pax7 and MyoD expression kinetics allow to follow the fate of the satellite cell progeny toward terminal differentiation or self renewal (from Zammit et al, 2004).

Since it is committed, but far from being fully differentiated, and it has the ability to undergo intensive proliferation thus maintaining the population by self-renewal, the satellite cell can be considered a stem cell, the resident stem cell of the skeletal muscle.

Satellite cell progeny down-regulating Pax7 and maintaining high levels of MyoD, continues with differentiation and expresses the transcription factor

myogenin. Once myogenin is expressed, the differentiative process is irreversible. Soon after, the expression of the myosin heavy and light chains (MyHC and MyLC), representing the main components of the contractile apparatus together with actin, is induced. In order to complete the formation of new myofibres, an irreversible cell cycle arrest is required. Fusion and a massive production of myosin follow.

The final result of this well regulated process is a myofibre basically identical to the one from which the satellite cells have originated, with its own pool of associated precursor cells, hundreds of post-mitotic myonuclei and the typical myosin-actin organization conferring contractility.

3. INTRODUCTION TO: "GHRELIN AND DES-ACYL GHRELIN PROMOTE DIFFERENTIATION AND FUSION OF C2C12 SKELETAL MUSCLE CELLS" (*Filigheddu N, Gnocchi VF, Coscia M, Porporato PE, Taulli R, Traini S, Baldanzi G, Chianale F, Cutrupi S, Arnoletti E, Ghe' C, Fubini A, Surico N, Sinigaglia F, Ponzetto C, Muccioli G, Crepaldi T and Graziani A.* **Molecular Biology of the Cell, 2007)**

3.1. Biological and therapeutical relevance of understanding the molecular mechanisms underlying muscle differentiation

A number of diseases directly or indirectly affects the skeletal muscle. The class of diseases more frequent directly affecting the skeletal muscle are the muscular dystrophies, genetic disorders causing the disruption of the contractile apparatus of the myofibre, with the progressive impairment of the muscular function (reviewed in Emery, 2002). The muscle involvement is often a consequence of another primary condition or disease; it's the case of trauma injuries, cancer or AIDS, these last two leading to a cachectic or atrophic condition.

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 order to replace missing or not functional ones. As summarized in paragraph 2.2., this well regulated process is carried out by the resident satellite cell pool. Although the main steps of their differentative programme have been elucidated, still little is known about the signals triggering satellite cell activation and about factors influencing its progression.

Thus, it would be highly useful, from a therapeutical point of view, to understand such signals and have the opportunity to influence the regenerative process all along.

3.2. Background and results

The anti-apoptotic activity of both ghrelin (GHR) and des-acyl ghrelin (des-acyl GHR) on cardiac and endothelial primary cultures and cell lines *in vitro* was demonstrated for the first time in the laboratory of Prof. Andrea Graziani (Baldanzi et al, 2002). Similarly, a number of *in vivo* evidences were earlier and thereafter collected both in humans and animal models, from other groups (Nagaya et al, 2001a; Nagaya et al, 2001b; Nagaya and Kangawa, 2003; Nagaya et al, 2004; Chang et al, 2004; Soeki et al, 2007).

Since cardiomyocytes are a specialized type of muscular cells and given the evidence of the ability of ghrelin to variously influence the proliferative and differentiative processes in a variety of cell lines (see paragraph 1.4.), we sought to investigate the possible role of ghrelin and des-acyl ghrelin in promoting differentiation of *in vitro* myoblasts.

Also to be taken in account, a number of evidences are accumulating proving the ability of ghrelin to counteract the cachectic state associated to a variety of pathologies, such as heart failure (Nagaya and Kangawa, 2003), cancer (reviewed in Ramos et al, 2004) and chronic obstructive pulmonary disease (Nagaya et al, 2005). Whether this beneficial anti-cachectic effect is due to increased appetite and GH release following ghrelin administration or to a direct action of ghrelin on the skeletal muscle cells, it still remains to be elucidated.

In order to evaluate the effects of GHR and des-acyl GHR on myoblasts, we used a highly validated *in vitro* model, the C2C12 cell line obtained in the late seventies from mouse injured hind limb (Yaffe and Saxel, 1977).

C2C12 are proliferating mouse myoblasts, still retaining 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 grow in adherent, not too confluent (<70%) cultures, actively proliferating in condition of high nutrient availability (10% foetal bovine serum). 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.

Firstly, they express early differentiation markers, such as MyoD and myogenin; irreversible cell cycle withdrawal follows. Once the proliferation has stopped, the contractile phenotype is induced, by the expression of MyHC and MyLC. Finally, C2C12 monocytes fuse together, giving rise to multi-nucleated myotubes (Andrés and Walsh, 1996), closely mimicking the myofibre (Figure 4).

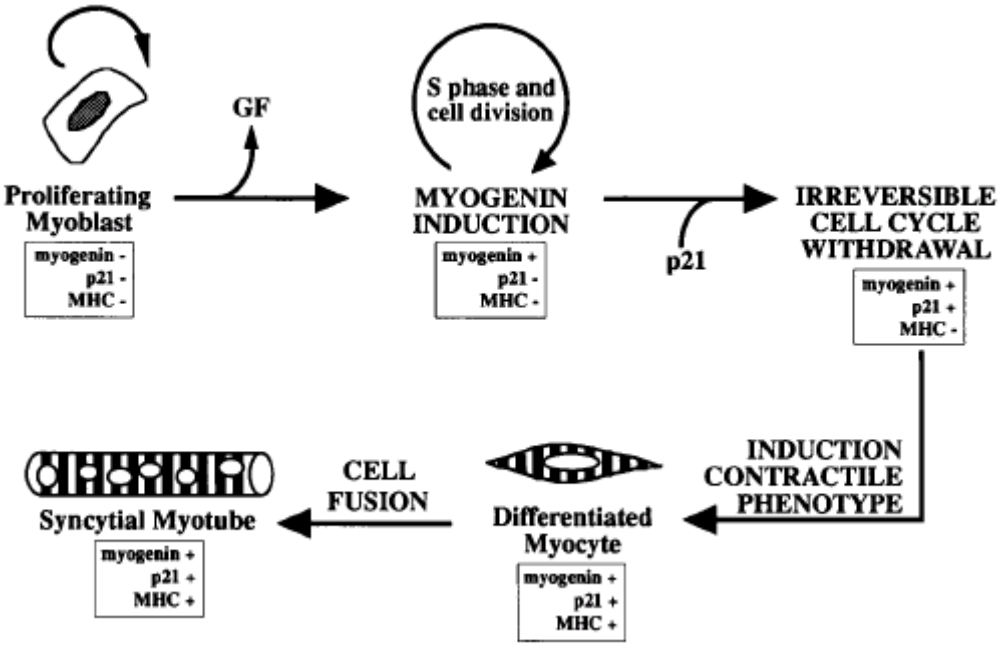

Fig. 4 C2C12 differentiation steps. Each step of C2C12 differentiation can be temporally separated and monitored by the expression of a number of different markers. In few days upon growth factors (GF) withdrawal, starting from actively proliferating C2C12 myoblasts, multi-nucleated myotubes are obtained, showing the typical actin-myosin sarcomeric organization (from Andrés and Walsh, 1996).

Indeed, C2C12 muscular differentiation process recapitulates the events physiologically occurring in satellite cells following a triggering signal and have been widely used to study the skeletal muscle *in vitro*, with all the advantages of using an immortalized cell line instead of primary cultures.

In the paper here presented, we demonstrated that both GHR and des-acyl GHR are able to promote differentiation and fusion of C2C12 myoblasts while they are maintained in high serum growth medium (GM) to an extent comparable to that induced by the low serum differentiation medium (DM).

In fact, both GHR and des-acyl GHR are able to induce expression of early and late differentiation markers (myogenin and MyHC) while inhibiting C2C12 proliferation. The extent of differentiation and fusion were measured calculating the differentiation (percentage of MyHC positive cells) and fusion (average number of nuclei/cells with at least 3 nuclei) indexes, respectively.

Moreover, both GHR and des-acyl GHR do improve differentiation and fusion of C2C12 myoblasts in DM.

From a biochemical point of view, we found out that the differentiative pathway triggered by GHR and des-acyl GHR lead to the activation of p38. This fact is relevant, since it has been shown that the constitutive activation of p38 itself is able to induce myogenin expression, cell cycle exit and terminal differentiation (Wu et al, 2000).

Importantly, after having demonstrated the absence of the GHSR-1a from both C2C12 myoblasts and myotubes, we conduced binding studies on C2C12 myoblasts membrane, finding the existence of high affinity binding sites displaced by both crude GHR or des-acyl GHR. These findings suggest that an unknown receptor, able to bind des-acyl GHR as well as GHR, should be responsible for the pro-differentiative activity of both isoforms on C2C12 myoblasts.

The experiments with GHR and des-acyl GHR were mainly conduced by treating the cells with the exogenous peptides at defined concentrations; nevertheless, to overcome the problems related to exogenous administration, we also produced a bidirectional lentiviral vector expressing GHR together with eGFP. The lentiviral vector backbone was developed and kindly provided by Naldini and colleagues (Amendola et al, 2005). We believe this lentiviral vector, that we called MA1-GHR, will be a useful tool for a number of future analysis and investigations.

4. GHRELIN: UNPUBLISHED RESULTS

4.1. A study of ghrelin's ability to protect myotubes from skeletal muscle atrophy *in vitro*

Skeletal muscle atrophy

Skeletal muscle atrophy is a catabolic, debilitating systemic response to some physiological condition, as fasting, or to a variety of diseases, such as cancer, diabetes mellitus, AIDS, renal failure; it also occurs in specific muscles upon denervation or disuse. 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. The loss of muscle proteins is the net result of a diminished protein synthesis coupled with increased protein degradation. Even if both processes occur at the same time, protein degradation via the ubiquitin-proteasome pathway is the major phenomenon responsible for muscle protein decrease and, in the end, for atrophy (reviewed in Lecker et al, 2006).

Recently, a study has been conduced to elucidate molecular changes at the transcriptional level during atrophy. cDNA microarrays experiments comparing four different atrophy-inducing conditions in mice or rats (fasting, cancer, renal failure, diabetes mellitus) to normal non atrophy-inducing conditions in pair-fed animals, revealed that in all types of atrophy-inducing conditions, a common set of 120 genes is co-ordinately regulated (Lecker et al, 2004), while 90% of the transcriptoma shows no changes.

This set of genes, named "atrogenes" because of their strict correlation with atrophy, includes genes coding for proteins of the ubiquitin-proteasome pathway (up-regulated), genes involved in energy production, genes coding for structural proteins of the extracellular matrix (down-regulated), and so on and so forth. Because of this highly conserved "atrophy program" it is possible to postulate a common signalling pathway influencing the same transcription factor(s) activated in the cell as an answer to all types of atrophying stimuli tested.

Among the proteins of the ubiquitin-proteasome degradation pathway, an E3 ubiquitin ligase is especially over-expressed: the Muscle Atrophy F-box (MAFbx) or Atrogin1 (Bodine et al, 2001). Atrogin1 is a useful marker at both the RNA and protein level to monitor the “atrophic state” of the cell.

Mainly responsible for the increased expression of Atrogin1, is the Fork-head box subgroup O (FoxO) family of transcription factors (Sandri et al, 2004). The FoxO factors are regulated both via transcriptional induction and via a classical mechanism of phosphorylation/dephosphorylation.

The phosphorylation of the FoxO factors inhibits their translocation in the nucleus and, consequently, the activation of the transcription of a number of genes, one being Atrogin1.

The phosphorylation of the FoxO factors is downstream the PI3K/Akt pathway, as shown in response to the growth factor IGF1 (Stitt et al, 2004; Nader, 2005; Léger et al, 2006; Li et al, 2005). The absence of mitogens or growth factors such as insulin or IGF1 blocks the PI3K/Akt signalling pathway and, as a consequence, the non-phosphorylated FoxO factors can enter in the nucleus and promote the transcription of Atrogin1 and others atrogenes. Furthermore, the activated Akt also promotes mTOR (activating) and GSK3β (inhbiting) phosphorylation, respectively, thus stimulating protein synthesis. In summary, the activation of the PI3K/Akt pathway results in reduced protein degradation and increased protein synthesis, while its inhibition leads to skeletal muscle atrophy (Figure 5).

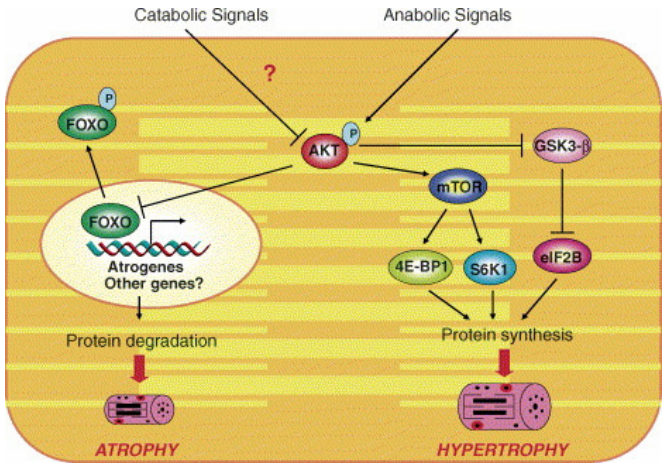

Fig. 5 Opposing anabolic (i.e. elevated nutrients, IGF1) and catabolic signals (i.e. dexamethasone, starvation) can trigger the atrophic or hypertrofic response in the muscle cell. The key regulatory molecule for switching from one condition to the other seems to be Akt. The other major molecular players are illustrated (From Nader, 2005).

Preliminary results

After considering the results obtained with GHR and des-acyl GHR on C2C12 myoblasts, the general positive effects on the whole body metabolism (appetite induction, GH release), and the beneficial effect of ghrelin on cachectic states *in vivo* (Nagaya and Kangawa, 2003; Ramos et al, 2004; Nagaya et al, 2005), we raised the hypothesis that ghrelin could be involved in directly counteracting atrophy on muscle cells.

To verify our hypothesis we set out an *in vitro* model of atrophy. C2C12 myoblasts were induced to differentiate and form myotubes (myotubes were obtained after 5 days of culture in mitogen-poor medium). The C2C12-derived myotubes were induced to undergo atrophy by a 24h treatment with dexamethasone 1 μM.

We verified that dexamethasone-treated myotubes were undergoing apoptosis by *i)* analyzing their morphology and quantitatively assessing the myotubes thickness *ii)* assaying by Western Blot the level of phosphorylation of Akt and FoxO-3 *iii)* measuring by Real Time PCR the expression of atrogenes such as Atrogin-1. All the analyses were carried out in parallel on myotubes not treated with dexamethasone or treated with IGF1 as controls.

The preliminary results we obtained indicate that both GHR and des-acyl GHR are able to protect C2C12-derived myotubes from dexamethasone-induced atrophy.

In particular, the morphology and the thickness of the myotubes, significantly affected by the dexamethasone treatment, are comparable to that of non-treated myotubes in the presence of GHR or des-acyl GHR.

Importantly, the expression of Atrogin-1 in dexamethasone-treated C2C12 myotubes, measured by Real Time PCR, is reduced by GHR and des-acyl GHR

in a dose dependent manner (ranging from 1 to 100 nM), suggesting a specific effect of the two hormones.

From a biochemical point of view, 100 nM GHR induces the phosphorylation of Akt in dexamethasone-treated myotubes, with a peak effect 10 minutes after the treatment and the return to basal level after 30 minutes. Moreover, the phosphorylation of FoxO-3 is also induced, suggesting that GHR could act through the described pathway involving Akt, the FoxO factors and the atrogenes.

In a recently published paper, Okimura and colleagues show that, in an almost identical experimental system, GHRP-2, a synthetic GHSR-1a agonist, can reduce the levels of Atrogin1 and attenuate atrophy in an IGF1-independent manner (Yamamoto et al, 2008). Their results, however, suggest that the attenuation of atrophy is achieved through the activation of GHSR-1a, while in our hands des-acyl GHR is also able to exert a similar effect, indicating that an alternative receptor should be involved.

Thus, further investigation will be required to fully characterize the mechanisms through which GHR and des-acyl GHR exert their effects on myotubes undergoing atrophy.

4.2. Two *in vivo* models of ghrelin transgenic over-expression

In order to verify the relevance of the effects of ghrelin we observed *in vitro* on cardiomyocytes and myoblasts (Baldanzi et al, 2002; Filigheddu et al, 2007), we decided to produce two *in vivo* models of ghrelin transgenic over-expression. In particular, we decided to produce a transgenic line of mice constitutively over-expressing ghrelin in the heart and another one over-expressing ghrelin in the skeletal muscle. Furthermore, the constitutive over-expression of ghrelin in the heart would be a useful tool to carefully assess the molecular mechanisms underlying its beneficial effect on the heart function, an effect widely observed *in vivo* following exogenous administration.

Although ghrelin is a secreted and circulating hormone, it has been widely shown that it can exert a variety of paracrine or autocrine activities as well (see paragraphs 1.2.-1.4.). Thus, we thought that a local ghrelin over-expression

would allow us to analyze the direct effects of ghrelin on site, possibly confirming our *in vitro* observations.

To obtain the heart-specific over-expression, we cloned the murine ghrelin cDNA under control of the promoter sequences of the β-MHC 3' UTR and the first three exons of the α-MHC, a cardiac isoform; the backbone plasmid was kindly provided by Dr. Marika De Acetis (Department of Genetics, Biology and Biochemistry, University of Torino). The construct obtained was microinjected in FVB donor oocytes that were then implanted in a pseudopregnant female. The procedures were carried out in the facility of Department of Genetics, Biology and Biochemistry of University of Torino in collaboration with Prof. F. Altruda and Dr. Sharmila Fagoonee.

On the other hand, to obtain the skeletal muscle-specific ghrelin over-expression we used a plasmid carrying the promoter and enhancer regions of the MLC1f (myosin light chain 1f) kindly provided by the laboratory of Prof. G. Cossu, (San Raffaele Institute, SCRI, Milan). However, while we were planning the production of the skeletal muscle-specific construct, it was discovered that from the same pre-pro ghrelin peptide another mature peptide was obtained, obestatin (Zhang et al, 2005). As initially for obestatin was proposed a role opposed to ghrelin's, we decided to insert a stop codon in the pre-pro peptide cDNA, downstream ghrelin and upstream obestatin, in order to avoid obestatin over-expression. The modified cDNA was then inserted under control of the skeletal muscle-specific promoter and the obtained construct was microinjected as described above.

We identified the founders of both transgenic mice lines performing genomic PCR specific for the transgenes: we got 5 founders for both transgenics.

Mating the founders with wild-type littermates we obtained the F1 and from heterozygous in the F1 we then obtained homozygous in the F2. We distinguished homozygous from heterozygous by semi-quantitative PCR of the transgene. We decided to carry on three transgenic lines for each transgenic, should they show differences due to the localization of the transgene insertion.

The permanent transgenic lines are now established and we started to characterize them.

We verified the ghrelin over-expression in the heart of cardiac over-expressing transgenic mice compared to control wild type by Real Time PCR and we found out that the heart-specific construct was effectively inducing an increase of ghrelin expression in the cardiac tissue. Moreover, analyzing the transgenic mice over-expressing ghrelin in the heart we found out by ELISA assay that the levels of circulating ghrelin and des-acyl ghrelin were higher compared to the wild type controls, suggesting that the hormone was correctly processed and secreted. Interestingly, the levels of circulating IGF1 were increased as well, a feature we should carefully take in account for our future analysis. Moreover, transgenic mice over-expressing ghrelin in the heart showed a growth rate comparable to wild types, suggesting that GH levels should not be significantly affected by the increased production of ghrelin.

Furthermore, evaluating the ratio between weight of the heart and total weight of transgenic mice over-expressing ghrelin in the heart in comparison with wild type, we found out that it was significantly increased. Histological and molecular investigations on the cardiac tissue of transgenic mice over-expressing ghrelin in the heart are now undergoing.

In the future, our plan is to evaluate the ability of the transgenic mice over-expressing ghrelin in the heart to be more resistant to heart damage; the heart insult will be obtained by administration of cardiotoxins or by inducing an ischemia-reperfusion damage.

On the other hand, unfortunately, the analysis of the transgenic mice over-expressing ghrelin in the skeletal muscle revealed that the transgene was not able to induce the expected over-expression (verified by Real Time PCR on the skeletal muscle tissue and by ELISA assay to assess the circulating levels of the hormone). We suspect that this is due to the modification we introduced in the ghrelin cDNA, inserting a stop codon in the pre-pro sequence downstream ghrelin/upstream obestatin. It is possible that the modified pre-pro peptide can not be correctly processed by the proteases and is degraded in the cell.

In conclusion, the work on our *in vivo* transgenic models of ghrelin expression is still ongoing. However, at least for the transgenic mice over-expressing ghrelin in the heart, the results seem to be promising; hopefully, the cardiac damage experiments would finally enlighten the relevance of ghrelin as a cardio-protective factor.

5. A YEAR IN LONDON: LEARNING TO WORK WITH SATELLITE CELLS

5.1. Isolation of single fibres and satellite cells

By working with myoblasts and muscle cell lines, I developed a deep interest in such systems and in the therapeutical possibilities offered by new cell-based technologies. Thus, I decided to deepen my knowledge in the muscle field, with special regard to satellite cells.

In order to learn how to work with satellite cells, during the last year of my PhD, I moved to London to work in the laboratory of Dr. Peter Zammit, in the Randall Division at King's College University. Moreover, having shown the effects of GHR and des-acyl GHR on C2C12 myoblasts, the next step was to investigate a possible role in promoting differentiation and fusion on primary muscle precursors, e.g. satellite cells.

The laboratory of Dr. Zammit is at the frontline in studying satellite cells, their physiologic role, regulation and involvement in pathologies such as the muscular dystrophies. In this section I will introduce some of the techniques to analyze satellite cells that I learned in Dr. Zammi's laboratory.

One of the most interesting techniques to study satellite cells in a highly physiological condition is the isolation of the single fibres with their associated satellite cells. Single fibre isolation allows the analysis of the satellite cell in its own niche, a protected environment underneath the basal lamina surrounding the fibre itself.

The basic technique to isolate single fibres firstly requires dissecting the muscle of interest avoiding to damage the fibres. Working with mice, the mostly used muscles are the hind limb ones, in particular the extensor digitorum longus (EDL), the tibialis anteriore (TA) and the soleus. More in general, the procedure can be carried out on virtually every muscle that can be integrally dissected.

The animal has to be sacrificed and the muscle of interest should be recovered as soon as possible to avoid post-mortem modification. The freshly isolated muscle has to be placed in a collagenase solution for a time that varies depending on the muscle, but usually in the order of 1-2 hours.

Following the inactivation of the collagenase, the single fibres can be isolated from the partially digested muscle by the use of a Pasteur pipette with rounded borders. Gently pipetting is required to avoid any damage to the basal lamina.

After few isolation passages and washes, the single fibres can be maintained in culture for as long as five days in a culture medium (DMEM + 10% horse serum + 0,5% chick embryo extract) that induces activation, proliferation and finally differentiation of the associated satellite cells.

Single fibres can then be fixed at various time points and analyzed for the expression of specific markers to determine the state of the satellite cells.

Single fibres can also be fixed and analyzed before the activation of the satellite cell, to observe the quiescent state.

As already discussed in paragraph 2.2., quiescent satellite cells express the transcriptional factor Pax7 and several other markers that allow a clear distinction from the myonuclei. When the fibre is maintained in the activating medium (plating medium, PM), satellite cells do express MyoD by 6 hours and after 24 hours are virtually all MyoD positive. The activated satellite cells exit from the quiescence and enter the cell cycle; a massive proliferation follows. By 48h from activation, the satellite cells progeny has already doubled at least once.

At this point, a choice has to be made between terminal differentiation or self renewal. Cells undergoing terminal differentiation would down-regulate Pax7, maintain MyoD and express myogenin, while cells going back to quiescence would maintain Pax7, down-regulate MyoD and would not express myogenin. Expressing myogenin is an irreversible step in the differentiation process.

The single fibre isolation technique is extremely useful to analyze the first steps of activation and differentiation of satellite cells, but is not suitable to analyze the latest.

To allow the analysis of the terminal steps of satellite cell differentiation, i.e. fusion, the cells have to be isolated from the fibre. In order to isolate the satellite cells, the single fibres have to be plated on a Matrigel surface. After 48-72h the activated, proliferating satellite cells migrate from the fibre and adhere to the Matrigel surface, allowing the removal of the fibre.

Satellite cells can be then maintained in culture for days, although their proliferation rate would slowly decrease in time and they would spontaneously differentiate at last. Indeed, it is possible to plate the satellite cells at a high density while they are still massively proliferating and induce fusion by switching them to a poor mitogen medium (2% horse serum), similarly to C2C12.

5.2. Effects of ghrelin on satellite cells

The first aim of my research period in Dr. Zammit's laboratory was to learn how to isolate single fibres and satellite cells, to stain them and follow their differentiation process. Being involved in a number of different research project in Dr. Zammit's laboratory allowed me to develop the needed skills to work with satellite cells. Moreover, I gave my contribution to several studies, which resulted in publications (see next paragraphs).

The second aim was to set up preliminary experiments to evaluate a biological role, if any, of GHR and des-acyl GHR on the activation, proliferation and differentiation of satellite cells.

A first group of experiments was designed to evaluate a possible effect of GHR and des-acyl GHR when added to the complete Plating Medium (DMEM + 10% HS + 0,5% CEE). The fibres, isolated as described, were maintained in Plating Medium with or without GHR or des-acyl GHR and fixed at different time points (T24 hrs, T48 hrs and T72 hrs). GHR and des-acyl GHR were added at a concentration of 100 nM at the beginning of the culture and, in an independent experiment, also added again after 12, 36 and 60 hrs to evaluate whether an initial trigger was sufficient to exert any effect or if a high, constant concentration of the hormone in the medium was necessary. No differences were noticed among these two conditions.

In this experimental setting we observed no significative differences on the total number of cells (considered as the sum of all the Pax7+ve/MyoD+ve, Pax7-ve/MyoD+ve and Pax7+ve/MyoD-ve cells) at both 24 or 48 hrs from the beginning of the culture, while we observed an increase when GHR was added to the medium after 72 hrs. A slightly increase was also observed in presence of des-acyl GHR, but further experiments are needed to eventually confirm these

observations. Importantly, at 72 hrs, while the total number of cells was increased, there were no differences in the percentages of cells adopting divergent fates when the fibres were cultured in presence of GHR or des-acyl GHR in comparison to controls.

We then set out to investigate the effects of GHR and des-acyl GHR added to a sub-optimal plating medium, i.e. with reduced nutrients.

We progressively reduced the amount of HS and CEE in the Plating Medium to 50%, 25% and finally 10% of normal.

Reducing the PM's serum concentration up to 50% slightly reduced the proliferation rate of the cells and both GHR and des-acyl GHR had no significant effect when added at a 100 nM concentration to this sub-optimal medium as observed after 48 and 72h.

Reducing the Plating Medium serum concentration to 25% surprisingly led to a proliferation increase after 48 hrs and des-acyl GHR 1 μM, but not GHR 1 μM, further - but slightly - increased the proliferation rate. Moreover, after 72h in culture, while the proliferation rate of the sub-optimal medium itself decreased in comparison to the complete medium, a clear effect of des-acyl GHR, but again not GHR, was to promote commitment toward differentiation, seen as a significant increase in the pool of cells only expressing MyoD.

Further reducing the Plating Medium serum concentration to 10% allowed us to observe a reduction of the proliferation rate already after 48h in culture; moreover, a number of cells only expressing MyoD were present; the possible explanation for this phenomenon is that in such a sub-optimal medium the satellite cell pool is induced to undergo a precocious differentiation.

In this experimental condition the ability of des-acyl GHR to further promote the cells to undergo differentiation was more evident, as it is already clear after 48h, while GHR is not effective. After 72h in culture it appeared that both GHR and des-acyl GHR are able to reduce the proliferation rate in order to enhance commitment to differentiation.

From these preliminary results we concluded that neither GHR or des-acyl GHR seem to be strong candidate as satellite cells activators, as they are not able to exert any relevant influence when added to the complete Plating Medium. The ablity of des-acyl GHR of driving satellite cells toward differentiation in a sub-optimal nutrients condition could be interesting as the

effect is not masked by other factors in the serum, but further investigation would be required to fully understand a possible relevance in the satellite cell physiology.

6. INTRODUCTION TO: "CONTROL OF MYF5 ACTIVATION IN ADULT SKELETAL MYONUCLEI REQUIRES ERK SIGNALLING" (Perez-Ruiz A, Gnocchi VF and Zammit PS. Cell Signalling, 2007)

6.1. Myf5

Myf5, MyoD, Myogenin and Mrf4 are basic helix-loop-helix transcription factors and form the myogenic regulatory factor (MRF) family (reviewed in Tajbakhsh and Buckingam, 2000).

In the embryo, *Myf5*, *MyoD* and *Mrf4* can individually direct muscle cell fate from somatic multipotent muscle progenitor cells (MPCs), and mice triple mutant for these genes totally lack differentiated fibres and myoblasts, thus identifying them as the determination genes for this lineage (Kassar-Duchossoy, 2004).

In the mouse embryo, Myf5 is expressed around E8.0 and in its absence myogenisis fails to occur until MyoD is expressed at E10.5 (Braun et al, 1994).

Using an elegant transgenic approach, it has been shown that the Myf5 locus is active also in the adult within satellite cells. $Myf5^{nlacZ/+}$ mice have been obtained by inserting the lacZ reporter sequence in the first intron of Myf5, thus disrupting Myf5 gene but producing a fusion protein formed by the first 13 aminoacids of Myf5 and β-galactosidase (Tajbakhsh et al, 1996). Homozigous transgenic mice die shortly after birth, while heterozigous are viable and can be therefore used to investigate Myf5 expression and regulation in the adult.

The analysis of $Myf5^{nlacZ/+}$ mice revealed that, although Myf5 is active in nuclei within muscle fibres at birth, the gene is switched off during early postnatal development. Indeed, Myf5 is not expressed in myonuclei at 6 weeks of age, but remains active in satellite cells (Beauchamp et al, 2000).

The use of $Myf5^{nlacZ/+}$ mice is also a useful tool to follow the fate of transplanted satellite cells, as following transplantation the myonuclei derived from such cells would be β-galactosidase positive (Heslop et al, 2001).

Also, muscle denervation results in the activation of the Myf5 locus in the myonuclei (Zammit et al, 2004b).

6.2. Approach and results

Little is known about the intracellular pathways leading to Myf5 activation and the signals triggering them, despite its importance in determining the skeletal muscle identity, the detailed analysis of its expression through development and adult life and a number of studies to define its genetic regulatory elements.

In the work presented inhere, we set out to investigate the possible role of calcium in Myf5 activation, as well as the upstream and downstream signalling molecules involved.

As a sensitive tool to analyze the activation of Myf5, we used the myonuclear activation model of the *Myf5* locus in myofibres from heterozygous $Myf5^{nlacZ/+}$ mice.

Single fibres with their associated satellite cells were isolated from EDL muscles of transgenic mice and cultured in suspension in DMEM alone or in a rich medium, the Plating Medium (DMEM + 10% horse serum + 0,5% chick embryo extract). When single fibres were maintained in DMEM, only satellite cells (typically 7-10 per fibre) resulted β-galactosidase positive, while culturing them in Plating Medium for 24h resulted in a robust induction of the Myf5 locus in virtually all the myonuclei in the fibre.

Therefore, it was possible to investigate both induction of Myf5 activation, by giving treatments to the fibres maintained in DMEM, and inhibition of Myf5 activation, by acting on the fibres cultured in Plating Medium.

We first assessed the effects of intracellular calcium on Myf5 activation artificially raising Ca^{2+} levels by exposing single fibres maintained in DMEM to thapsigargin or ionomycin. The results obtained indicate that an increase in intracellular calcium results in activation of the Myf5 locus. To confirm the result, we treated myofibres maintained in Plating Medium with dantrolene, which blocks the release of Ca^{2+} from intracellular stores; indeed, the exposure to dantrolene inhibited the activation of Myf5. Similar results were obtained using EGTA, which depletes intracellular stores as well as prevents extracellular Ca^{2+} influx.

We then investigated if calmodulin, an important intracellular Ca^{2+}-interacting protein, was involved in Myf5 activation. The inhibition of the Ca^{2+}-

calmodulin interactions by use of W7 resulted in a significant reduction of the myonuclear activation of Myf5 in fibres cultured in Plating Medium.

Ca^{2+}-dependent activation of calmodulin does, in turn, lead to the activation of the calmodulin kinase and calcineurin (reviewed in Klee, 1991). Treating myofibres maintained in Plating Medium with the calmodulin kinase inhibitor KN-93 also reduced Myf5 myonuclear activation in a context normally inducing it, though not to the extent observed with W7. Similarly, the blocking of calcineurin by FK506 or Cyclosporine A inhibited myonuclear Myf5 activation to a lesser extent than W7. A well known downstream target of calcineurin is NFAT; inhibiting NFAT with Inca-6 resulted in a reduced Myf5 activation.

Although thapsigargin or ionomycin were able to revert the effects of FK506 on Myf5 activation, none of them was able to revert the effect induced by Inca-6, suggesting that the Ca^{2+}-dependent Myf5 activation is mainly achieved through calmodulin kinase than through calcineurin signalling.

We then investigated the involvement of ERK signalling downstream Ca^{2+} intracellular level increase by treating the fibres with the MEK1/2 inhibitor U0126. Inhibiting ERK resulted in a significant decrease in the myonuclear activation of Myf5 in fibres in Plating Medium. Such inhibition can be overcome only partially by the use of phenylephrine or isoproterenol that activate ERK signalling through G protein-coupled receptors. Moreover, phenylephrine or isoproterenol can induce myonuclear activation of Myf5 when the fibres are maintained in DMEM. Using specific agonists or inhibitors, we also show that PKA is not involved in Myf5 activation, thus confirming the observed effects were ERK-specific.

Inhibiting ERK while increasing the intracellular calcium levels in fibres in DMEM did not resulted in Myf5 activation, suggesting that ERK is downstream the Ca^{2+} in the signalling pathway. Decreasing cytosolic Ca^{2+} in the presence of ERK agonists in fibres in Plating Medium resulted in Myf5 activation. Moreover, ERK agonists could overcome the blocking of Ca^{2+}-calmodulin interactions but not the inhibition induced by suppressing calmodulin kinase activity, indicating that calmodulin kinase activity is necessary for ERK signalling. ERK signalling to activate Myf5 in myonuclei does not require NFAT, as ERK agonists can overcome NFAT-dependent inhibition of Myf5 activation.

P38 and JNK MAPKs involvement in NFAT-dependent activation of Myf5 was explored; both p38 and JNK can phosphorylate NFAT and block its translocation to the nucleus. Using specific inhibitors as well as agonists for both kinases, we demonstrate that their activity, thus NFAT phosphorylation, block Myf5 myonuclear activation.

Finally, we decided to briefly investigate possible factors triggering the Ca^{2+} increase in the myofibre and consequently Myf5 myonuclear activation. We identified in IGF1 a potent substitute for Plating Medium, while other candidates such as HGF or bFGF failed to trigger Myf5 activation.

EGTA-mediated calcium depletion, as well as NFAT inhibition, resulted in IGF1 failure to induce Myf5 activation. To dissect the involvment of the PI3K/GSK3β and the ERK pathway downstream IGF1 in inducing Myf5 activation, we used the PI3K inhibitor LY294002 and the ERK inhibitor U0126 to treat fibres maintained in DMEM and stimulated with IGF1. Our results indicate that IGF1 can activate Myf5 through both these pathways.

7. INTRODUCTION TO "MUSCLE SATELLITE CELL FATE CHOICE IS INFLUENCED BY β-CATENIN" (Perez-Ruiz A, Ono Y, Gnocchi VF and Zammit PS Journal of Cell Science, 2008)

7.1. Satellite cell fate choice

As repeatedly pointed out, satellite cells are the resident stem cells of the adult skeletal muscle and have the capacity to originate a progeny undergoing terminal differentiation and, at the same time, self-renew (Zammit et al, 2004a). The result of this highly regulated process is the maintenance of a small, quiescent, undifferentiated population after each round of intense proliferation and differentiation having the same properties of the original cells.

To date, little is known about the mechanisms underlying the satellite cell fate choice as well as the signalling molecules involved.

It has recently been shown that asymmetrical divisions, as well as symmetrical, are occurring during satellite cell proliferation following activation (Conboy et al, 2007; Kuang et al, 2007), suggesting a possible mechanism through which the progeny of a single satellite cell can adopt divergent fates. Asymmetrical divisions result in a different segregation of factors in the daughter cells, possibly influencing their following choices.

In investigating possible molecules involved in the fate choice, the Notch signalling pathway has been implicated (Conboy and Rando, 2002); in particular Delta-1, a well known Notch agonists, has been identified as one of the factors segregating with the daughter cell terminally differentiating (Kuang et al, 2007).

Wnt signalling has an important role in developmental myogenesis, with different Wnts able to activate muscle specific genes such as Myf5 and MyoD at various developmental stages in the embryo (reviewed in Parker et al 2003). Wnts are secreted signalling molecules which bind different Frizzled Receptors of the Serpentine family and Low-Density Lipoprotein Receptor-related protein family. Wnts act through distinct canonical and non-canonical pathway; the activation of the Wnt canonical pathway involves the stabilization of β-catenin and its subsequent translocation in the nucleus where it acts as coactivator of

the TCF/LEF transcription factors (reviewed in Willert and Jones, 2006) (Figure 6).

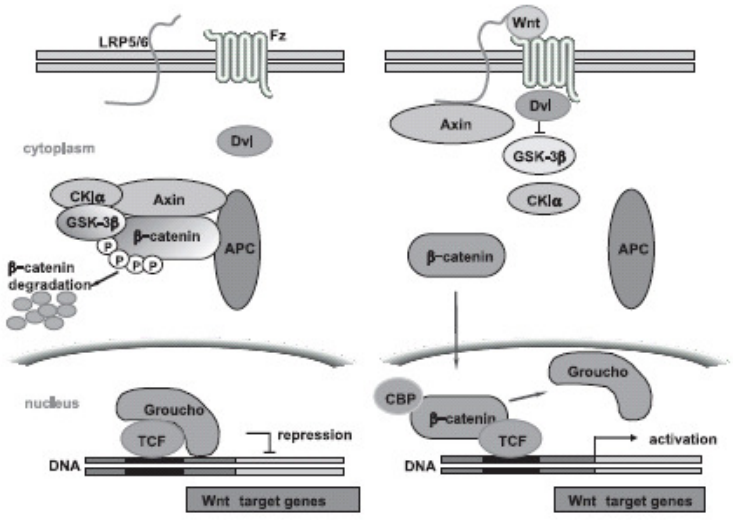

Fig. 6 The Wnt/β-catenin canonic pathway. Wnts, binding to their Frizzled receptors and their LRP5/6 co-receptor, sequestrate axin from the cytoplasm and activate Dvl which, in turn, phosphorylates and deactivates Gsk3β. In such a condition, β-catenin is not phosphorylated by the axin/Gsk3β complex and can thus enter the nucleus, promoting transcription acting as a co-activator for the TCF/LEF transcription factors (from Takahashi-Yanaga and Sasaguri, 2007).

Importantly, it has recently been demonstrated that Wnt activation of Myf5 in the somites involves the canonical pathway and thus β-catenin (Borello et al, 2006).

β-catenin was firstly discovered for its role in cell adhesion, specifically for being a key component of adherens junctions. In fact, β-catenin promotes cell adhesions by connecting the intracellular domain of cadherin to the actin cytoskeleton through the adaptor protein α-catenin (reviewed in Bienz, 2004).

β-catenin is thus a critical component of two important processes, cell adhesion and cell signalling. Because of its key role in these two distinct -but often linked- processes, not surprisingly a β-catenin de-regulation can lead to cancer in a variety of tissues such as lung or gut (Perez-Moreno, 2006).

7.2. Results

In the paper presented inhere we set out to investigate the role of β-catenin in the satellite cell fate choice between stemness and terminal differentiation.

β-catenin, as part of the Wnt canonical signalling pathway, has been implicated in the process of fate choice between stemness and differentiation in a variety of cellular systems, such as the hepatic stellate cells (Kordes et al, 2008) and murine embryonic stem cells (Anton et al, 2007).

Importantly, it has recently been shown that in aged mice the conversion of satellite cells from a myogenic to a fibrogenic lineage, a physiological effect of aging, is mediated by Wnt signalling, indicating once more the importance of this signalling pathway in the muscle stem cell fate choice (Brack et al, 2007).

First of all we investigated β-catenin expression in quiescent and activated satellite cells using the single fibre isolation technique. Indeed, β-catenin is expressed in satellite cells during myogenic progression and slightly variation in the cellular distribution were observed, from a more cytoplasmic distribution in freshly isolated satellite cells to a more superficial or peri-nuclear localization after 48-72h from activation.

To evaluate the effects of constitutive β-catenin expression in satellite cell fate choice, we produced retroviral constructs encoding wild-type β-catenin or a stabilized version as well as eGFP to follow the infection. The stabilized version of β-catenin has been modified with specific aminoacid substitution to prevent phosphorylation and subsequent degradation (Barth et al, 1999). We used the retroviruses obtained by packaging the constructs in 293T cells co-transfected with a helper virus to infect satellite cells associated to their single fibre, C2C12 cells and satellite cells isolated from the myofibre.

We followed the fate choice of the infected cells by immunostaining for Pax7, MyoD, myogenin and MyHC, as the maintenance of Pax7 together with the down-regulation of the other markers is a distinctive feature of stemness while the down-regulation of Pax7 associated to the expression of MyoD, myogenin and finally MyHC indicates terminal differentiation.

Results obtained in each of the above listed cellular systems clearly indicate that the constitutive over-expression of both wild-type β-catenin or its

stabilized version lead to an increased number of cells expressing Pax7 and failing to differentiate and fuse to form myotubes compared to the controls.

The same results were obtained by inhibiting the phosphorylation and subsequent proteasomal degradation of β-catenin. β-catenin phoshorylation, resulting in ubiquitination and degradation, is carried out by Gsk3β. Therefore, inhibiting Gsk3β by exposing the single fibres to SB216763, resulted in constitutive β-catenin activity.

We then decided to investigate whether β-catenin down-regulation would have the opposite effect. β-catenin levels were reduced using silencing RNA (*si-RNA*) transfection, as verified by Western Blot analysis. Plated satellite cells transfected with β-catenin *si-RNA* formed myotubes with a significantly higher fusion index and originated a significantly smaller number of Pax7 expressing cells compared to controls, confirming our hypothesis.

As next step we investigated whether the increased differentiation observed by reducing β-catenin levels was due to a reduction in its transcriptional activity, as β-catenin also acts through interaction with adhesion molecules such as members of the cadherin family. In order to uncouple the transcriptional from the non-transcriptional effects, we used a dominant negative construct encoding a fusion protein in which the activator domain of β-catenin was replaced by the Drosophila Engrailed Repressor Domain (ERD). The β-catenin ERD construct is no longer able to activate the transcription targets downstream β-catenin but is still able to associate with the cadherin complex, allowing to examine the two process separately (Montross et al, 2000).

Plated satellite cells infected with the β-catenin ERD retrovirus showed a higher fusion index compared to controls, indicating that the influence on satellite cell fate choice elicited by β-catenin is mediated by its transcriptional activity.

Established the ability of β-catenin to influence satellite cell fate choice through its transcriptional activity, it will be very interesting to identify the transcriptional targets downstream β-catenin itself to elucidate the molecular mechanism controlling such fascinating process.

PART II

8. DIACYLGLYCEROL KINASES: KEY ENZYMES IN A VARIETY OF CELL PROCESSES

8.1. Diacylglycerol kinases

Diacylglycerol kinases (Dgks) phosphorylate diacylglycerol (DAG) to obtain phosphatidic acid (PA). In the cell, DAG is obtained by inositol phospholipids and other phospholipids by the action of phospholipase C (PLC) and phospholipase D/PA phosphatase upon cell stimulation by a variety of agonists such as growth factors, cytokines, and so on (reviewed in Nishizuka, 1992).

To date, ten members of the Dgk family have been identified in mammals (Dgkα-θ); common features of all Dgks are two or three characteristic PKC-like C1 domains and the catalytic region, then they are classified in 5 different subgroups on the basis of the presence of different regulatory domains in their primary sequences (reviewed in Topham and Prescott, 1999) (Figure 7).

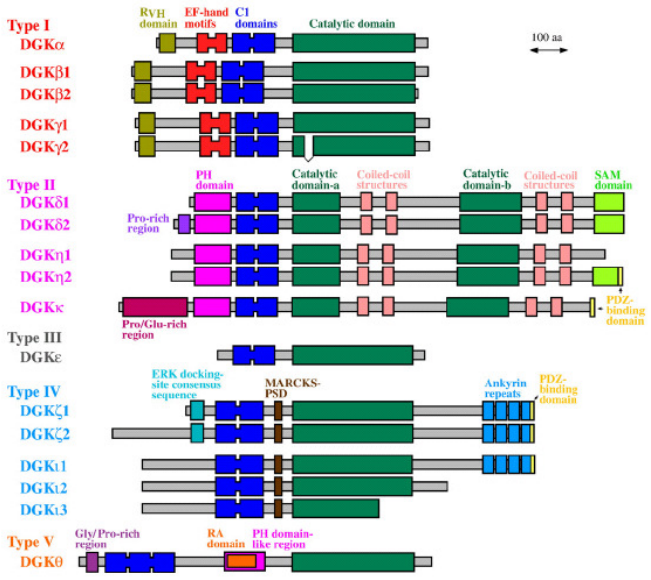

Fig. 7 Schematic rapresentation of DGK isozymes (from Sakane et al, 2007)

Moreover, a number of Dgks undergo alternative splicing, increasing the complexity of the Dgk landscape. Such a high number of different isoforms reflects the importance of a tightly balanced equilibrium between DAG and PA, as well as the fact that different Dgks have specific roles (reviewed in Mérida et al, 2008).

In fact, both DAG and PA are extremely important bioactive lipids, able to interact with a variety of other cell components and to exert a number of cellular functions such as neural and immune responses, cytoskeleton reorganization and carcinogenesis. Briefly, DAG regulates protein kinase C (PKC) isoforms, Ras guanyl nucleotide-releasing protein (RasGRP), chimaerins (Rac-specific GTPase-activating proteins or Ras-GAP), the transient receptor potential channel TRPC2 and other molecules. On the other hand, PA is involved in vesicle trafficking and modulates the activity of several enzymes as PIP-5-kinase, RacGAP, RasGAP, PKCζ, mTOR and many others (reviewed in Topham and Prescott, 1999). Thus, it is likely that Dgks act as DAG consumers as well as PA generators.

Due to the presence of different domains and the tissue distribution, individual Dgk isoforms act in isoform-specific complexes and exert different functions in a variety of cellular systems. Also to be taken in account are the regulatory sequences and the subcellular localization, both likely contributing to regulate Dgks function (reviewed in Sakane et al, 2007).

8.2. Dgkα

Dgkα belongs to the type I family of Dgks, characterized by a N-terminal recoverin homology domain and a pair of calcium binding EF-hand motifs (Sakane et al, 1990). Although the primary structures of the type I Dgks are quite similar to each other, recent studies highlighted that their physiological functions are markedly different.

Importantly, Dgkα is a cytosolic enzyme which associates to the plasma membrane upon growth factor stimulation (Flores et al, 1996).

T lymphocytes

Due to its abundance in lymphocytes, Dgkα has been widely studied in these cells. In particular, it has been shown that Dgkα negatively regulates T-lymphocytes activation. In fact, following T-cell receptor (TCR) antigen-mediated triggering, PLCγ1 is activated and the consequent PLCγ1-derived DAG production is essential for the localization and activation of several proteins involved in T-cell activation. To guarantee adequate control of signal intensity and duration, negative regulatory pathways have to be activated. Among them, the translocation of Dgkα to the cell membrane following TCR triggering has been shown to modulate PLCγ1-derived DAG level, so negatively regulating PKCθ and Ras signalling, by reversing PKCθ and RasGRP membrane translocation, respectively (Diaz-Flores et al, 2003; Sanjuan et al, 2003). Importantly, the translocation of Dgkα to the plasma membrane correlates with elevation of its enzymatic activity (Sanjuan et al, 2003).

The importance of Dgkα in anergy induction in T lymphocytes has recently been demonstrated *in vivo* using the knock out mouse model (Olenchock et al, 2006). Negative regulation of TCR-dependent signalling by Dgkα is relevant not only for effective T-cell primary response, but also for processes such as self-recognition, immune tolerance and autoimmune response. Each of these processes is characterized by the necessity of achieving T-cell clonal anergy and, accordingly, Dgkα has been identified as an anergy-induced gene (Macian et al, 2002).

Moreover, in T lymphocytes, in contrast with its negative role in the regulation of TCR-dependent activation, Dgkα-dependent PA production is necessary for IL-2-induced G1 to S transition (Flores et al, 1999). IL-2 promotes proliferation through the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway independently of PLCγ activation and Dgkα act as a positive regulator when activated T-cell are undergoing IL-2-dependent clonal expansion.

Epithelial and endothelial cells

Beside being expressed at high levels in lymphocytes, Dgkα is also expressed in endothelial and epithelial cells, fibroblasts and oligodendrocytes.

In the laboratory of Prof. Graziani the main research focus is on the study of Dgkα in endothelial and epithelial cells.

Importantly, Cutrupi et al. from Graziani's laboratory firstly demonstrated the involvement of Dgkα in tyrosine kinase receptor signalling in both endothelial and epithelial cells (Cutrupi et al, 2000). In particular, it has been shown that the binding of the c-Met receptor by its ligand HGF (hepatocyte growth factor) triggers the activation of Dgkα. Dgkα activation is achieved downstream HGF through its association with Src, a non-receptor tyrosine kinase. Such association results in the phosphorylation of Dgkα and its subsequent activation (Cutrupi et al, 2000).

Src is one of the 11 members of the Src-family of protein kinases (SFKs) in humans. SFKs are proto-oncogenes and play key roles in a variety of processes such as cell proliferation, survival and motility.

v-Src is the viral counterpart of Src; encoded by the avian cancer-causing oncogene of Rous sarcoma virus, v-Src is constitutively activated. Indeed, transforming fibroblasts and other cells with v-Src is a useful validated tool to investigate the effects of constitutive active Src on mammals cell lines.

Src contains an SH2 and an SH3 domains allowing interaction with a variety of proteins. Also, a myristylation sequence is present to allow its docking to the plasma membrane, as well as a protein tyrosine kinase domain (SH1). Src is regulated by autophosphorylation as well as by phosphorylation from others tyrosine kinases.

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

9. INTRODUCTION TO "DIACYLGLYCEROL KINASE-α PHOSPHORYLATION BY SRC ON Y335 IS REQUIRED FOR ACTIVATION, MEMBRANE RECRUITMENT AND HGF-INDUCED CELL MOTILITY" (Baldanzi G, Cutrupi S, Chianale F, Gnocchi VF, Rainero E, Porporato PE, Filigheddu N, van Blitterswijk WJ, Parolini O, Bussolino F, Sinigaglia F and Graziani A. Oncogene, 2008)

Despite the evidence that Dgkα was activated along the signalling pathway triggered by HGF binding to c-Met on epithelial and endothelial cells through a Src-mediated mechanism (Cutrupi et al, 2000), the details of such activation were missing for a long while. In the paper presented inhere, we now provide the molecular determinants for Src-mediated Dgkα activation and we highlight the importance of both phosphorylation and membrane recruitment as interdependent key steps in the process.

Fundamental tools for reaching our goals were the Myc-tagged Dgkα mutants we produced in order to separately analyze the importance of specific portion of the sequence in interacting with Src and in the phosphorylation process, as well as the relevance of membrane recruiting. First of all, we mutated tyrosines 60 and 335, as they were preceded by an isoleucine, suggesting substrate selection for Src (Songyang and Cantley, 1995), obtaining Myc-Dgkα-Y60F and Myc-Dgkα-Y335F, respectively. We also obtained two Dgkα mutants lacking the entire C-terminal half of the protein (Myc-Dgkα-STOP) or the last 13 aminoacids (Myc-Dgkα-ΔP); in fact, at its C-terminus, Dgkα contains a proline-rich sequence highly resembling the proline-rich motif allowing interaction of a number of molecules with the Src SH3 domain. Finally, we produced a Dgkα carrying a myristylation sequence (Myr-Dgkα) to investigate the effect of constitutive membrane recruitment.

As first important result, comparing the anti-phosphotyrosine (anti-PY) Western Blot of anti-Myc immunoprecipitates obtained by transfecting COS cells with Myc-Dgkα-wt, Myc-Dgkα-Y60F or Myc-Dgkα-Y335F it appeared that Y335 was the one phosphorylated by Src.

We then investigated whether Dgkα interacts with the SH2 and SH3 domains of Src, as a number of Src substrates do, by *in vitro* pull-down assays using immobilized GST-Src-SH2 or GST-Src-SH3 incubated with cell lysates of COS cells transfected with Myc-Dgkα-wt, Myc-Dgkα-Y60F, Myc-Dgkα-Y335F, Myc-Dgkα-STOP or Myc-Dgkα-ΔP. The results obtained from pull-down experiments indicate that Dgkα interacts with both SH2 and SH3 Src domains, and that interaction with Src-SH2 is dependent on Y335 phosphorylation while interaction with Src-SH3 is dependent on the C-terminus proline-rich region.

As next step we verified whether the proline-rich tail of Dgkα -and so the consequent association with the SH3 domain- was required for Src-mediated tyrosine phosphorylation. Indeed, the co-expression in 293T cells of the Dgkα mutants together with Src-Y527F, an activated form of Src, revealed that the proline-rich region is required for Src-mediated Dgkα phosphorylation, suggesting a mechanism in which Dgkα, in order to be phosphorylated and activated, has to physically interact with Src through docking at SH2 and SH3 domains.

We then set out to investigate whether the phosphorylation on Y335 is responsible for HGF-dependent activation of Dgkα; in fact, although the activation of Dgkα has been extensively demonstrated in response to HGF and other growth factors, the role of the phosphorylation has been elusive so far.

Using Dgk enzymatic assays we showed for the first time a direct correlation between Dgkα phosphorylation and its enzymatic activity; moreover, it was assessed that both Y335 and the prolin-rich domain are essential to the full enzymatic activation.

As Dgkα is a cytosolic enzyme recruited to the plasma membrane, where it encounter its substrate and elicit its function, we investigated the relationship between phosphorylation, activation and membrane recruitment. In order to analyze Dgkα subcellular localization we generated GFP-fused version of Dgkα-wt as well as mutated. By using the GFP constructs we confirmed that Dgkα-wt is mainly diffuse in the cytoplasm and associates with the membrane following HGF administration. Surprisingly, we found out that both GFP-Dgkα-Y335 and GFP-Dgkα-ΔP were restricted to a vesicular intracellular localization and failed to translocate to the plasma membrane upon HGF stimulation. These

results indicate that both Y335 and the proline-rich domain are required for Dgkα localization to the plasma membrane upon stimulation. Moreover, the peculiar localization in intracellular vesicle of both mutants, suggests that the recruitment of Dgkα to the membrane would occur through vesicular trafficking. To verify such hypothesis, we treated GFP-Dgkα-wt transfected cells with Brefeldin A, a specific inhibitor of vesicular traffic. Indeed, following Brefeldin A administration, GFP-Dgkα-wt accumulated in intracellular vesicles, confirming our hypothesis.

Furthermore, we investigated whether Y335 phosphorylation is a requirement to induce cell movement following HGF stimulation. In fact, we already demonstrated that Dgkα activity is necessary to transduce the HGF- and VEGF-mediated migratory signalling (Cutrupi et al, 2000; Baldanzi et al, 2004). Using a transwell chemotaxis quantitative assay to compare the migratory behaviour of Myc-Dgkα-wt- and Myc-Dgkα-Y335F-transfected COS-7 cells, we verified that Y335 phosphorylation is necessary to transduce the migratory signal, as the Y335F mutant is unable to promote migration in response to HGF.

Finally, transfecting the mutant carrying a myristylation sequence (Myr-Dgkα) in COS-7 cells, we showed that the constitutive localization of Dgkα to the plasma membrane is sufficient to induce a migratory phenotype in the absence of HGF stimulation.

10. CELL MIGRATION: A BRIEF OVERVIEW

10.1. Epithelial to mesenchimal transition (EMT) and two-dimensional cell movement

Epithelial cells are characterized by a well defined basal-apical polarization and have tight connections one to the other in order to collectively act as a barrier. Nevertheless, during embryonic development, tissue repair or through cancer progression, polarization and contacts are lost in a process known as epithelial to mesenchimal transition (EMT) (reviewed in Thiery and Sleeman, 2006) (Figure 8).

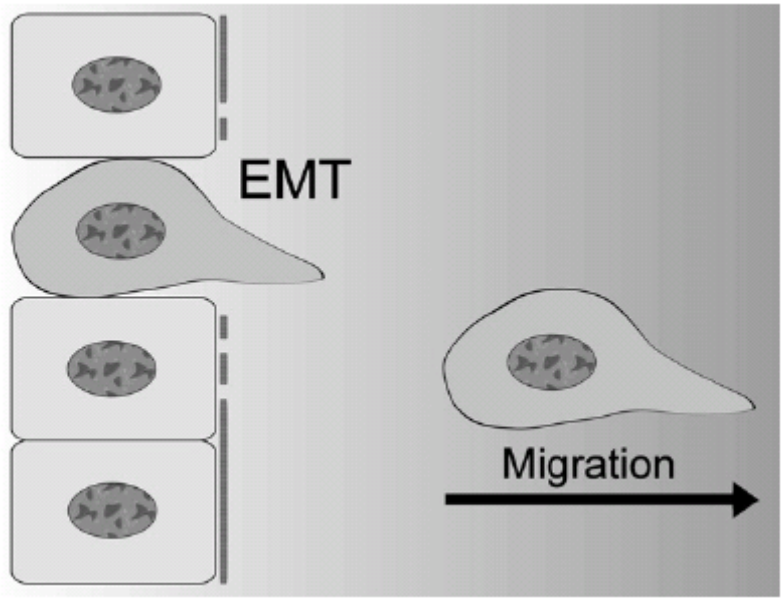

Fig. 8 A schematic representation of EMT: a polarized and tightly connected epithelial cell loses the cell-cell contacts and acquires a motile, amoeboid phenotype (From Guarino, 2007).

Cells undergoing EMT are highly motile and invasive, positive features in a developmental or healing context, but negative when tumoral cells are involved. Importantly though, the molecular mechanisms underlying cell migration seem to be common to both neoplastic and non-neoplastic cells,

indicating that, instead of the process itself, the misregulation of the process is responsible for cancer progression.

Epithelial cells cultured *in vitro* form polarized colonies; the dispersion of a colony following the loss of intercellular adhesions and the acquisition of a migrating phenotype, a process known as cell scattering, basically recapitulates the steps of EMT, allowing a detailed *in vitro* analysis.

Epithelial cell scattering can be triggered *in vitro* by growth factors or oncogenes activation. One of the most important growth factors inducing epithelial cell scattering is HGF (reviewed in Maulik et al, 2002).

In vitro, once cell-cell adhesions are lost and a motile phenotype is acquired, epithelial cell scatter randomly on the culture surface in the absence of a specific motogenic source.

Briefly, two-dimensional cell movement can be temporally and spatially regulated in four steps: 1) front-membrane protrusion, 2) adhesion of the protrusion to the substrate, implying the formation of new adhesion sites known as focal adhesions, 3) movement of the cell body and 4) retraction of the rear part of the cell. The front-membrane protrusions can be finger-like structures or sheet-like structures and are known as filopodia and lamellipodia respectively. Curling up lamellipodia are referred to as ruffles.

At a molecular level, both filopodia and lamellipodia are concentrated arrays of polar actin filaments; localized actin polymerization at the leading edge of these structures, as well as acto-myosin contractions, push the membrane forward (Small et al, 2002). At the rear end of the cell, the opposite process occurs, i.e. a regulated actin de-polymerization.

10.2. Molecular regulation of cell migration

The actin reorganization necessary to cell movement is a highly regulated process. The Rho family of small GTPases, including Rho, Rac and Cdc42, is the main regulator downstream growth factor-induced cell scattering.

Rho family small GTPases cycle between a GDP-bound inactive state in the cytoplasm to a GTP-bound active state in the plasma membrane, at the focal complexes, where they meet their interactors.

Rho family small GTPases activation and localization are strictly regulated by a number of molecules. In particular, guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP to GTP, thus promoting GTPases activation; GTPase activating proteins (GAPs) accelerate GTP hydrolysis, thus negatively regulating small GTPases activity; a negative regulation is exerted by GDP dissociation inhibitors (GDIs) as well, as they act as chaperones maintaining Rho small GTPases in the cytosol, bound to GDP.

Briefly, downstream Rho family of small GTPases the formation of filopodia and lamellipodia is obtained by monomeric actin polymerization by the Arp2/3 complex. The Arp2/3 complex is composed of 7 different proteins and is activated by WASP (Wiskott-Aldrich syndrome protein) family protein. WASP proteins, and particularly N-WASP, are the key link between phosphoinositides on the plasma membrane, the Arp2/3 complex and the Rho family of small GTPases (through direct binding to Cdc42). The Arp2/3 complex is also activated by interaction of Rac with Scar/WAVE proteins. The interaction between Rac and Scar/WAVE is not direct, but mediated by the adaptor protein Irsp53 (insulin receptor substrate protein 53 or IRS-58).

Other important effectors and direct interactors of Rac and cdc42 are members of the p21-activated kinase (PAK) protein family.

In the complex landscape of cell migration, transmembrane integrines are fundamental players. Integrins link the external extracellular matrix environment to the intracellular signalling. Moreover, they intracellularly bind modulators of actin dynamics, exerting an active role in remodelling processes.

In particular, integrins can activate the focal adhesion kinase FAK/Src complex of signalling proteins, including the regulator Paxillin.

Paxillin is a relatively small protein containing many protein-binding modules and is therefore classified as a molecular adaptor or scaffold protein. Its interaction with and subsequent phosphorylation by FAK, Src and other molecules lead to its activation and promotes its interaction with other proteins.

In conclusion, following growth factor stimulation of receptor tyrosine kinases or the engagement of integrins with the extracellular matrix, a number of regulatory proteins and adaptors are recruited to specific locations within the cell where they build finely regulated signalling complexes in order to reorganize the actin cytoskeleton and allow efficient cell migration.

11. INTRODUCTION TO "DIACYLGLYCEROL KINASE-α MEDIATES HEPATOCYTE GROWTH FACTOR-INDUCED EPITHELIAL CELL SCATTER BY REGULATING RAC ACTIVATION AND MEMBRANE RUFFLING" (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. Molecular Biology of the Cell, 2007)

In previous works from Graziani's laboratory we demonstrated that Dgkα activity is necessary to transduce the HGF- and VEGF-mediated migratory signalling through a Src-dependent mechanism (Cutrupi et al, 2000; Baldanzi et al, 2004). Moreover, we recently showed that both Dgkα phosphorylation on Y335 and its C-terminal prolin-rich domain are necessary to promote migration in response to HGF (Baldanzi et al, 2008).

In the paper presented inhere we investigated the role of Dgkα downstream HGF along the signalling pathway leading to cell scattering and epithelial cells migration.

As *in vitro* model for studying cell migration we used HGF- or v-Src-induced cell scattering on Madin-Darby canine kidney (MDCK) cells. To study the role of Dgkα activity in the molecular events eventually leading to cell scattering, we analyzed the effect of its inhibition by a variety of approaches: using a pharmacological isoform-specific Dgk inhibitor (R59949), expressing a kinase-defective dominant negative mutant (Dgkα-DN) or by transient expression of specific siRNA directed toward the Dgkα canine isoform.

We performed wound-healing and quantitative chemotaxis assays to analyze HGF- or v-Src-induced cell scattering and migrating ability in MDCK cells, comparing control cells to cells in which Dgkα was inhibited. The results obtained by these experiments clearly showed that Dgkα activity is required for HGF- and v-Src-induced cell scatter and migration, since its inhibition by any of the above mentioned methods resulted in significantly impaired movement.

As already briefly discussed, the process of cell scattering requires both the dissolution of the cell-cell contacts and the acquisition of the migrating

phenotype. Cell-cell contact loss is obtained by internalization of E-cadherins, which quickly follows the scattering triggering signal.

We investigated whether Dgkα activity was required upon HGF stimulation to internalize E-cadherins. Interestingly, inhibiting Dgkα activity by R59949 administration or by expressing Dgkα-DN, did not have any effect on the efficiency of E-cadherin internalization. On the other hand, colony spreading, lamellipodia formation and focal adhesion remodelling were severely affected, indicating that different signalling pathways are directing the coordinated processes leading to an efficient cell migration and that Dgkα activity is not involved in promoting cell-cell adhesions loss.

As next step, we set out to investigate the role of Dgkα activity in the early molecular rearrangements preluding to cell migration, i.e. the formation of ruffles and, more specifically, focal adhesion sites within them. The formation of ruffles and focal adhesions can be monitored by following F-actin re-organization and condensation at the ruffle edge, as well as by analyzing the accumulation of focal adhesion proteins such as FAK or Paxillin at the new adhesion sites.

We analyzed the distribution of F-actin and Paxillin by immunostaining in untreated, HGF-stimulated and Dgkα-activity-inhibited MDCK cells. Our results show that Dgkα activity is necessary to rearrange the actin cytoskeleton upon HGF stimulation and to form ruffles. Moreover, the accumulation of focal adhesion protein at the focal adhesion sites is highly impaired when Dgkα is inhibited. However, Paxillin phosphorylation on Y31 and Y118, required for Paxillin-mediated growth factor-induced migratory signals, is unaffected by inhibition of Dgkα.

These results strongly suggest that Dgkα activity is involved in the early phases of acting reorganization, ruffle and focal adhesions formation, as its inhibition is later translated in migration impairment.

Finally, we identified the small GTPase Rac as one of the molecules whose activation is induced downstream HGF through a Dgkα-dependent mechanism. In fact, conducing pull down assays using GST-PAK to purify active GTP-bound Rac, we found that inhibiting Dgkα by either R59949 administration

or by Dgkα-DN expression, caused a dramatic reduction in the levels of GTP-bound Rac obtained upon HGF treatment.

Moreover, as Rac activation and plasma membrane localization are closely related events, we verified by immunostaining and confocal microscopy analysis if Rac translocation was affected as well as its activation by Dgkα inhibition. Indeed, when Dgkα was inhibited, Rac failed to localize to the plasma membrane in response to HGF stimulation, confirming not only the strong relationship between its activation and its cellular localization, but underlying the importance of Dgkα in mediating the early molecular events conferring a migratory phenotype to epithelial cells.

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ORIGINAL ARTICLE

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

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Diacylglycerol (DAG) kinases (Dgk), which phosphorylate DAG to generate phosphatidic acid, act as either positive or negative key regulators of cell signaling. We previously showed that Src mediates growth factors-induced activation of Dgk-α, whose activity is required for cell motility, proliferation and angiogenesis. Here, we demonstrate that both hepatocytes growth factor (HGF) stimulation and v-Src transformation induce tyrosine phosphorylation of Dgk-α on Y335, through a mechanism requiring its proline-rich C-terminal sequence. Moreover, we show that both proline-rich sequence and phosphorylation of Y335 of Dgk-α mediate: (i) its enzymatic activation, (ii) its ability to interact respectively with SH3 and SH2 domains of Src, (iii) its recruitment to the membrane. In addition, we show that phosphorylation of Dgk-α on Y335 is required for HGF-induced motility, while its constitutive recruitment at the membrane by myristylation is sufficient to trigger spontaneous motility in absence of HGF. Providing the first evidence that tyrosine phosphorylation of Dgk-α is required for growth-factors-induced activation and membrane recruitment, these findings underscore its relevance as a rheostat, whose activation is a threshold to elicit growth factors-induced migratory signaling.
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Keywords: hepatocyte growth factor; Src; diacylglycerol kinase; tyrosine kinase; signal transduction; cell migration

Introduction

Diacylglycerol (DAG) kinases (Dgk), which phosphorylate DAG to generate phosphatidic acid (PA), comprise a family of 10 distinct enzymes, grouped in 5 classes each featuring distinct regulatory domains and a highly conserved catalytic domain preceded by two cysteine-rich C1 domains (Topham and Prescott, 1999). Recent evidence showed that α, ζ and θ Dgk isoforms are regulated by extracellular ligands and play a role in signal transduction (reviewed by van Blitterswijk and Houssa, 2000; Luo *et al*., 2003). Dgk-α is activated by several growth factors: vesicular endothelial growth factor (VEGF) and hepatocytes growth factor (HGF) in endothelial and epithelial cells (Cutrupi *et al*., 2000; Baldanzi *et al*., 2004), and interleukin (IL)-2 in T cells (Flores *et al*., 1999; Cipres *et al*., 2003). Both *in vitro* and *in vivo* experiments in knockout mice, showed that in T cells Dgk-α and -ζ regulate cell sensitivity to T-cell receptor (TCR) activation by negatively modulating the intensity and the kinetic of DAG-mediated recruitment of both RasGRP and protein kinase C (PKC)-θ (Jones *et al*., 2002; Zhong *et al*., 2003; Carrasco and Merida, 2004; Olenchock *et al*., 2006; Zha *et al*., 2006).

Conversely, we previously showed that inhibition of Dgk-α activity, obtained either pharmacologically or by expression of dominant-negative mutant or by RNA interference, impairs HGF-, VEGF- and anaplastic lymphoma kinase (ALK)-induced chemotaxis and proliferation in several cell types (Cutrupi *et al*., 2000; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005), as well as *in vitro* angiogenesis in endothelial cells (Baldanzi *et al*., 2004). Similarly in T cells, pharmacological inhibition of Dgk-α severely impairs IL-2-induced G1–S phase transition (Flores *et al*., 1999).

Activation of Dgk-α by tyrosine-kinase receptor and IL-2, requires Src-family tyrosine kinase activity and involves association of Dgk-α with either Src or Lck (Cutrupi *et al*., 2000; Cipres *et al*., 2003; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005). Furthermore, either pervanadate treatment of endothelial cells or constitutive activation of Lck in T cells result in tyrosine phosphorylation and activation of Dgk-α (Cutrupi *et al*.,

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2000; Cipres *et al*., 2003). Despite these data strongly suggest that Dgk-α is regulated by tyrosine phosphorylation, no tyrosine phosphorylation of Dgk-α had been detected upon stimulation with either HGF, VEGF, IL-2 or upon activation of the ALK receptor in different cell types (Cutrupi *et al*., 2000; Cipres *et al*., 2003; Baldanzi *et al*., 2004; Bacchiocchi *et al*., 2005).

Several evidences suggest that Dgk-α is activated upon its recruitment to the plasma membrane, through a mechanism requiring multiple steps. For instance, Cipres *et al*. (2003) showed that activation and recruitment of Dgk-α by IL-2 is mediated by binding to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and requires the C1 domains of Dgk-α. However, these authors suggested that the lipid-binding domain is masked in the three dimensional structure of Dgk-α, and that other molecular events, for instance calcium binding to the EF-hand domain, would unmask it (Sanjuan *et al*., 2001; Cipres *et al*., 2003).

Here, we identify Y335 and the proline-rich C-terminal sequence as the molecular determinants of Dgk-α responsible for: (i) its tyrosine phosphorylation and activation upon HGF stimulation or upon oncogenic Src expression, (ii) its recruitment to the membrane and (iii) its ability to transduce HGF chemotactic signaling. These results fully prove the biological relevance of tyrosine phosphorylation of Dgk-α in signaling pathways leading to cell migration elicited by growth factor or oncogenic Src.

Results

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

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

As optimal protein-substrate sequences for Src tyrosine kinase activity provides optimal consensus sequences for binding of SH2 domain of Src itself (Songyang *et al*., 1993; Songyang and Cantley, 1995), we decided to investigate the ability of Y335 of Dgk-α to mediate interaction with Src-SH2 domain in an *in vitro* pull-down assay. Immobilized GST-Src-SH2 fusion protein was incubated with cell lysates obtained from serum cultured COS cells transfected with either empty vector or Myc-Dgk-α wt or mutants. Myc-Dgk-α wt was pulled down by GST-SrcSH2, but not by GST alone, indicating that Dgk-α interacts with Src-SH2 domain (Figure 3a). The interaction between Dgk-α and the SH2 domain is specific, as the GST-Src-SH2 R175L mutant, unable to recognize the phosphorylated tyrosine (Yeo *et al*., 2006), does not interact with Myc-Dgk-α (Table 1). Furthermore, Myc-Dgk-α-Y335F, which shows a dramatically reduced phosphorylation upon coexpression with Src, fails to associate with GST-Src-SH2 in the pull-down assay, while Myc-Dgk-α-Y60F interacts with GST-Src-SH2 as well as Myc-Dgk-α wt. In summary, these experiments demonstrate that Src-SH2 domain interacts selectively with the phosphorylated Y335 of Dgk-α. The interaction of Dgk-α is not limited to Src-SH2 domain, as, at least *in vitro*, Dgk-α interacts also at similar or lower efficiency, with SH2 domains of Bruton's tyrosine kinase (Btk), c-phospholipase C (PLC)γ, Grb2 and Lck, but not with SH2 domains of Abl, n-PLCγ and p85n (Table 1).

As several Src substrates, such as p130Cas, become tyrosine phosphorylated upon interaction of their proline-rich motif with Src-SH3 domain (Pellicena and Miller, 2001), we verified whether Dgk-α interacts with Src-SH3 domain in a pull-down assay. Immobilized GST-Src-SH3 was incubated with cell lysates obtained from serum cultured COS cells, either control or expressing Myc-Dgk-α-wt or mutants. Myc-Dgk-α-wt and Myc-Dgk-α-Y335F were specifically pulled down by immobilized GST-Src-SH3, but not by GST alone (Figure 3b), indicating that indeed Dgk-α interacts with Src-SH3 domain. The interaction between Dgk-α and the SH3 domain is specific, as the GST-Src-SH3-D99N a SH3 mutant, which is impaired in poly-proline binding (Weng *et al*., 1995), does not interact with Dgk-α. Although Dgk-α does not contain a consensus sequence for SH3 interaction (PxxP), it features a highly conserved C-terminal proline-rich sequence (PMLMGPPPR, Figure 2). Thus, we generated two deletion mutants lacking respectively the entire C-terminal half of Dgk-α (Myc-Dgk-α-STOP) or the last 13 amino acids PPPRSTNFFGFLS (Myc-Dgk-α-ΔP). Both mutants were assayed in the GST-Src-SH3 pull-down assay. Figure 3b shows that both Myc-Dgk-α-ΔP and Myc-Dgk-α-STOP

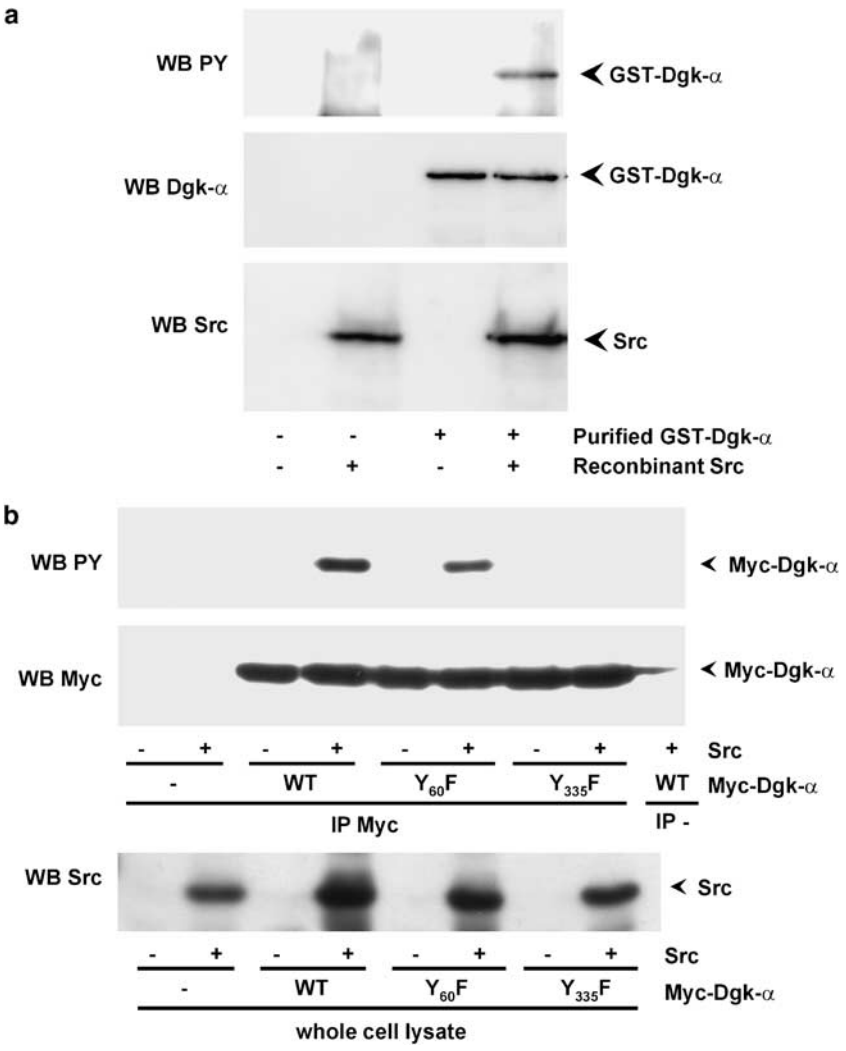

Figure 1 Dgk-α phosphorylation by Src on Y335. (**a**) Partially purified GST-Dgk-α or GST were incubated with or without recombinant Src in kinase buffer for 10 min at 30°C. Samples were split and analysed by western blot with antibodies against phosphotyrosine (upper panel), Dgk-α (middle panel) or Src (lower panel). (**b**) Growing COS-7 cells co-transfected with the indicated Myc-Dgk-α and Src constructs were lysed in detergent-containing buffer A. Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed by western blot with anti-phosphotyrosine (upper panel), and anti-myc antibodies (lower panel). Total cell lysates were analysed with anti-Src antibodies (right panel). Dgk, diacylglycerol kinase; GST, glutathione-*S*-transferase.

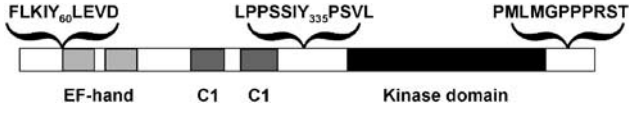

Figure 2 Dgk-α structure. The structure of Dgk-α contains three structurally defined domains: 2 EF-hand motifs, a double atypical C1 domain and a catalytic domain. Y60 lies upstream of the EF-hand motif, Y335 between the second C1 domain and the catalytic domain and the proline-rich region at the C-terminal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

mutants, different from Myc-Dgk-α-wt and Myc-Dgk-α-Y335F, are not pulled down by immobilized GST-Src-SH3 fusion protein. These data indicate that the proline-rich region is required for Dgk-α interaction with Src-SH3 (Figure 3a). The interaction of Dgk-α is not limited to the SH3 domain of Src, but SH3 domains of both Lck and Abl interact as well with Dgk-α (Table 1).

Based on the model proposed for tyrosine phosphorylation of p130Cas (Pellicena and Miller, 2001), we verified whether proline-rich tail of Dgk-α is required for Src-mediated tyrosine phosphorylation. We coexpressed in 293 T cells Myc-Dgk-α either wt, ΔP or Y335F with Src-Y527F, an activated form of Src. Tyrosine phosphorylation of Myc-Dgk-α in anti-myc immunoprecipitates was assayed by anti-phosphotyrosine western blot. Figure 4 shows that Myc-Dgk-α-ΔP and Myc-Dgk-α-Y335F mutants are not tyrosine phosphorylated upon coexpression with Src-Y527F, while Myc-Dgk-α-wt is tyrosine phosphorylated. Anti-myc and anti-Src western blots confirm uniform expression of transfected proteins, either wt or mutant.

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

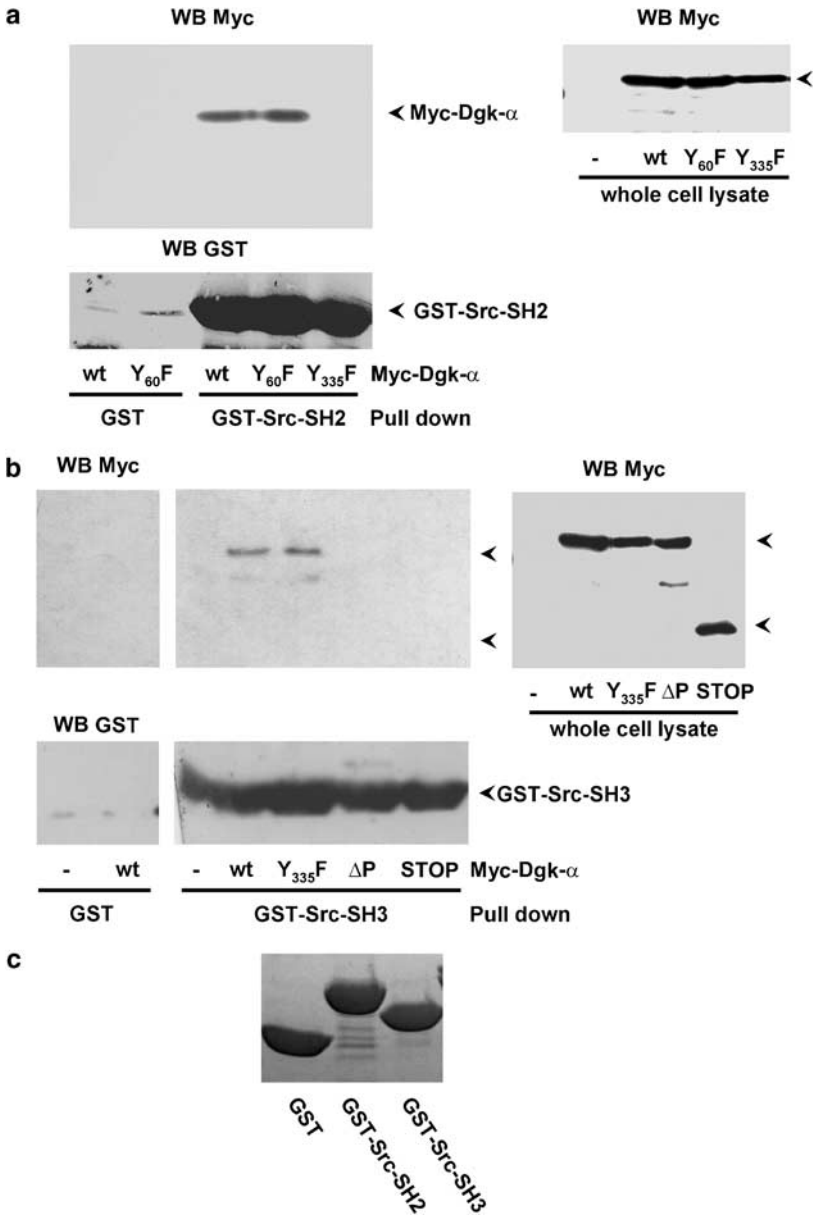

Figure 3 Dgk-α interaction with Src-SH2 and Src-SH3 domains. (**a**) Growing COS-7 cells, transfected with indicated Myc-Dgk-α constructs, were lysed in buffer A. Cell lysates were incubated with agarose-bound purified GST or GST-Src-SH2 for a pull-down assay. Pulled down Myc-Dgk-α (left panel) and Myc-Dgk-α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

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

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

The data presented so far clearly indicate that Y335 and the pro-rich C-terminal sequence of Dgk-α are the major determinants for its Src-mediated tyrosine phosphorylation, and provide the reagents to investigate whether phosphorylation of Y335 is required for Src- and HGF-induced enzymatic activation of Dgk-α. Indeed, while several evidence have firmly showed that activation of Dgk-α by growth factors depends on Src family tyrosine kinases, the putative role of its tyrosine phosphorylation in growth factor-induced enzymatic activation has been elusive (Cutrupi *et al*., 2000; Cipres

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

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

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

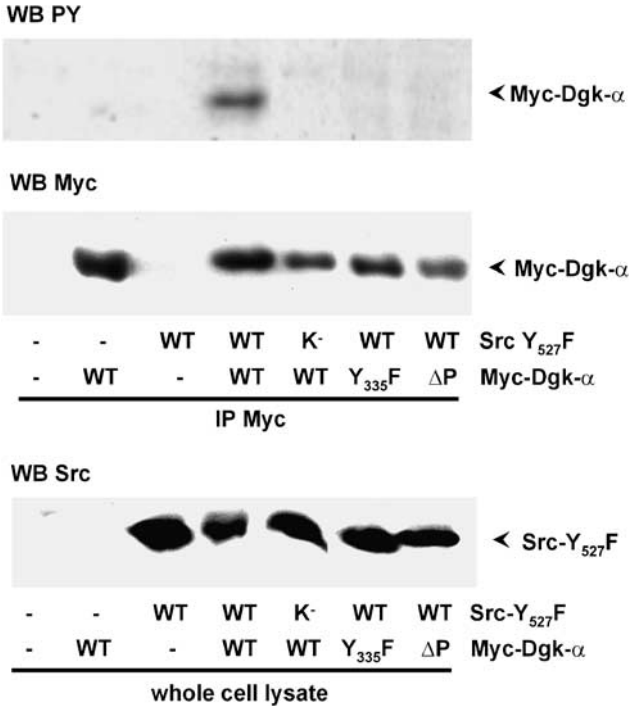

Figure 4 Dgk-α phosphorylation by Src requires Y335- and proline-rich C-terminal sequence. Growing HEK 293T co-transfected with the indicated Myc-Dgk-α and Src-Y527F constructs were lysed in detergent-containing buffer A. Myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed by western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

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

The enzymatic activity of Myc-Dgk-α either wt, Y335F or ΔP, were assayed upon co-incubation with Src, in an *in vitro* activation assay performed with crude lysates obtained from either Src- or Dgk-α-transfected cells. Through this assay, we had previously shown that enzymatic activity of Myc-Dgk-α wt is significantly increased upon co-incubation with Src cell lysates (dark column), as compared with control lysates (white columns) (Cutrupi *et al*., 2000; Figure 5). Conversely, the enzymatic activities of either Myc-Dgk-α–Y335F or Myc-Dgk-α–ΔP mutant are not significantly stimulated upon co-incubation with Src *in vitro* (Figure 5). This finding provides the first direct demonstration that both Y335 and proline-rich sequence are required for activation of Dgk-α *in vitro*.

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

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

To provide further evidence for the role of Y335 and proline-rich sequence as major determinants of Src-mediated activation of Dgk-α in intact cells, we investigated tyrosine phosphorylation and activation of Myc-Dgk-α either wt, Y335F or ΔP in transiently transfected Madin–Darby canine kidney (MDCK)-*ts*-v-Src epithelial cells (Figure 7). In these cells, *ts*-v-Src tyrosine kinase activity is impaired at 40°C, and is activated upon shifting the cell culture to 35°C (Behrens *et al*., 1993). Under these conditions, differently from COS and 293T cells, Myc-Dgk-α is expressed at low level, and it does not significantly affect total Dgk activity assayed in whole-cell lysates (Figure 7b).

Shifting MDCK-*ts*-v-Src cells to the permissive temperature results in both tyrosine phosphorylation (Figure 7a) and enzymatic activation (Figure 7b) of Myc-Dgk-α wt, as evaluated respectively by anti-phosphotyrosine western blot of anti-myc immunoprecipitates and *in vitro* Dgk-α assay. Next, we verified whether v-Src induces tyrosine phosphorylation and stimulates enzymatic activity of both Myc-Dgk-α–Y335F and Myc-Dgk-α–ΔP. Activation of *ts*-v-Src fails to induce tyrosine phosphorylation of both Myc-Dgk-α−Y335F and Myc-Dgk-α–ΔP (Figure 7a), and fails to stimulate their enzymatic activity (Figure 7b). Expression of both mutants is comparable to the wild type (Figure 7).

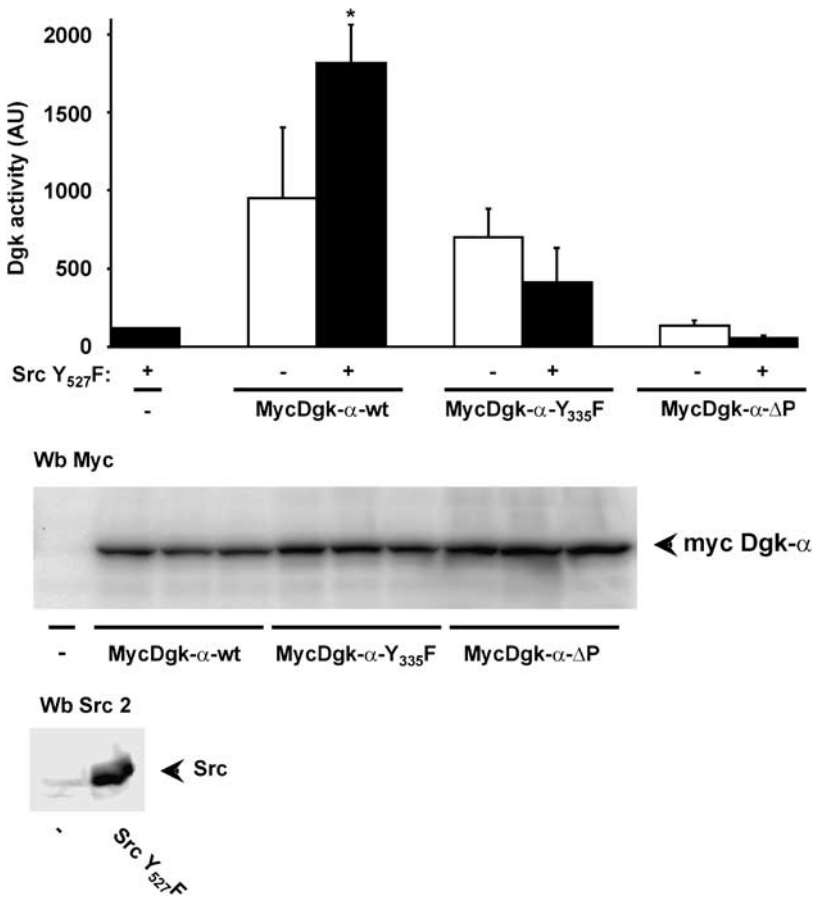

Figure 5 Dgk-α activation by c-Src *in vitro* requires Y335- and proline-rich C-terminal sequence. COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP or Src were homogenized with buffer B in absence of detergent. Cell extracts were mixed as indicated in presence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

In summary, these results, providing the first evidence *in vivo* that Dgk-α is a target of oncogenic Src, demonstrate that Src regulates Dgk-α *in vivo* through phosphorylation of Y335. In addition, as both enzymatic activation and tyrosine phosphorylation of Dgk-α depend on its proline-rich sequence, these data suggest that interaction of Dgk-α proline-rich sequence with Src-SH3 domain is a prerequisite for its phosphorylation and enzymatic activation.

Y335 and proline-rich C-terminal sequence are required for HGF-induced membrane recruitment of Dgk-α

As Dgk-α is a cytosolic enzyme which associates to the plasma membrane upon growth factor stimulation (Flores *et al*., 1996; Sanjuán *et al*., 2003), we investigated whether phosphorylation of Dgk-α on Y335 regulates its recruitment to the membrane upon HGF stimulation. To address this question, we investigated the subcellular localization of GFP tagged Dgk-α wt, Y335F and ΔP mutants, transiently transfected in MDCK cells. We observed that in most of control transfected cells, GFP-Dgk-α wt is localized exclusively in the cytosol, and that upon HGF stimulation it translocates at the plasma membrane in the majority of transfected cells (70%) (Figures 8a and 9c). In addition, the kinase dead mutant (GFP-Dgk-α-k−) behaves as the wild type, being diffuse in the cytoplasm in control cells and associates to the plasma membrane in HGF-stimulated cells (Figure 8b). HGF-induced membrane recruitment was dependent on Src activity, as it was reduced of 50% by pharmacological inhibition of Src with 10 μM PP2 (Figure 8).

To verify whether tyrosine phosphorylation of Dgk-α mediates HGF-induced membrane recruitment of Dgk-α, we investigated the subcellular localization of both Y335F and ΔP mutants. Surprisingly, in most of control-transfected cells, GFP-Dgk-α-Y335F is associated to intracellular vesicles. Similarly, GFP-Dgk-α-ΔP is also associated to intracellular vesicles, albeit of different shape and size, in all transfected cells. Upon HGF stimulation, neither mutant translocates at the plasma membrane, while their vesicular localization is not affected (Figure 9).

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

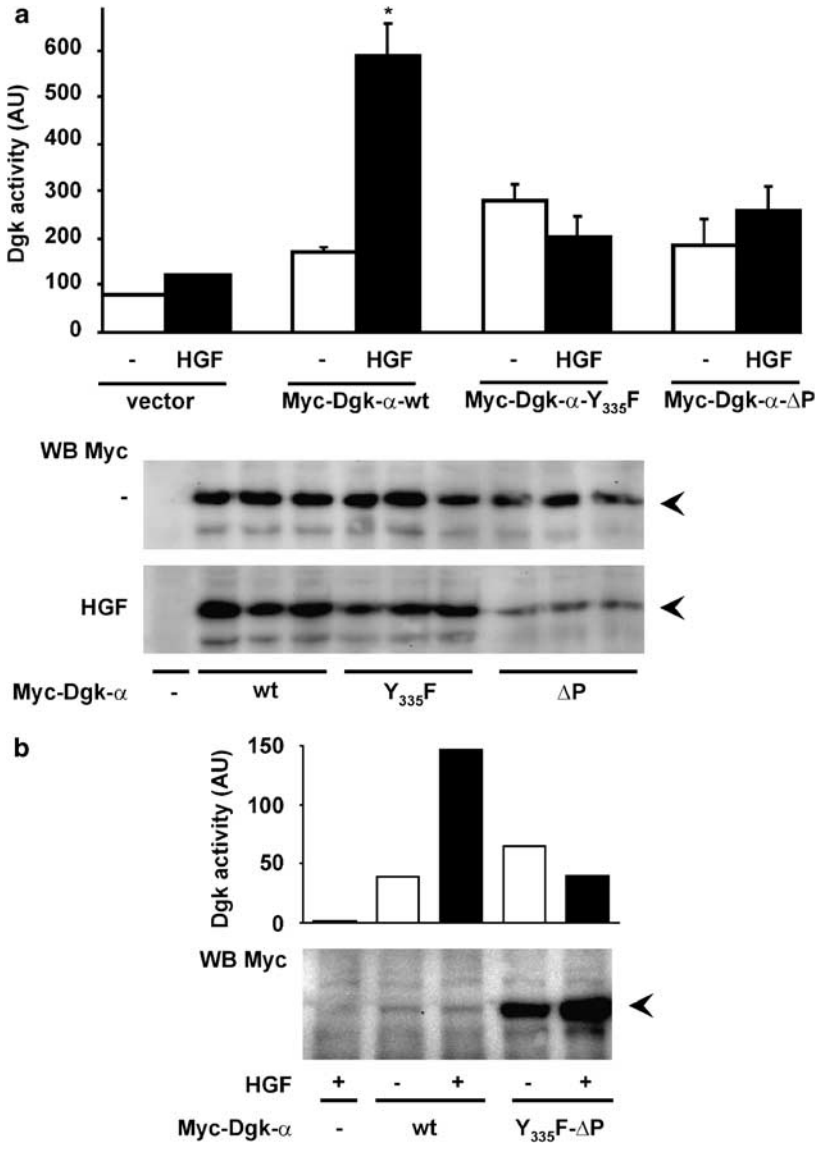

Figure 6 Dgk-α activation by HGF *in vivo* requires Y335- and proline-rich C-terminal sequence. (**a**) COS-7 cells transfected with either empty vector, Myc-Dgk-α wt, Myc-Dgk-α-Y335F, Myc-Dgk-α-ΔP were stimulated with HGF (100 μg/ml, 15 min), homogenized with buffer B in absence of detergent and analysed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

that the recruitment of Dgk-α to the plasma membrane may occur through vesicular traffic. If this holds true, we should expect that specific inhibition of vesicular traffic between the inner cytosol and the plasma membrane by Brefeldin A (BFA) treatment, would result in accumulation of GFP-Dgk-α-wt in intracellular vesicles (Lippincott-Schwartz *et al*., 1989). Indeed, upon 15 min of treatment with 10 μM BFA, even GFP-Dgk-α-wt associates to intracellular vesicles in unstimulated cells and fails to translocate to the membrane following HGF stimulation (Figure 10).

These observations strongly suggest that HGF-induced recruitment of Dgk-α to the plasma membrane depends on the integrity of the vesicular transport network, requires phosphorylation of Y335 by Src, but does not require its enzymatic activity.

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

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

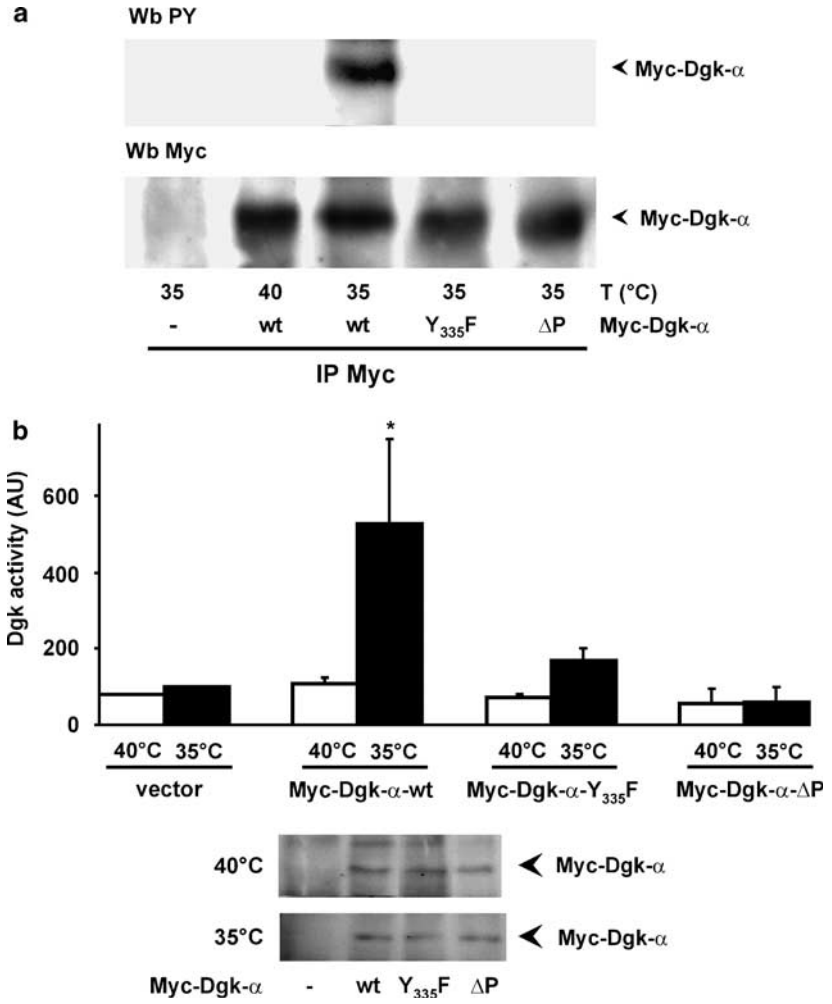

Figure 7 Dgk-α phosphorylation and activation by v-Src requires Y335- and proline-rich C-terminal sequence. Ts-v-Src/MDCK cells transfected with the indicated Myc-Dgk-α constructs, were cultured at nonpermissive temperature (40°C), and, where indicated, shifted at the permissive temperature (35°C) for 1 h. (**a**) After lysis in detergent-containing buffer A, myc-Dgk-α was immunoprecipitated with anti-myc antibodies and analysed by western blot with anti-phosphotyrosine antibodies (upper panel), and anti-myc antibodies (lower panel). (**b**) Cells were homogenized in buffer B, not containing detergent, and homogenates were assayed for Dgk activity (upper panel). Values are mean ± s.e. of triplicates (*t-test, $P<0.05$). Myc-Dgk-α protein expression was verified by anti-myc western blot (lower panel). Dgk, diacylglycerol kinase; MDCK, Madin–Darby canine kidney.

does not stimulate chemotaxis of COS-7 cells, transient overexpression of Myc-Dgk-α-wt makes COS-7 cells able to migrate in response to HGF in a transwell chemotaxis quantitative assay (Figure 11a). This observation provides a functional assay to verify the requirement for phosphorylation of Y335 to transduce HGF-induced migratory signaling. Figure 11 indicates that the expression of Myc-Dgk-α-Y335F mutant impairs HGF-induced motility of COS cells, as compared with wild type. These data lend further support to the hypothesis that activation and membrane recruitment of Dgk-α, occurring through its phosphorylation on Y335, are required for HGF-induced migratory signaling.

Next, we asked whether Dgk-α constitutive recruitment to the plasma membrane provides sufficient signaling to stimulate cell motility. Sanjuan *et al*. (2001) had previously shown that myristylated Dgk-α is constitutively active and associated to the plasma membrane. Transient expression of myr-Dgk-α in COS cells, enhances threefold spontaneous migration of serum-starved COS cells in absence of HGF in transwell chemotaxis assay and enhanced spontaneous cell migration in a wound healing assay (Figures 11b and c). These observations carried out in two different migration assays indicate for the first time that constitutive activation of Dgk-α at the cell membrane provides rate limiting intracellular signals, both necessary and sufficient to stimulate cell migration.

Discussion

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

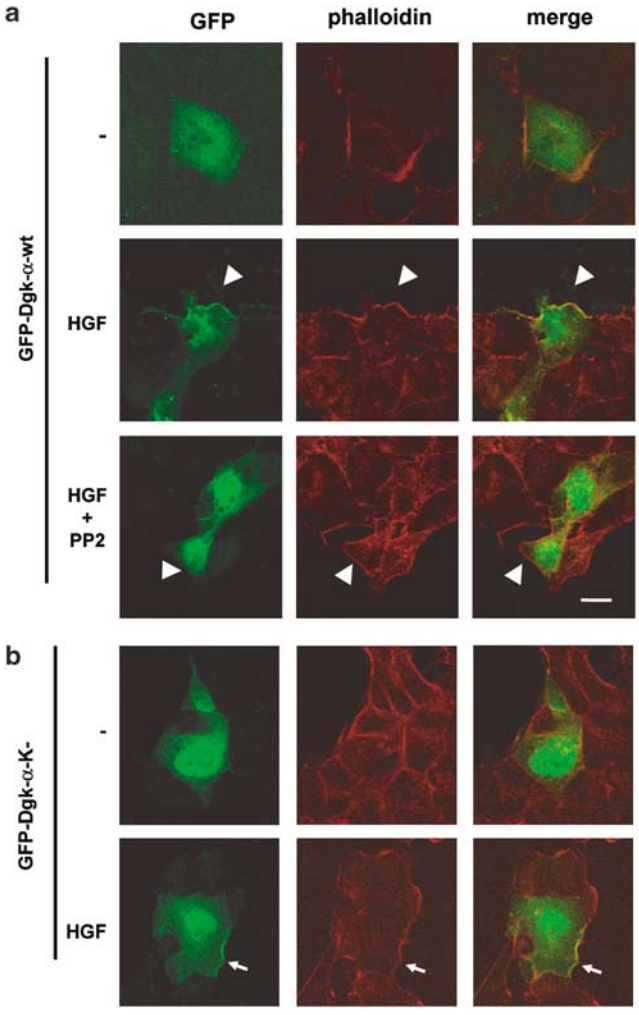

Figure 8 Dgk-α is recruited at cell membrane upon HGF treatment. MDCK cells transfected with GFP-Dgk-α wt or GFP-Dgk-α-K− were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with PP2 (10 μM PP2 for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal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

through Src-family tyrosine kinases, although the significance of tyrosine phosphorylation for its growth factors-induced enzymatic activation, translocation to the plasma membrane, and for its role in growth factors cell signaling, has not proved yet.

Dgk-α contains at least two conserved tyrosine residues, Y60 and Y335, both featuring Ile in −1 position, a signature for putative Src substrates (Schmitz *et al*., 1996). By phenylalanine substitution of either one of the two tyrosines, we showed that Y335, rather than Y60, is the major site of phosphorylation upon coexpression of Dgk-α with Src or v-Src, and is responsible for the association of Dgk-α with Src-SH2 domain. However, our data cannot rule out that upon phosphorylation of Y335, Dgk-α may be then phosphorylated on other sites. The substitution of Y60, differently from Y335, does not affect either Src-induced tyrosine phosphorylation of Dgk-α, either its ability to interact with Src-SH2 domain. These observations suggest either that Y60 is not a phosphorylation site of Dgk-α, or that its phosphorylation is secondary to Y335 occurring at lower stoichiometry.

The observation that C-terminal proline-rich sequence of Dgk-α is required for its interaction *in vitro* with Src-SH3, suggests that such interaction may participate in the mechanism leading to its phosphorylation by Src. Indeed, we showed that the proline-rich sequence is required for phosphorylation and activation (see below) of Dgk-α by Src and HGF, both *in vitro* and in intact cells. These data are highly consistent with the current model for the interaction of Src with its targets, such as p130Cas (Kanemitsu *et al*., 1997; Scott and Miller, 2000). According to this model, Src would first interact with Dgk-α through its SH3 domain, and then it would phosphorylate it on Y335. Subsequently, phosphorylated Y335 would become a docking site for Src-SH2 domain, and may lead to the stabilization of the Dgk-α/Src complex, and eventually to the phosphorylation of multiple secondary sites, providing additional docking sites for SH2-containing proteins. Alternatively, phosphorylation of Y335 itself, may allow interaction of Dgk-α with other SH2-containing proteins.

Phenylalanine substitution of Y335 abrogates both HGF- and v-Src-induced activation of Dgk-α in intact cells, while it does not affect its basal activity. In addition, even deletion of proline-rich sequence of Dgk-α, which impairs its tyrosine phosphorylation, significantly reduces enzymatic activation without affecting its basal activity. Both these observations support our conclusion that phosphorylation of Y335 dictates the ability of Dgk-α to be stimulated by both growth factors and v-Src activation.

Y335 lies in a linker sequence between the second C1 and the kinase domain, which according to the surface exposition plot (http://scansite.mit.edu/), features high surface accessibility. We may speculate that phosphorylation of Y335 acts as a molecular switch, which by unfolding an intramolecular interaction, shifts Dgk-α toward an open active configuration and/or a configuration able to interact with an activator. A similar model has been demonstrated for growth factors-induced activation of Raf-1, whose activity is stimulated by Src-mediated phosphorylation of Y340, which, similar to Y335of Dgk-α, is placed in a linker region between the C1 domain and the catalytic domain (Mason *et al*., 1999; Tran and Frost, 2003).

Activation of soluble enzymes acting on lipid substrates is tightly coupled to their recruitment to the membrane, where they encounter their substrates as well as their regulators. Phosphatidylinositol (PI) 3-kinase, PLC-γ, PI4P 5-kinase and phospholipase D are mostly cytosolic proteins which are recruited to the membrane through their ability to interact with tyrosine phosphorylated receptors, membrane-bound small GTPases, lipids and other membrane-associated scaffolding proteins (Santarius *et al*., 2006). The data presented here clearly demonstrate that both Y335- and proline-rich

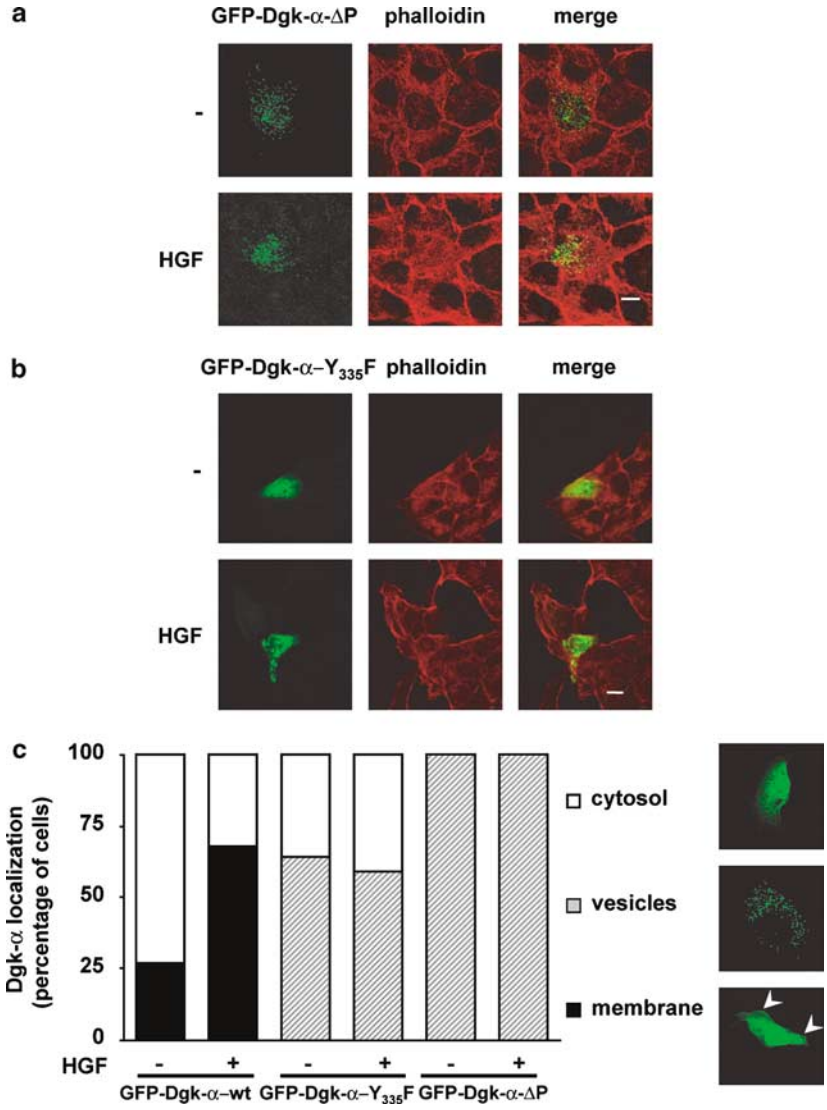

Figure 9 Recruitment of Dgk-α at cell membrane requires Y335- and proline-rich C-terminal sequence. MDCK cells tranfected with either GFP-Dgk-α-wt, GFP-Dgk-α-ΔP (**a**) or GFP-Dgk-α-Y335F (**b**) were stimulated with HGF (50 ng/ml, 15 min). Cells were stained with phalloidin-TRITC and images acquired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

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

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

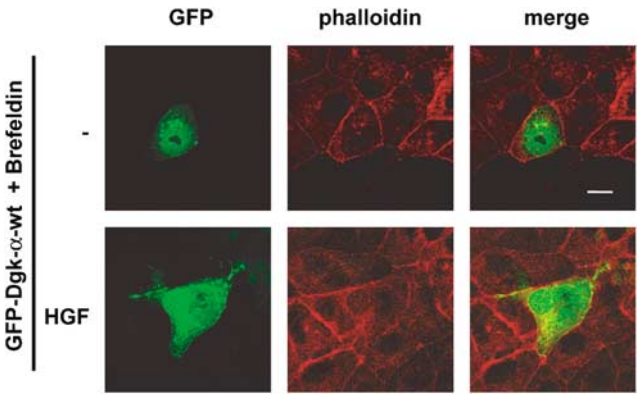

Figure 10 Brefeldin causes accumulation of Dgk-α on cytoplasmic vesicles. MDCK cells transfected with GFP- Dgk-α wt were treated with HGF (50 ng/ml, 15 min). Where indicated cells were pre-treated with BFA (10 μM BFA for 15 min). Cells were stained with phalloidin-TRITC and images acquired by confocal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

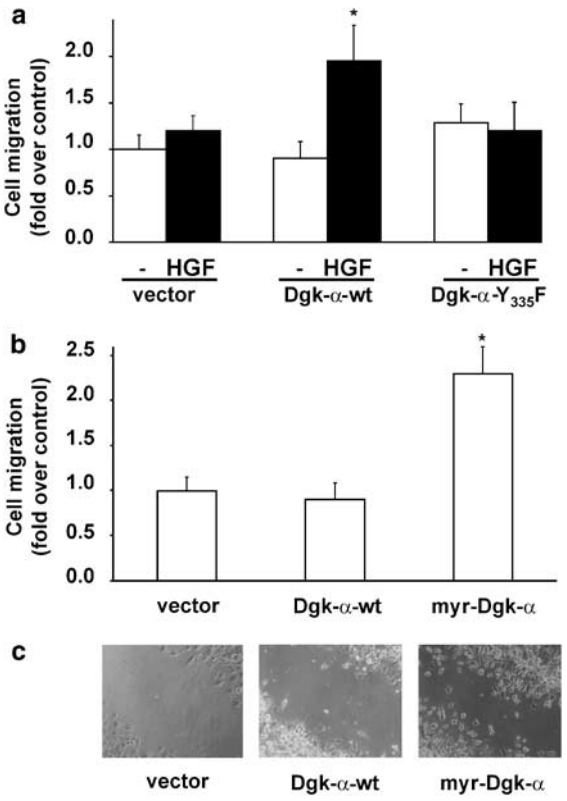

Figure 11 Membrane recruitment of Dgk-α is necessary and sufficient for cell motility. (**a**) COS cells transfected with either Dgk-α wt, or Y335 mutant or empty vector were stimulated to migrate by HGF (100 ng/ml) in a transwell chemotaxis assay. Data are expressed as fold increase over control, values are mean ± s.e. of four independent experiments (*paired *t*-test, $P = 0.07$). (**b**) Spontaneous cell migration of COS cells transiently transfected with myr-Dgk-α in a transwell chemotaxis assay. Data are expressed as fold increase over control, values are mean ± s.e. of four independent experiments (*paired *t*-test, $P = 0.06$). (**c**) Spontaneous cell motility of COS cells transiently transfected myr-with Dgk-α was assayed in a wound healing assay. A representative field of multiple experiments is shown. Dgk, diacylglycerol kinase; HGF, hepatocytes growth factor.

when the protein is still active, suggest that transient phosphorylation of Y335 would act as a switch allowing the direct interaction of atypical C1 domain with either a membrane protein or lipid. This model is consistent with our finding that activation of Dgk-α *in vitro* by Src requires the presence of intact membranes, as it does not occur by co-incubating the two purified proteins (data not shown). Moreover, according to this model, activation of Dgk-α would generate a coincidence signal derived from time- and space-co-incidence of two independent signals, Src activation and a still unidentified membrane signal, either lipidic or proteic.

Alternatively, the surprising observation that both Myc-Dgk-α-Y335F and Myc-Dgk-α-ΔP mutants are associated to intracellular vesicles rather than being diffuse in the cytosol, may suggest that they are mislocalized and segregated from Src, resulting in defective tyrosine phosphorylation. However, different from the wild type, neither mutant is activated by Src in the *in vitro* assay with whole-cell extracts (Figure 3), and becomes tyrosine phosphorylated upon co-incubation with Src in an *in vitro* assay with purified recombinant proteins (data not shown). Moreover, in intact cells Src and Dgk-α mutants do not appear to be segregated from each other, as observed in immunofluorescence (data not shown). These observations make unlikely that the defective phosphorylation and activation of either mutant may depend on their mislocalization.

The inability of both Dgk-α mutants to be activated and/or tyrosine phosphorylated by Src, and their vesicular localization, might also depend on their putative misfolding. However, as both protein mutants feature a basal enzymatic activity, it is unlikely that their inability to be activated by Src, depends on their putative misfolding. Moreover, even wild-type Dgk-α localize to similar vesicles upon cell treatment with low doses of BFA (Figure 10; see below).

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

Intriguingly, upon growth factor stimulation, Src itself is recruited from the perinuclear area to the plasma membrane through Rab11-dependent endosomal traffic (Sandilands *et al*., 2004). Based on these observations, we may speculate that SH3- and SH2-mediated interaction with Src, may couple Dgk-α to the endosomal traffic machinery responsible for Src targeting from the perinuclear region to the plasma membrane. This speculation is consistent with previous data reporting arachidonate-induced association of Dgk-α to the Golgi in CHO cells

(Shirai *et al*., 2000), and that Dgk-α associates with the trans Golgi network and late endosomal compartments, regulating the secretion of FAS-L bearing lethal exosomes (Alonso *et al*., 2005). In addition, overexpression of Dgk-δ, bearing distinct regulatory domains from α-isoform, suppresses endoplasmic reticulum (ER) to Golgi traffic, and inhibits Golgi reassembly following BFA treatment and washing (Nagaya *et al*., 2002). However, the investigation of the role of Dgk-α in endosomal traffic and the characterization of intracellular vesicles associated to Myc-Dgk-α-Y335F and Myc-Dgk-α-ΔP mutant are beyond the scope of this communication.

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

This finding demonstrates that phosphorylation of Y335 is indeed required to transduce HGF chemotactic signaling and suggests that activation of Dgk-α may finely tune threshold signals coordinating the function of downstream targets.

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

Materials and methods

Cells culture

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

Reagents

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

Construction of expression vectors and site-directed mutagenesis

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

Y60F GGGAACATTATCCACTTCGAGGAAGATTTT CAGGAATTGCTG and CAGCAATTCCTGAAAATCTTC CTCGAAGTGGATAATGTTCCC;

Y335F GGCCAGGACACTGGGAAAGATGGAAGATG GAGG and CCTCCATCTTCCATCTTTCCCAGTGTCCT GGCC;

STOP GATGATTTAAATTAGAGCACCTCTGAGGCT and AGCCTCAGAGGTGCTCTAATTTAAATCATC;

K− CGGATTGGTGTGTGGTGACGACGGCACAGTA GGC and GCCTACTGTGCCGTCGTCACCACACACCAA AATCCG.

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

D99N AGTCCCGGACTGAAACGAACTTGTCCTTCA AGAAA and TTTCTTGAAGGACAAGTTCGTTTCAGTC CGGGACT.

R175L GAACCTTCTTGGTCCTGGAGAGCGAGACGA and GTCGTCTCGCTCTCCAGGACCAAGAAGGTT.

Transfection with plasmid vectors and stimulation

COS-7 AND HEK 293T cells were transiently transfected with Cell-Phect Transfection kit (Amersham-Pharmacia, Milan, Italy) using respectively DEAE-dextrane or calcium phosphate method. MDCK and MDCK-*ts*-v-Src were transfected by lipofectamine 2000 (Invitrogen). Cells were lysed after 48 h from transfection and expression of transfected protein verified by western blot. For HGF stimulation experiments, cells were serum starved for 16 h and then stimulated for 15 min with recombinant HGF (100 ng/ml). MDCK-*ts*-v-Src were made quiescent by culturing in 0.1% serum at the nonpermissive temperature of 40°C for 16 h and then switched to the permissive temperature of 35°C for 1 h. When inhibitors were

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

Preparation of cell lysates, homogenates, immunoprecipitation, western blotting
Cells were lysed in buffer A (25 mM Hepes (pH 8), 1% NP-40, 10% glycerol, 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 2 mM ethylene glycol-bis(beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM $ZnCl_2$, 50 mM ammonium molibdate, 10 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma)) (Lippincott-Schwartz *et al*., 1989). Cells homogenates were prepared by collecting the cells with a rubber scraper in buffer B (buffer A without NP-40), homogenizing them with a 23 G syringe (Sigma) and by spinning at 500 *g* for 15 min. Protein concentration was determined by the bicinchoninic acid method (Pierce, Milan, Italy) and equalized for each point using buffer. Immunoprecipitation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blots were performed as described previously (Lippincott-Schwartz *et al*., 1989). Western blot results were acquired and quantified with Versadoc system (Bio-rad, Milan, Italy).

Dgk-α assay
Dgk-α activity in cell homogenates (25 μl) was assayed by measuring initial velocities (5 min at 30°C) in presence of saturating substrates concentration (1 mg/ml diolein (Fluka, Milan, Italy), 5 mM ATP, 3 μCi/μl [α^{32}P]-ATP (Amersham), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM EGTA in 25 mM Hepes pH 8, final reaction volume 50 μl). Lipids were extracted as described previously (Graziani *et al*., 1991), and PA was separated by thin layer chromatography (TLC) in chloroform:methanol:water:25% ammonium hydroxide (60:47:11:4). TLC plates had been previously coated with (potassium oxalate 1.3%, EDTA 5 mM):(methanol) 3:2 and desiccated. [^{32}P]-PA was identified by co-migration with nonradioactive PA standards stained by incubation in iodine chamber. Radioactive signals were detected and quantified by GS-250 Molecular Imager and Phosphor Analyst Software (Bio-Rad). The experiments of activation *in vitro* were carried out by co-incubating the homogenates (10 μg protein) for 15 min at 15°C in presence of 1 mM ATP and 5 mM $MgCl_2$, as reported previously (Cutrupi *et al*., 2000).

Purification of GST fusion proteins
SH3 and SH2 domains fused to GST were expressed in *Escherichia coli* and purified according to standard protocol. In brief, protein synthesis was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside and cells were harvested 4 h later by centrifugation. Pellets were resuspended in buffer G (50 mM Tris–HCl, 100 mM NaCl, 5% glycerol, pH 8) and cells disrupted by sonication (Branson, Danbury, CT, USA). Supernatants were collected by centrifugation (15 min at 12 000 *g*) and purified on glutathione-sepharose column (Amerscham Pharmacia). The matrix with the attached proteins was removed from the column and used for the subsequent pull-down experiments. Purity and quantity of proteins were determined by SDS–PAGE and Coomassie-blue staining, usually purity was $\geqslant$80%.

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

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

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

Cell staining and confocal microscopy
MDCK cells were seeded on glass coverslips (Marienfeld, Germany) settled at the bottom of the wells of 24-well cell culture plates, cultured to appropriate confluence and then transfected. Before stimulation, cells were serum starved overnight in DMEM and then stimulated with HGF 50 ng/ml. Where indicated, cells were pre-treated with 10 μM PP2 for 15 min. After stimulation, cells were washed twice in phosphate-buffered saline (PBS) and fixed with fixing solution (3% paraformaldehyde-4% sucrose in PBS) for 5 min at room temperature. After two washes in PBS, cells were permeabilized with a Hepes-Triton Buffer (20 mM Hepes pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) for 5 min at 4°C. Cells were then washed three times with PBS containing 0.2% BSA and incubated for 15 min with PBS containing 2% BSA. TRITC-phalloidin (1:100 in PBS-2% BSA) was added directly onto the glass plates in the humidified chamber for 30 min and the excess was washed away by three wash with PBS-0.2% BSA. Each glass coverslip was washed briefly in water and blocked onto a glass microscope slide with Mowiol (20% Mowiol 4-88 in PBS 1 × pH 7.4). Images were acquired with a × 63 objective using a Leica TCS SP2 Confocal Microscope.

Cell migration assay
Cos cells, transfected and serum starved as indicated, were seeded (10^7 cells/ml in 200 μl suspension 0,1% FCS) in 8 μm pore size transwell (Corning-Constar, Milan, Italy). The lower chamber was filled with 0,1% FCS medium with or without HGF (50 U/ml) and incubated at 37°C in air with 5% CO_2 for 8 h. Cells remaining in the insert were then mechanically removed and the lower surface of filters stained with crystal violet and counted at the inverted microscope.

Statistical analysis
Statistical test used is two tails *t* test. Data on graph are shown as mean ± s.e.m.

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Diacylglycerol Kinase-α Mediates Hepatocyte Growth Factor-induced Epithelial Cell Scatter by Regulating Rac Activation and Membrane Ruffling

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Diacylglycerol kinases (Dgk) phosphorylate diacylglycerol (DG) to phosphatidic acid (PA), thus turning off and on, respectively, DG-mediated and PA-mediated signaling pathways. We previously showed that hepatocyte growth factor (HGF), vascular endothelial growth factor, and anaplastic lymphoma kinase activate Dgkα in endothelial and leukemia cells through a Src-mediated mechanism and that activation of Dgkα is required for chemotactic, proliferative, and angiogenic signaling in vitro. Here, we investigate the downstream events and signaling 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 small interfering RNA-mediated down-regulation of endogenous Dgkα, impairs 1) HGF- and v-Src-induced cell scatter and migration, without affecting the loss of intercellular adhesions; 2) HGF-induced cell spreading, lamellipodia formation, membrane ruffling, and focal adhesions remodeling; and 3) 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 remodeling of actin cytoskeleton and focal contacts in migrating epithelial cells.

INTRODUCTION

Epithelial tissues are characterized by monolayers of highly polarized cells, whereas 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 and Sleeman, 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).

Hepatocyte growth factor (HGF) and oncogenic Src induce in vitro cell scatter of several epithelial cells, whereas 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-Miagkova 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 remodeling of cortical actin and focal adhesions (Beherens *et al*., 1993; Lamorte *et al*., 2002). The signaling 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 phosphatidylinositol (PI) 3-kinase, phospholipase C (PLC)γ, Ras, and Rac are required (Lamorte *et al*., 2002, and references therein). Src plays a crucial role in HGF signaling because 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 down-regulation of cadherin-mediated cell–cell adhesions, and of several focal adhesion proteins required for cell motility and invasiveness, such as focal adhesion kinase (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 10

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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 protein kinase C (PKCs), Ras guanyl nucleotide releasing proteins (RasGRPs), and chimaerins. Similarly, several signaling 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, Rho guanine nucleotide dissociation inhibitor protein (RhoGDI), Ras- and Rho-GTPase–activating proteins (GAPs); and signaling lipid-metabolizing enzymes, such as phosphatidylinositol 4-phosphate 5-kinase [PI(4)P 5-kinase] and PLCγ (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, diacylglycerol kinase (Dgk) enzymes may act as terminators of DG-mediated signals as well as activators of PA-mediated signals.

Recent evidence showed that α, ζ, and θ Dgk isoforms are regulated by extracellular ligands and that they 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 T cell receptor (TCR) activation, leading to overactivation of the Ras pathway and a defect in anergy, whereas overexpression of Dgkα in T cells impairs TCR signaling (Olenchock *et al*., 2006a). Evidence in T cells indicates 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 signaling (Luo *et al*., 2003; Sanjuan *et al*., 2003; Zhong *et al*., 2003). Conversely, mast cells derived from Dgkζ −/− mice feature a diminished high-affinity IgE receptor-mediated degranulation, correlating with impaired PLCγ activation and calcium response, both likely dependent on PA production (Olenchock *et al*., 2006b).

We have previously shown that in endothelial and leukemia cells, activation of Dgkα downstream from tyrosine kinase receptors, such as HGF-receptor, vascular endothelial growth factor (VEGF) receptor-2, and anaplastic lymphoma kinase (ALK), is required for either chemotactic or proliferative signaling induced by their respective ligands and for cell proliferation upon interleukin-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 signaling 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 Madin Darby canine kidney (MDCK) cells, and particularly in those mechanisms leading to cell spreading and F-actin cytoskeleton and focal adhesions remodeling. By further investigating the role of Dgkα in HGF early signaling, we show that upon 15 min 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 and MDCK-*ts*-v-Src (Baldanzi *et al*., 2004) are a kind gift of W. Birchmeier (Max-Delbrück-Centrum, Robert-Rössle-Str. 10, 13125 Berlin). Cells were cultured in high glucose DMEM GlutaMAX medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen) and antibiotic-antimicotic solution (Sigma-Aldrich, St. Louis, MO), in humidified atmosphere with 5% CO_2. MDCK cells were cultured at 37°C, whereas 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 (Rocky Hill, NJ), and R59949 (diacylglycerol kinase inhibitor II) was from Sigma-Aldrich. Dimethyl sulfoxide, vehicle for R59949, was always used in control samples at the same dilution as R59949. Anti-Myc and anti-Rac1 were from Upstate Biotechnology (Charlottesville, VA); anti-paxillin was from BD Biosciences Transduction Laboratories (Lexington, KY); anti-paxillin $pTyr^{31}$ and $pTyr^{118}$ and anti-Akt $pSer^{473}$ were from BioSource International (Camarillo, CA); anti-Akt was from Cell Signaling Technology (Beverly, MA); anti-α-tubulin was from Sigma-Aldrich; anti-vinculin was from Novus Biologicals (Littleton, CO); anti-FAK was from Calbiochem (San Diego, CA); and Alexa Flour 546/633-phalloidin was from Invitrogen. Anti-Dgkα was kindly provided by W. J. van Blitterswijk (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Secondary horseradish peroxidase-conjugated antibodies were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA); and secondary fluorescein isothiocyanate (FITC)- and tetramethylrhodamine B isothiocyanate (TRITC)-conjugated antibodies were purchased from Dako Denmark (Glostrup, Denmark).

Expression Vectors, Transfections, and Infections with Retroviral Vectors

Myc-Dgkα cDNA cloned into pMT2 expression vector has been described previously (Cutrupi *et al*., 2000). Green fluorescent protein (GFP)-Dgkα-wild type (WT) was obtained by cloning Dgkα in pcDNA-DEST53 (Invitrogen) by using Gateway kit (Invitrogen) according to manufacturer's instructions. Briefly, Dgkα cDNA was inserted in pDONOR 2.11 vector by polymerase chain reaction (PCR) and BP recombination (recombination of an *att*B substrate with an *att*P substrate to create an *att*L-containing entry clone). LR recombination (recombination of an *att*L substrate with an *att*R substrate to create an *att*B-containing expression clone) 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, La Jolla, CA) as described previously (Cutrupi *et al*., 2000). PINCOS retroviral vector, PINCOS/Dgkα-DN and PINCOS/Dgkα-WT, expressing both GFP and the inserted gene, have been described previously (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, Mountain View, CA; kindly provided by R. Piva, University of Torino) was transiently cotransfected, by Lipofectamine 2000 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 was removed by centrifugation at 1500*g*, and the supernatant was filtered by a 0.45-μm pore filter and added with Polybrene (8 μg/ml). Cells were plated in a six-well plate and infected by adding 2 ml of retroviral supernatant and 1 ml of growth medium. The day after the first infection cells were reinfected as described briefly. Sixteen hours later, cells were placed and maintained in growth medium. Efficiency of infection was ~80%, as measured by fluorescence-activated cell sorting (FACS) analysis and/or observation with fluorescence microscope of GFP-expressing cells.

The murine Dgkα, resistant to canine Dgkα small interfering RNAs (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, Austin, TX). Sequences were as follows: C1, sense GCUCAGAAGUGGACAGGAUtt and antisense AUUCUGUCCACUUCUGAGCtg; C2, sense CCCAGACAUCCUGAAAACCtt and antisense GGUUUUCAGGAUGUCUGGGtc; C3, sense CCUUCCACACCACAAAAACtt and antisense

GUUUUUGUGGUGUGGAAGGtg. A glyceraldehyde-3-phosphate dehydrogenase scramble siRNA (Ambion) was used as negative control.

The BLOCK-iT Fluorescent Oligo (Invitrogen) is a fluorescein-labeled double-stranded RNA 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 previously (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-Aldrich]), lithium chloride buffer (500 mM LiCl and 25 mM Tris-HCl, pH 8) and TNE (25 mM Tris, pH 8, 150 mM NaCl, and 1 mM EDTA), all supplemented with fresh 1 mM Na_3VO_4, the immunocomplexes were assayed at room temperature for 10 min by incubation with 1 mg/ml diolein (Fluka, Buchs, Switzerland; 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 (GE Healthcare, Chalfont St. Giles, United Kingdom), 10 mM $MgCl_2$, and 1 mM $ZnCl_2$. Lipids were then extracted as described previously (Graziani *et al*., 1991), and PA was separated by thin layer chromatography (TLC) in chloroform:methanol:water:32% ammonium hydroxide (60:47:10:3). TLC plates had been coated previously with (1.3% potassium oxalate, 5 mM EDTA):methanol (3:2). [^{32}P]PA was identified by comigration with nonradioactive 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 (Bio-Rad, Hercules, CA). One-half of immunoprecipitated lysates was assayed for Dgk activity, whereas the other half was heat-denatured in Laemmli buffer, separated in SDS-polyacrylamide gel electrophoresis (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 they were allowed to growth in small colonies. Cells were stimulated in serum-free medium with 2 ng/ml HGF for 24 h, in presence or absence of 1 μM R59949, fixed with 3% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS), and then photographed with phase-contrast optics with a 20× objective (Carl Zeiss, Jena, Germany). For v-Src–induced cell scatter, MDCK-*ts*-v-Src cells were shifted to the permissive temperature of 35°C in 0% fetal bovine serum (FBS) medium for 24 h, in presence or absence of 1 μM R59949.

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

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

Invasion

Invasion assays were performed in serum-free medium in 6.5-mm Transwells with 8-μm pore size membranes. The Transwell membrane was precoated with 10 μg of Matrigel (BD Biosciences, San Jose, CA) in 50 μl of cold serum-free medium and dried overnight at room temperature. Cells (10^5) were seeded in the upper chamber of the Transwell apparatus. The lower chamber was filled with DMEM and 2% FBS in presence or absence of 100 ng/ml HGF, and cells were allowed to migrate for 48 h. After washing with PBS, the cells on the upper surface of the Transwell membrane were removed using a cotton-tipped swab, whereas those cells onto the lower surface 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, Lauda-Königshofen, Germany) in 24-well cell culture plates. Cells were overnight starved and then stimulated with 10 ng/ml HGF for the indicated times. R59949 (1 μM) was given as pretreatment in short-time HGF experiments (15 min), whereas in long-time experiments (from 4 h onward), it 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 $MgCl_2$, and 0.5% Triton X-100), washed with PBS containing 0.2% bovine serum albumin (BSA), and incubated for 15 min with PBS containing 2% BSA. Then, 15 μl of primary antibody (1:100 in PBS containing 2% BSA) was added directly onto each glass coverslip in a humidified chamber for 30 min, and excess antibody was washed away with PBS containing 0.2% BSA. Cells were then incubated for an additional 15 min with PBS containing 2% BSA and FITC-/TRITC-conjugated secondary antibodies and/or Alexa Fluor 546/633-phalloidin (1:30 and 1:200 in PBS containing 2% BSA, respectively) was added for 30 min 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% 1,4-diazabicyclo[2.2.2]octane in PBS, pH 7.4) and let polymerize. Confocal images were acquired with the Leica confocal microscopy TSP2 and LCS Leica confocal software (Leica, Wetzlar, Germany). Basal planes are shown.

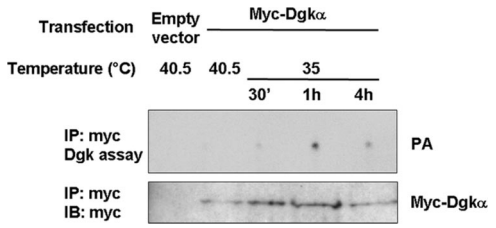

Figure 1. v-Src activates Dgkα. MDCK-*ts*-v-Src maintained at the nonpermissive temperature of 40.5°C were transiently transfected with Myc-Dgkα, starved overnight in 0% FBS medium, and shifted to the permissive temperature of 35°C for the times indicated. Cell lysates were immunoprecipitated with an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

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-Aldrich]). 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 (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 min on a rotating wheel at 4°C. The lysates were centrifuged at 10,000*g* for 30 min, 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 min on a rotating wheel at 4°C. The lysates were then centrifuged at 10,000*g* for 30 min, 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*. (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 min. R59949 (1 μM), when used, was added with a 30 min pretreatment and maintained during the subsequent HGF stimulation. Cells were then washed in ice-cold PBS and lysed with glutathione transferase (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 dithiothreitol) and harvested by scraping. The clarified lysates were incubated for 45 min with purified GST-PAK-BD at 4°C, precoupled to glutathione-Sepharose beads (GE Healthcare). After three washes with GST-fish buffer, samples were resuspended in Laemmli buffer, heat-denatured, and separated by SDS-PAGE in a 12% polyacrylamide 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 analyzed when quantification was performed. Couples of conditions were compared using Student's *t* test. Histograms represent means ± SEs.

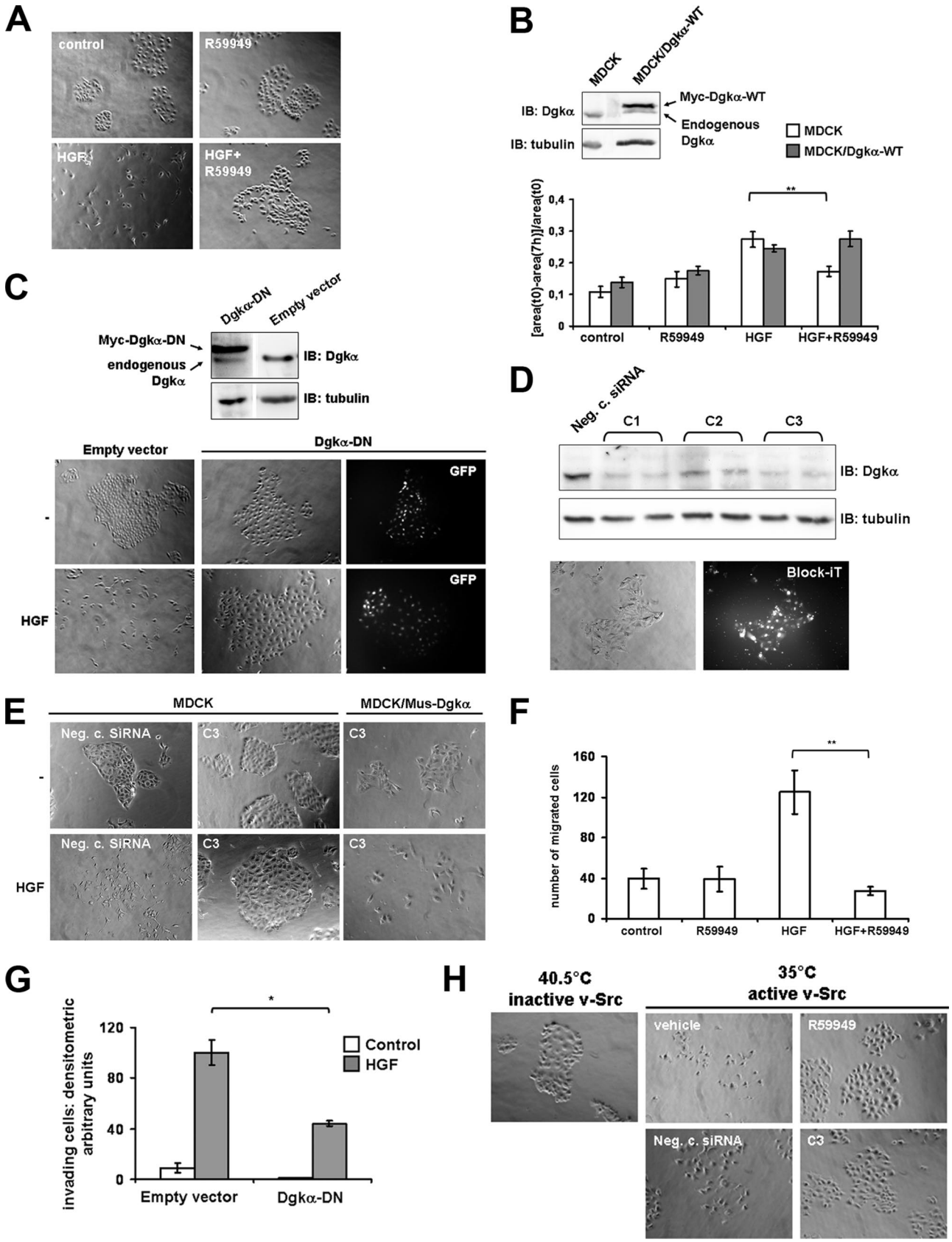

Figure 2. Dgkα is required for HGF-induced cell scatter and migration of MDCK cells. (A) MDCK cell colonies were treated, in 0% FBS medium, with 2 ng/ml HGF in presence or absence of 1 μM R59949 for 24 h. Representative fields are shown. (B) Control or MDCK/Dgkα-WT cells were allowed to migrate into the wounded area in 0% FBS medium with 2.5 ng/ml HGF in presence or absence of 1 μM

RESULTS

Dgkα Activation Mediates HGF-induced Scatter and Migration of MDCK Cells

We showed previously 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 signaling pathways involved.

MDCK cells express endogenous Dgkα and feature an R59949-sensitive Dgk activity associated to anti-phosphotyrosine immunoprecipitates upon HGF stimulation (data not shown). On 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 1 h (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 reported previously 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 intercellular adhesions and migration of cells away from one another (Beherens *et al*., 1993; Weidner *et al*., 1993; Palacios and D'Souza-Schorey, 2003).

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 1 μM 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 Dgkα in MDCK cells fully reestablishes HGF-induced cell migration even in presence of 1 μM R59949 (Figure 2B).

Figure 2 (cont). R59949 for 7 h. Quantification was performed as described in *Materials and Methods*. Means of at least four experiments with SEs 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 2 ng/ml HGF for 24 h. 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 they 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 2 ng/ml HGF for 24 h. Representative fields are shown. (F) MDCK cells were seeded in the top part of a chemotaxis chamber and induced to migrate in presence of 50 ng/ml HGF in the bottom part, in presence or absence of 1 μM R59949. The histograms represent the number of migrated cells, means of eight different wells with SEs. **p < 0.005. A representative experiment is shown. (G) MDCK/empty vector or MDCK/Dgkα-DN cells were seeded in the top chamber of a Transwell apparatus. Invasion through a Matrigel-covered porous membrane was induced in 48 h in 2% FBS medium by the presence of 100 ng/ml HGF in the bottom chamber. Fixed cells on the Transwells lower surface were stained with crystal violet, photographed, and quantified by optical densitometry. Means of three experiments are shown, with SEs; *p ≤ 0.05. (H) MDCK-*ts*-v-Src cell colonies, maintained at the nonpermissive 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 h. The same experiment was performed with untransfected cells, in presence or absence of 1 μM R59949, as indicated. Representative pictures are shown.

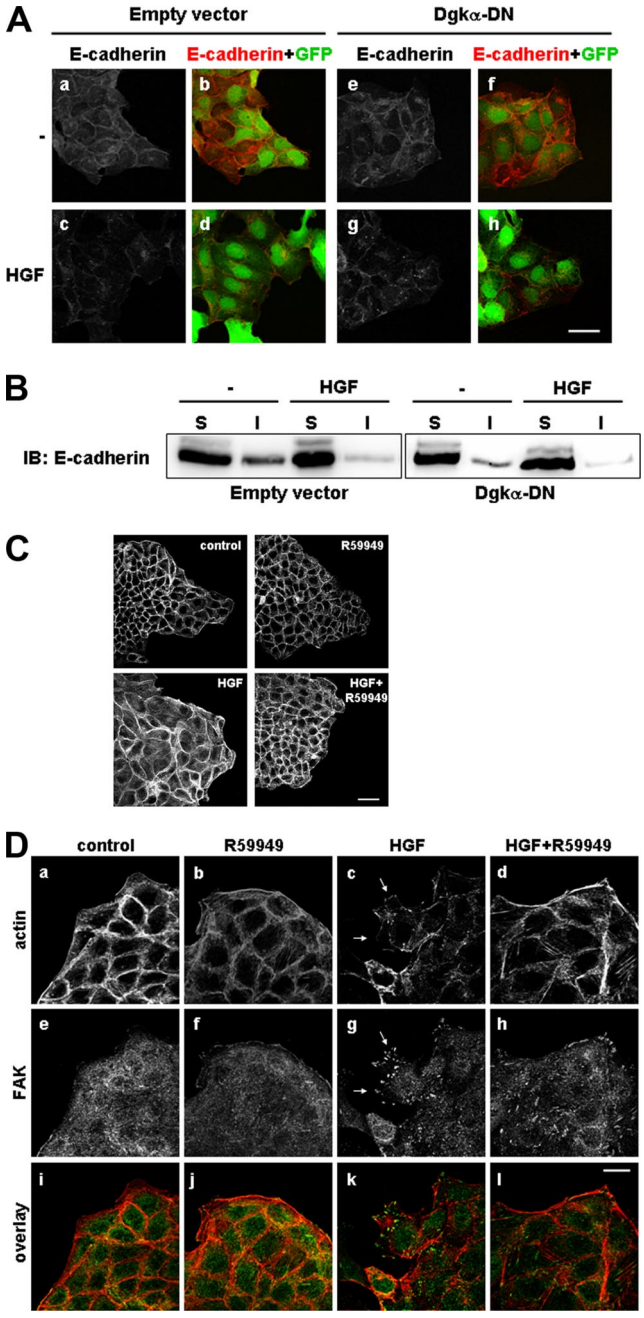

Figure 3. Dgkα is required for HGF-induced cell spreading and lamellipodia formation, but not for down-regulation of E-cadherin–mediated intercellular adhesions. (A) MDCK/empty vector or MDCK/Dgkα-DN cells were treated with 10 ng/ml HGF for 6 h, fixed, and stained for E-cadherin. Representative pictures are shown. Bar, 20 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were treated with 10 ng/ml HGF for 6 h. Cell lysates were fractionated into an 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 10 ng/ml HGF for 4 h, in presence or absence of 1 μM R59949. Fixed cells were stained for actin filaments with phalloidin. Bar, 40 μm. (D) MDCK cells treated as described in C were fixed and stained for actin (red, a–d) and FAK (green, e–h). Bar, 16 μm. Representative pictures are shown.

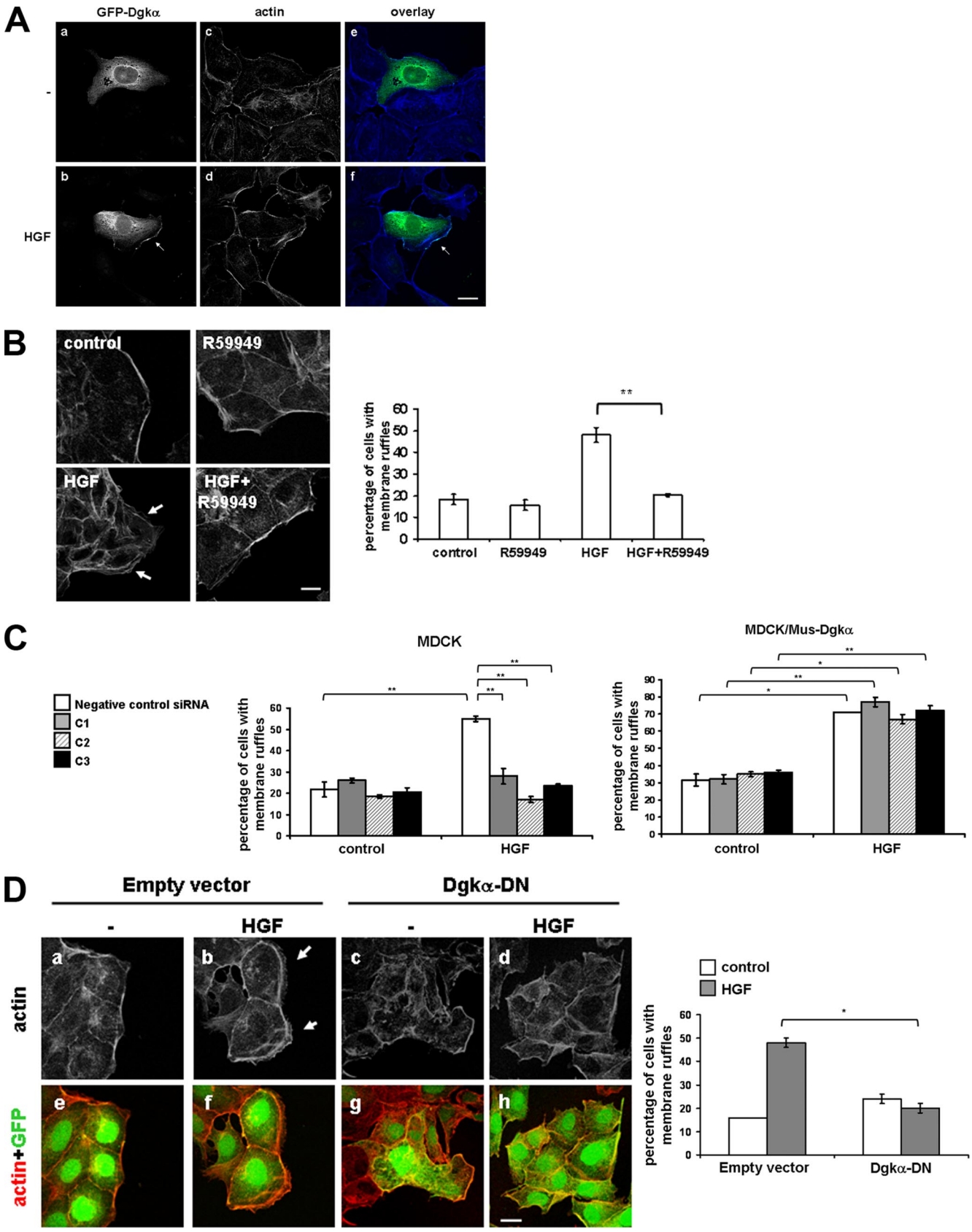

Figure 4. Dgkα is required for HGF-induced membrane ruffling of MDCK cells. (A) MDCK cells were transiently transfected with GFP-Dgkα, starved overnight in 0% FBS medium, stimulated with 10 ng/ml HGF for 15 min, fixed, and stained for actin. Bar, 8 μm. (B) MDCK cell colonies were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, fixed, and stained for actin. Bar, 16 μm. Confocal acquired images were observed and cells at the edge of colonies were scored for

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).

To further verify the specificity of Dgkα requirement in HGF-induced cell scatter, the endogenous protein was down-regulated by transient transfection of specific siRNAs. Three siRNAs were designed (C1, C2, and C3), transiently transfected in MDCK cells, and they 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 down-regulation of endogenous Dgkα inhibits HGF-induced MDCK cell scatter (Figure 2E). Similar results were obtained with C1 and C2 (data not shown). 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, 1 μM R59949 abolishes HGF-induced chemotaxis of MDCK cells toward the HGF-filled lower chamber (Figure 2F), whereas 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 signaling 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 down-regulation 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 Remodeling, and Lamellipodia Formation from Down-Regulation 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 h from HGF stimulation (Beherens *et al*., 1993; Potempa and Ridley, 1998; Kimura *et al*., 2006), which occurs concomitantly to colony spreading, so that the area covered by each colony increases two- to threefold. 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 FAK, are recruited at new sites of adhesion and at the tips of stress fibers (Weidner *et al*., 1993; Ridley *et al*., 1995; Palacios and D'Souza-Schorey, 2003).

We observed that inhibition of Dgkα, either by 1 μM 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 3Ag), occurring upon 6 h of cell stimulation. In addition, we performed fractionation of MDCK cell lysates in NP-40–soluble and NP-40–insoluble fractions. On 6 h 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 1 μM R59949 results in a remarkable reduction of HGF-induced colony spreading upon 4 h 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 3Da–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 3De–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 down-regulates E-cadherin–mediated intercellular adhesions and that its inhibition uncouples HGF-induced events, leading to loss of intercellular adhesions from the signaling pathways mediating cell spreading, F-actin remodeling, lamellipodia formation, and eventually cell migration.

Dgkα Is Required for HGF-induced Membrane Ruffle Formation and Focal Adhesions Remodeling

On 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 min of HGF treatment it accumu-

Figure 4 (cont). presence of membrane ruffles (arrows). The percentage of cells with membrane ruffles was calculated. Means of three experiments with SEs 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 10 ng/ml HGF for 15 min. 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 SEs 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 10 ng/ml HGF for 15 min, fixed, and immunostained for actin (red, a–d). The arrows indicate membrane ruffles in empty vector-infected cells. 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 SEs are presented. $^{*}p < 0.05$.

lates 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 out to investigate earlier changes in F-actin cytoskeleton organization in response to HGF. On 15 min 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 <20% in control cells (vehicle- and R59949-treated cells) to ~50% in HGF-treated cells. In presence of 1 μM R59949, the percentage of membrane ruffle-displaying cells upon HGF stimulation is reduced to almost the control value (Figure 4B). 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 with cells expressing the vector alone (Figure 4D). In conclusion, these data demonstrate that the formation of membrane ruffles occurring upon 15 min of HGF treatment depends on stimulation of Dgkα activity.

Membrane ruffle 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 signaling molecules, is required for HGF-induced signaling 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, whereas in cells at colony edge it is also localized in focal adhesions along the outer plasma membrane (Figure 5, Aa and Ba). On 15 min of HGF stimulation, paxillin condensates to the newly formed focal complexes in correspondence of membrane ruffles (Figure 5Ac and Bb). On inhibition of Dgkα by either 1 μM R59949 (Figure 5Ad) or by expression of Dgkα-DN (Figure 5Bf), Paxillin accumulates along the outer plasma membrane instead of being recruited in the area of ruffling, whereas ruffle 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.

To verify that paxillin indeed accumulates in structures identifiable as focal complexes, we analyzed its colocalization with vinculin, a resident protein whose function is to stabilize them (Ziegler *et al*., 2006). In unstimulated cells vinculin and paxillin colocalize 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, although impairing HGF-induced neoformation of ruffles and focal complexes at membrane ruffles, does not affect vinculin and paxillin colocalization (Figure 5C).

On growth factor stimulation Src- and FAK-mediated phosphorylation of paxillin is required to recruit and coordinate multiple signaling complexes, regulating events at the leading edge of migrating cells (for review, see Brown and Turner, 2004). Phosphorylation of paxillin on tyrosine 31 and 118 mediates its association with Crk, and it is required for growth factor-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 it 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 remodeling 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 signaling 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 and Suetsugu, 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 1 μM 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 multimolecular complexes at the cell leading edge. Because several Rac guanine nucleotide exchange factors (GEFs) are regulated through their pleckstrin homology 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 signaling (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, whereas only ~20% of colony-edge cells feature Rac at the outer plasma membrane (Figure 7Aa). After 15 min of HGF stimulation, the percentage of colony-edge cells featuring Rac at the outer plasma membrane raises to >40% (Figure 7Ac), whereas localization of Rac at cell–cell contacts is not affected. Inhibition of Dgkα by 1 μM R59949 treatment abolishes HGF-induced Rac membrane targeting (Figure 7Ad), whereas it does not significantly affect Rac localization in unstimulated cells (Figure 7Ab) nor Rac localization at cell-cell contacts. Similar results were obtained when Dgkα was inhibited upon expression of Dgkα-DN (Figure 7B). On HGF stimulation Rac is properly membrane localized in cell infected with the empty vector (Figure 7Bb), whereas it

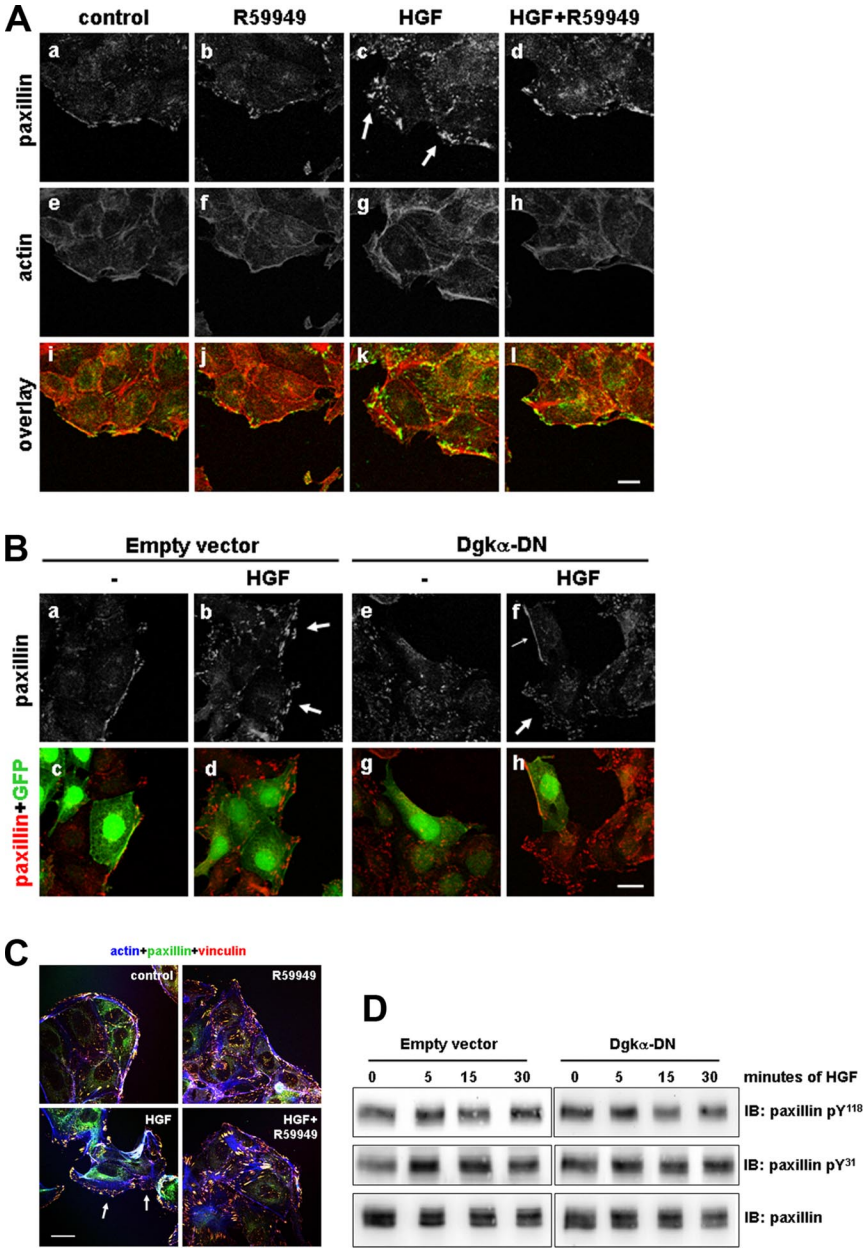

Figure 5. Dgkα is required for HGF-induced paxillin localization to newly formed focal complexes. (A) MDCK cell colonies were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, fixed, and stained for paxillin (green, a–d) and actin (red, e–h). Representative pictures are shown. Bar, 16 μm. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min, fixed, and immunostained for paxillin (red, a, b, e, and f). Thick arrows indicate paxillin localization at focal adhesions in the areas of membrane ruffling, whereas the thin arrow indicates paxillin localization at cell periphery in a Dgkα-DN–infected cell, without membrane ruffles. 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. Bar, 16 μm. (D) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium and treated with 50 ng/ml HGF for 15 min. Whole cell lysates were separated by SDS-PAGE and probed with anti-paxillin pTyr[31] and pTyr[118] and anti-paxillin.

remains predominantly cytoplasmic in Dgkα-DN–expressing cells (Figure 7Bf).

In summary, these data demonstrate that Dgkα is required for HGF-induced activation and targeting of Rac to the plasma

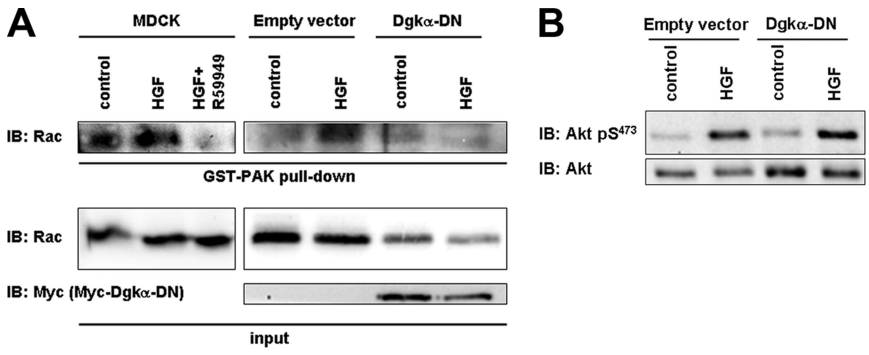

Figure 6. Dgkα is required for HGF-induced Rac activation. (A) MDCK cells were starved overnight in 0% FBS medium, treated with 100 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, and lysed. GTP-bound active Rac was purified in each sample by pull-down with GST-fused PAK CD domain. MDCK/empty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

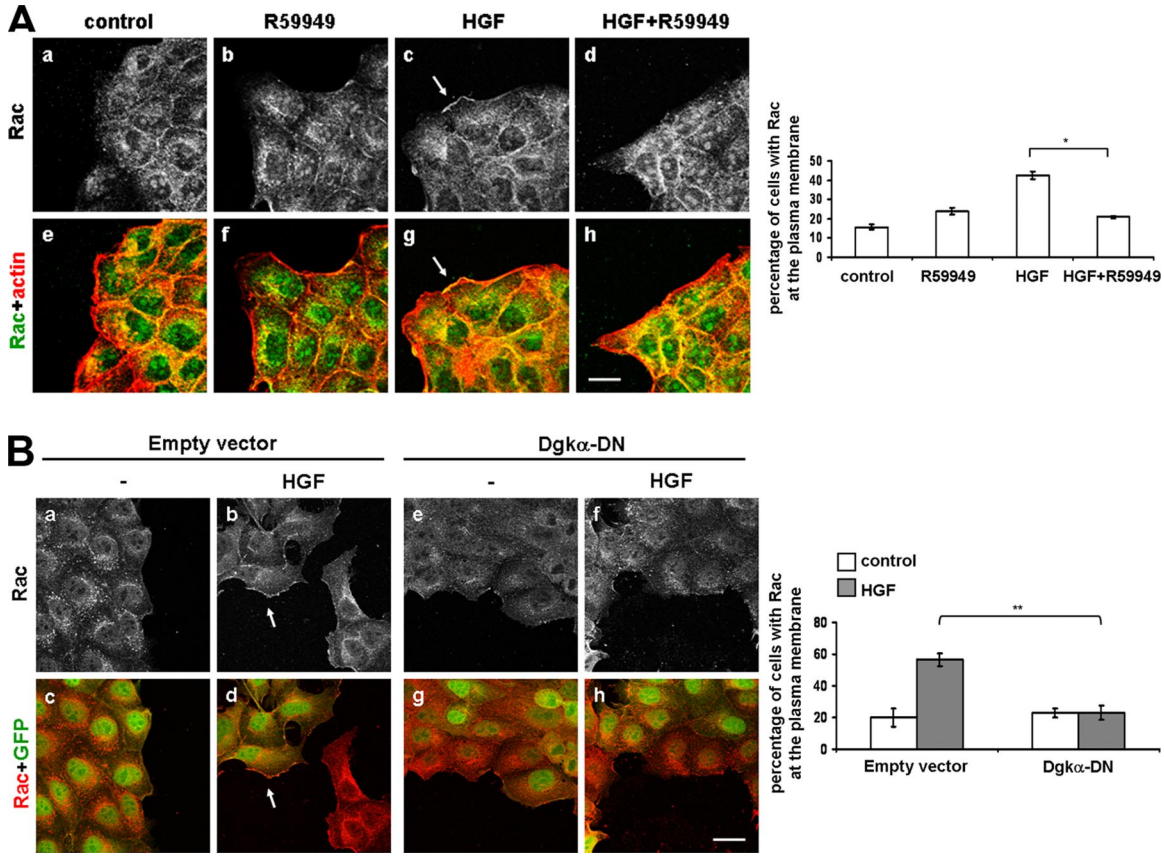

Figure 7. Dgkα is required for HGF-induced Rac localization to the plasma membrane. (A) MDCK cell colonies were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min in presence or absence of 1 μM R59949, fixed, and stained for Rac (green, a–d) and actin (red). Representative pictures are shown. 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 SEs are shown. *$p < 0.05$. (B) MDCK/empty vector or MDCK/Dgkα-DN cells were starved overnight in 0% FBS medium, treated with 10 ng/ml HGF for 15 min, fixed, and immunostained for Rac (red, a, b, e, and f). Representative pictures are shown. 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 SEs are presented. **$p < 0.005$.

membrane and for the following formation of membrane ruffles, thus strongly suggesting that Dgkα is involved in the signaling pathways regulating Rac function and targeting upon activation of HGF receptor. These data demonstrate that Dgkα plays a pivotal role in the migratory signaling downstream HGF, being involved in early molecular events such as Rac activation, membrane ruffle 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 down-regulation of the endogenous protein, impairs both HGF- and 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, 2007; Bacchiocchi *et al.*, 2005). Moreover, these data suggest that Dgkα represents a crucial node in the signaling 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 signaling 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 signaling 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 herein that upon Dgkα inhibition, no cell spreading, lamellipodia extension, and remodeling 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, although 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. Together, these data provide the first circumstantial evidence that Dgkα may act in growth factors signaling at the leading edge of migrating cells.

Ruffle 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 signaling 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*., 2006a; Topham and Prescott, 2001; Zha *et al*., 2006). However, in epithelial cells, neither the overexpression nor the down-regulation of Dgkα affects the Ras pathway, as detected by extracellular signal-regulated kinase-1/2 phosphorylation (our unpublished data). In addition Dgkγ, but not Dgkα, upon its recruitment to the plasma membrane, negatively regulates platelet-derived growth factor (PDGF)- and epidermal growth factor (EGF)-induced Rac activation and membrane ruffling, by enhancing the activity of β2-chimaerin, a Rac GAP containing a C1 and a Src homology 2 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 controls 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 colocalize 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). Because 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 speculation that Dgkα also may regulate Rac activation through this mechanism. Together, these data strongly indicate that distinct Dgk isoforms act as regulators of Rac membrane targeting and activation through multiple mechanisms, whose complexity 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 phosphatidylinositol 3,4,5-trisphosphate (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 herein, we may discuss several hypotheses, 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 ruffle formation upon PDGF, EGF, and fibroblast growth factor 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 it 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).

On 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 remodeling and membrane ruffle formation.

Several Rac and Arf GEFs are regulated by phosphatidylinositol 3,4,5-trisphosphate, the product of PI 3-kinase. However, we can rule out that Dgkα mediates Rac activation by regulating phosphatidylinositol trisphosphate (PIP_3) synthesis, because 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) allows speculation 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 ruffle 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), events that we showed to not be regulated by Dgkα, making Tiam1 an unlike target of Dgkα activity.

In conclusion, herein 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 signaling by mediating Rac targeting and activation, thus revealing a

novel signaling pathway linking tyrosine-kinase receptors and Src to small GTPases in the context of cell migration.

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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

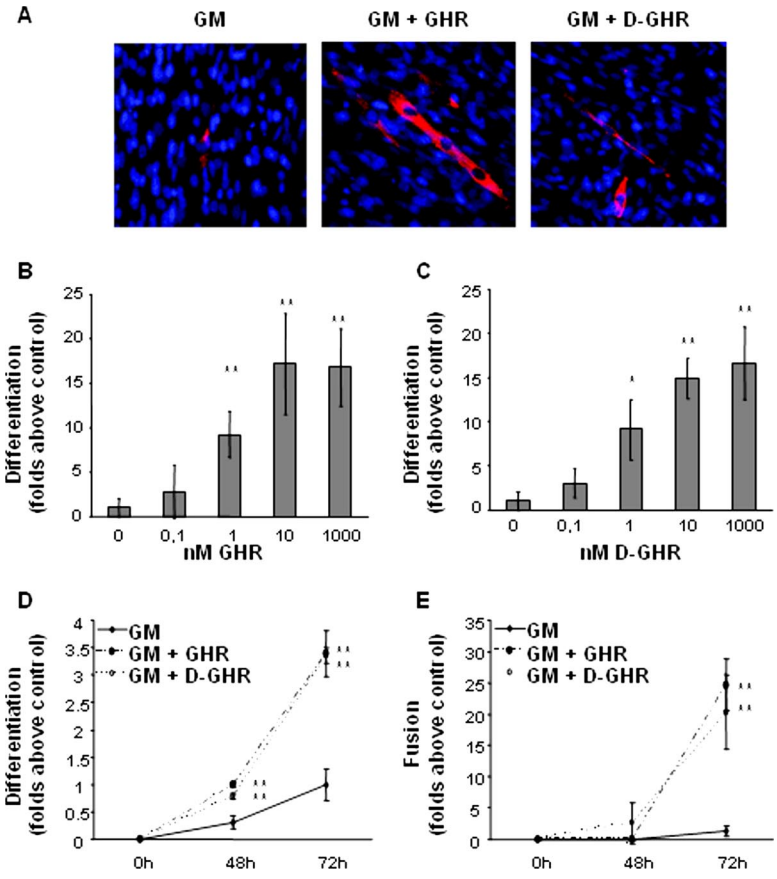

Figure 1. GHR and D-GHR induce differentiation and fusion of C2C12 myoblasts in GM. Cells were treated either with GHR or D-GHR in GM and fixed for staining with anti-MHC antibody and DAPI. (A) Representative immunofluorescences of C1C12 myoblasts treated for 72 h with 10 nM GHR or D-GHR stained with 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

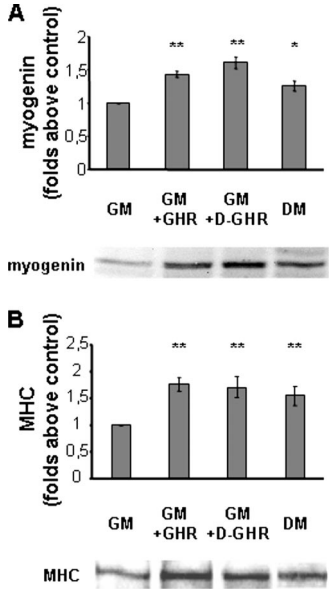

Figure 2. Western blot analysis of myogenic markers expression. C2C12 myoblasts were incubated in GM for 24 or 72 h with 10 nM GHR or D-GHR. The content of myogenin and MHC was measured by Western blot of whole cell lysates and the intensity of the bands quantified. Cells differentiated in DM were considered 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.

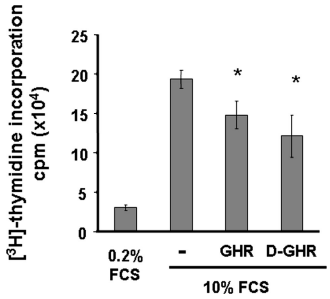

Figure 3. Effect of GHR and D-GHR on basal incorporation of [^{3}H]thymidine into DNA by C2C12 myoblasts. DNA synthesis was estimated by incorporation of [^{3}H]thymidine after a 24-h incubation with or without 10 nM GHR or D-GHR in GM. *$p < 0.05$ versus control.

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

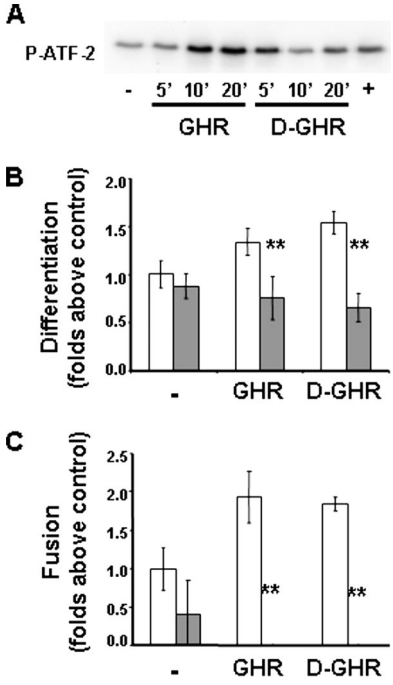

Figure 4. GHR and D-GHR induce differentiation and fusion of C2C12 myoblasts by activation of p38. (A) Kinase activity of p38 on ATF-2 induced by 1 μM GHR and D-GHR in C2C12 myoblasts. Cells were starved in 0.2% FCS overnight and then treated for the indicated times. Treatment with 10 μg/ml lysophosphatidic 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.

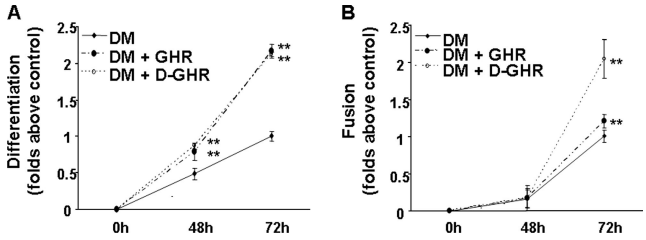

Figure 5. GHR and D-GHR enhance differentiation and fusion of C2C12 myoblasts in differentiation medium. Cells were treated with 10 nM GHR or D-GHR in DM and fixed after 48 and 72 h for staining with anti-MHC antibody and DAPI in order to determine the differentiation index. ** $p < 0.01$ versus control.

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).

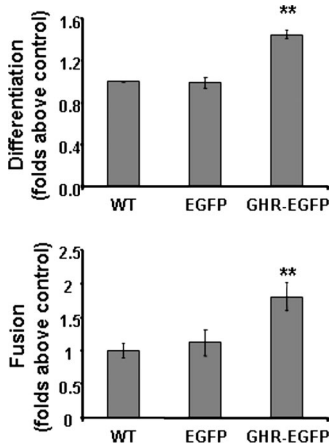

Figure 6. Increase of differentiation and fusion of C2C12 myoblasts overexpressing murine GHR. Cells transfected with a lentiviral vector expressing both GHR and EGFP under control of a bidirectional promoter, a lentiviral vector expressing EGFP only, and C2C12 wild-type cells were induced to differentiate 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.

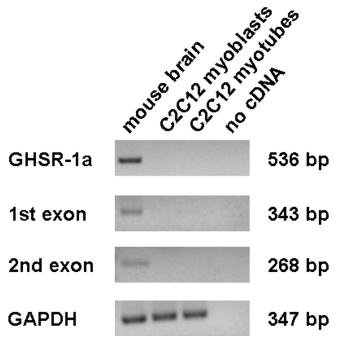

Figure 7. Expression of GHSR-1a, by RT-PCR, in C2C12 myoblasts and myotubes. Mouse brain was used as positive control for amplification of complete receptor, first exon, and second exon. Qualitative controls for samples were performed using mouse GAPDH.

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

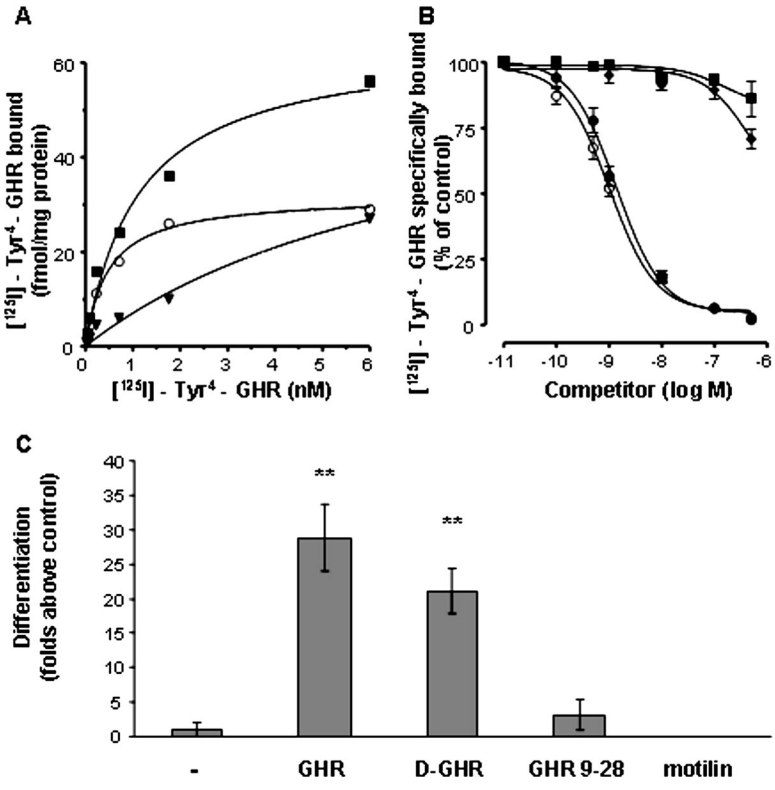

Figure 8. C2C12 myoblasts contain a GHR receptor distinct from GHSR-1a. (A) Specific binding (○) was determined by incubation of crude membranes with increasing concentrations of radiolabeled GHR (^{125}I-Tyr^4-GHR) in the absence (total binding, ■) or in the presence (nonspecific binding, ▼) of 1 μM 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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Control of *Myf5* activation in adult skeletal myonuclei requires ERK signalling

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Abstract

Myf5 plays a central role in determination of the myogenic lineage, yet the signalling pathways that control its activation remain unclear. In adult muscle, *Myf5* is expressed in satellite cells and muscle spindles but not by myonuclei. However, *Myf5* expression is activated in myonuclei in response to muscle denervation. This can be modelled in culture using $Myf5^{nlacZ/+}$ mice, allowing signalling pathways controlling *Myf5* to be readily examined. We found that mitogen-rich medium induces activation of the *Myf5* locus through calcium, which interacts with calmodulin to promote calcineurin and calmodulin kinase. Calcineurin activates NFAT to control *Myf5* activation, while p38/JNK activity prevents activation by this route. Calmodulin kinase however, acts predominately through ERK signalling to activate *Myf5*. Interestingly, we found that IGF-1 can substitute for mitogen-rich medium and activates *Myf5* through calcium, PI3K and ERK pathways. Together these observations show that *Myf5* activation in adult muscle is accomplished by a complex signalling pathway, and provides candidates that can be examined for their role in *Myf5* regulation during development.

Keywords: *Myf5*; Skeletal muscle; ERK; IGF-1; Ca^{2+}; Calcineurin; Calmodulin

1. Introduction

Myogenic cells first arise in the somites, transitory mesoderm-derived structures formed in pairs on either side of the neural tube. Mesodermal cells within somites become specified as skeletal muscle precursors and form the myotome, the earliest site of differentiated muscle [1]. Integral to the initiation of the skeletal muscle programme are the myogenic regulatory factors, with *Myf5*, MyoD and *Mrf4* involved in determining the myogenic lineage [2,3]. The final member of this basic helix–loop–helix transcription factor family is myogenin, which is essential for the final steps of muscle cell differentiation [4]. *Myf5* is first expressed around E (embryonic day) 8.0 of mouse development [5] and in its absence, initiation of myogenesis fails to occur until the myogenic programme is rescued by MyoD at ~E10.5 [6].

Considering the pivotal role that *Myf5* plays in determining the myogenic lineage, it is crucial to identify the transcription factors and signalling pathways that control its expression. The main approach to date has been to identify the *cis* acting regulatory elements in the *Mrf4*/*Myf5* locus that are responsible for developmental expression using transgenesis [7–14]. The elements necessary for the complete expression pattern of *Myf5* however, are distributed over ~140 kb of genomic sequence upstream of the *Myf5* transcription initiation site [8]. This transgenic analysis is beginning to delimit regions able to direct expression at specific locations during development. This has in turn, lead to the factors that control *Myf5* expression at these sites being found by the presence of their consensus binding sites, such as sonic hedgehog/Gli for epaxial somite [15,16] and Pax3 in the developing limb [17].

The *Myf5* locus is also active in normal adult muscle. Skeletal muscle is composed of syncytial myofibres with hundreds of postmitotic myonuclei maintaining the contractile apparatus. New myonuclei are produced by satellite cells, located beneath the basal lamina that surrounds each myofibre [18]. Using the $Myf5^{nlacZ/+}$ mouse, we have shown that the locus is active in satellite cells and muscle spindles [18,19] while others have

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shown that there is activation of the locus during muscle regeneration [20]. β-galactosidase is also present in myonuclei of *Myf5*$^{nlacZ/+}$ mice during the perinatal period [21], probably as a result of perdurance of the *Myf5*/β-galactosidase fusion protein, but is subsequently undetectable in myonuclei of mature myofibres [19]. However, muscle denervation results in the *Myf5* locus being activated in myonuclei [18], consistent with observations that *Myf5* transcripts, together with those of MyoD and myogenin, are elevated in denervated muscle [22–24]. This activation of myogenic transcription factors maybe involved in the re-distribution of muscle genes such as acetylcholine receptor [25] and N-CAM [26] in response to denervation.

We have begun to identify regions in which elements responsible for adult expression of *Myf5* reside but at present, ~140 kb upstream of the *Myf5* transcription initiation site is also required for driving all aspects of *Myf5* expression in adult [18]. The regions necessary for myonuclear activation of *Myf5* however, reside between 59 and 8.8 kb upstream of the *Myf5* promoter [18] and delimiting the elements within this ~50 kb of genomic sequence responsible requires extensive transgenic analysis.

This activation of *Myf5* in myonuclei can be modelled in culture though: while myonuclei of freshly isolated fast myofibres from *Myf5*$^{nlacZ/+}$ mice do not express *nlacZ*, certain culture conditions result in the rapid and robust activation of the *Myf5* locus in virtually all myonuclei [27]. This provides an accessible system with which to explore control of *Myf5* activation, allowing specific targeting of particular signalling pathways to be performed. Thus providing an alternative to delimiting ever smaller genomic elements to define consensus binding sequences for identification of factors responsible. This model is also useful, considering how relatively little material can be obtained from appropriate embryonic/foetal sources to study control of *Myf5*.

In this study we have used myonuclear activation of the *Myf5* locus in *Myf5*$^{nlacZ/+}$ mouse as a sensitive indicator with which to explore the signalling pathways that control this gene in adult muscle. We found that a crucial factor in mitogen-rich Plating Medium that results in activation of the *Myf5* locus is insulin-like-growth factor 1 (IGF-1) which can act through calcium in a complex signalling pathway. Ca^{2+} interacts with calmodulin promoting calmodulin kinase and calcineurin activation, which subsequently operates through separate, though not independent, pathways. Calcineurin activates NFAT to control *Myf5*, while p38/JNK activity prevents *Myf5* activation, suggesting that p38/JNK are inhibiting NFAT phosphorylation. Calmodulin kinase however, acts predominately through ERK signalling to regulate *Myf5*. We have also found that IGF-1 activates the *Myf5* locus through both the PI3K/GSK3 and ERK pathways. Together these observations show that a complex signalling pathway interacts to regulate *Myf5* in adult muscle.

2. Materials and methods

2.1. Tissue preparation and isolation of single fibres

Adult *Myf5*$^{nlacZ/+}$ mice (~8–12 weeks) were used in this study. Mice were killed by cervical dislocation and extensor digitorum longus (EDL) muscles were removed and myofibres isolated as described previously [19].

2.2. Myofibre culture

Isolated myofibres were cultured in suspension in 6-well plates in DMEM or Plating Medium containing 10% horse serum (PAA Laboratories) and 0.5% Chick Embryo Extract (CEE; ICN Biomedicals), and incubated at 37 °C in 5% CO_2 for 24 h. When cultured in mitogen-rich Plating Medium, myonuclei of EDL myofibres activate the *Myf5* locus, while culture in DMEM (basal medium) alone does not [18,27]. Thus, Plating Medium and DMEM were used as positive and negative controls respectively in each experiment. Where used, inhibitors or agonists were added to either Plating Medium or DMEM as appropriate for 24 h.

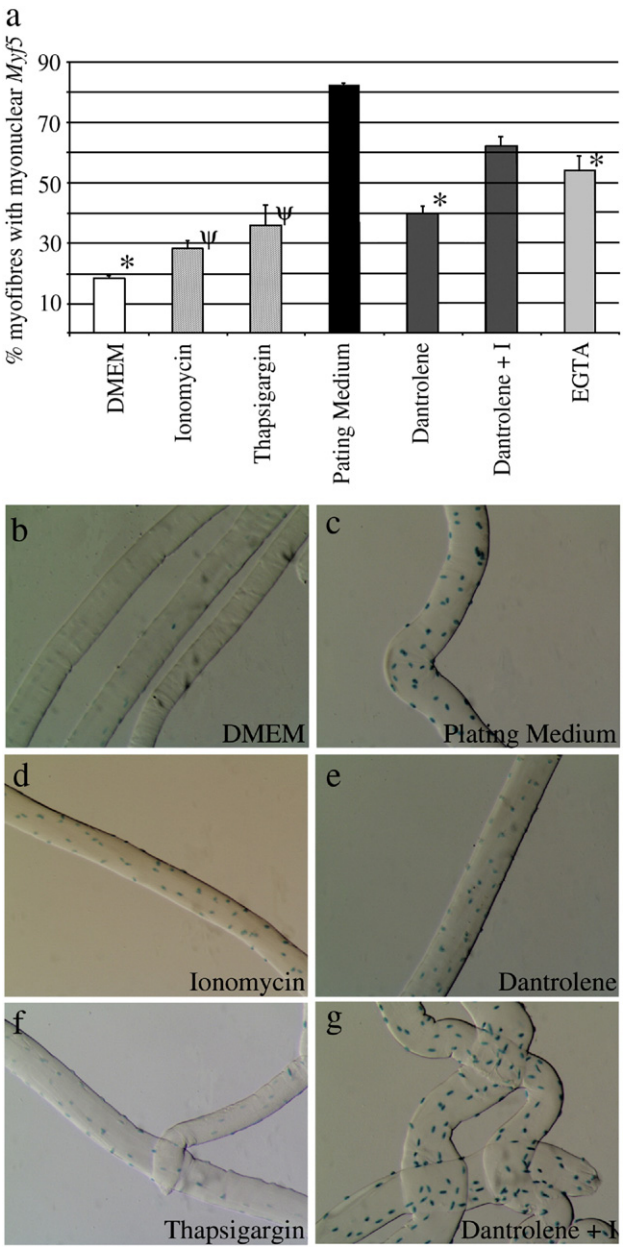

Fig. 1. Calcium activates the *Myf5* locus in EDL myonuclei. (a) Bar graph depicting the percentage of myofibres with activated *Myf5* after altering calcium levels for 24 h. Ionomycin (I; 1 μM) and thapsigargin (TG; 1 μM) were added to DMEM, while dantrolene (DAN; 50 μM) and EGTA (2 mM) were added to Plating Medium. Values are the population mean±SEM of pooled data from approximately 20 fibres from each of at least 3 *Myf5*$^{nlacZ/+}$ mice. ($p<0.05$, comparing each treatment to myofibres cultured in control Plating Medium (*) or DMEM (ψ)). (b–g) Typical myofibres after exposure to the reagents indicated on the panels and incubation in X-Gal solution.

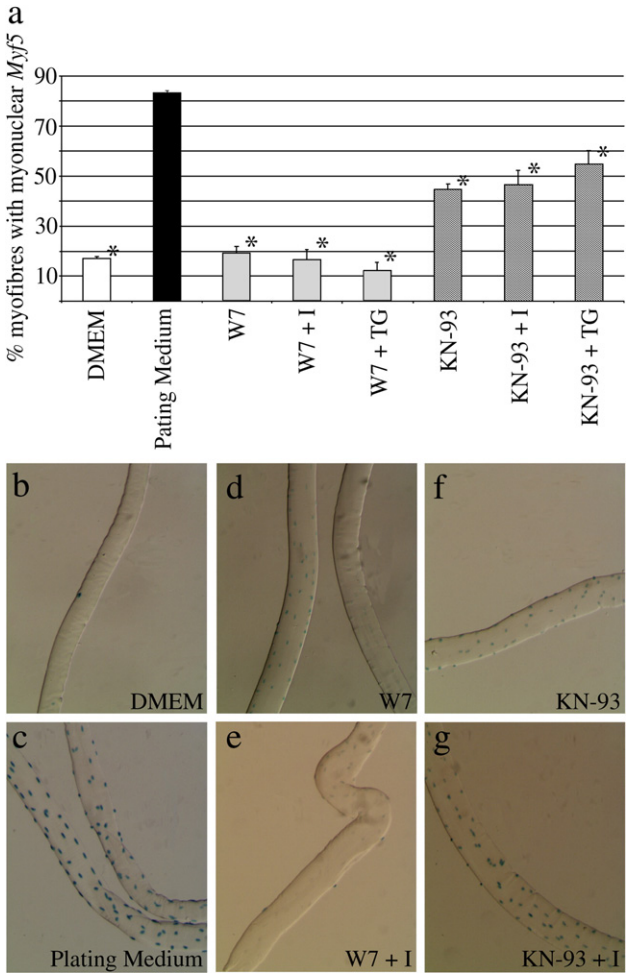

Fig. 2. Ca^{2+}–calmodulin interaction regulates *Myf5* activation through calmodulin kinase. (a) Bar graph showing the percentage of total myofibres assayed that had activated the *Myf5* locus when calmodulin kinase was blocked using either W7 (30 μM) or KN-93 (20 μM) in Plating Medium. Values are the mean ± SEM of data pooled from approximately 20 fibres from each of at least 3 $Myf5^{nlacZ/+}$ mice. (*$p<0.05$, comparing each treatment to myofibres cultured in control Plating Medium). (b–g) Examples of myofibres after exposure to the reagents indicated on the panels and incubation in X-Gal solution to reveal β-galactosidase activity in myonuclei. I, Ionomycin (1 μM); TG, thapsigargin (1 μM).

Then, myofibres were fixed in 2% formaldehyde/PBS for 5 min. Since calcium is central to control of skeletal muscle contraction, altering calcium levels (with ionomycin, thapsigargin or EGTA) often results in myofibre hypercontraction, from which they do not recover. Therefore, only viable, non-hypercontracted myofibres were included in this study.

2.3. Histochemistry

To visualise β-galactosidase activity, fixed myofibres were incubated overnight at 37 °C in X-Gal solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, 400 μg/ml X-Gal and 0.02% IPEGAL in PBS). Myofibres were rinsed in PBS and mounted in Faramount mounting medium.

2.4. Inhibitors and agonists

Ionomycin, thapsigargin, Cyclosporine A, dantrolene, phenylephrine, anisomycin and EGTA were obtained from Sigma. KN93, U0126, H89, Protein Kinase A Inhibitor 14–22 Amide (PKI), SB202190, JNK inhibitor II, isoproterenol, 8-Br-AMPc and LY294002 were obtained from Calbiochem. FK506 was obtained from Fujisawa Pharmaceutical Co. and SB216763 from Tocris Bioscience. Drugs were used according to manufacturer's instructions.

2.5. Quantification of myonuclear Myf5 re-activation

Viable myofibres with *nlacZ* expression in virtually all myonuclei were considered to have activated the *Myf5* locus and the data expressed as a percentage of the total myofibres analysed for that condition (between 15–20 fibres). Data from at least three independent experiments were pooled to give a population mean (±SEM). Statistical differences between conditions were analysed by Kruskal–Wallis Student's *t* or *U*-Mann–Whitney tests.

3. Results

Our experimental model for examining the signalling pathways responsible for mediating *Myf5* expression is the $Myf5^{nlacZ/+}$ mouse [28]. In adult myofibres, only satellite cells have β-galactosidase activity. When cultured in mitogen-rich Plating Medium for 24 h however, myonuclei of EDL myofibres activate the *Myf5* locus, as shown by the presence of nuclear located β-galactosidase. Exposure to Plating Medium has to be for at least 18 h to elicit *Myf5* activation (data not shown). However, culture in DMEM (basal medium) for 24 h does not result in myonuclear *Myf5* expression. This system therefore, provides a model where *Myf5* activation can be readily studied.

3.1. Calcium levels affect the Myf5 locus in myonuclei

Since calcium signalling is central to many aspects of muscle regulation and becomes de-regulated following denervation [29], we initially explored its role in myonuclear activation of *Myf5*. Myofibres were cultured in the presence of drugs that alter cytosolic Ca^{2+} levels. Artificially raising cytosolic Ca^{2+}

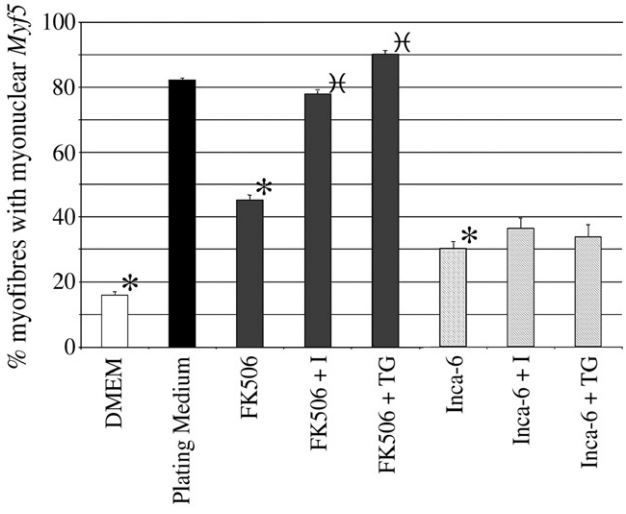

Fig. 3. Calcineurin and NFAT affect myonuclear *Myf5* activation. DMEM and Plating Medium were used as negative and positive controls, with calcineurin inhibition using FK506 (20 μM) and NFAT inhibition with Inca-6 (6.25 μM) in Plating Medium. All drugs were added in Plating Medium and analysed after 24 h in culture. Values are the mean ± SEM of data pooled from approximately 20 fibres from each of at least 3 $Myf5^{nlacZ/+}$ mice. (*$p<0.05$, comparing each treatment to myofibres cultured in Plating Medium; ⋇$p<0.05$ vs FK506). I, Ionomycin (1 μM); TG, thapsigargin (1 μM).

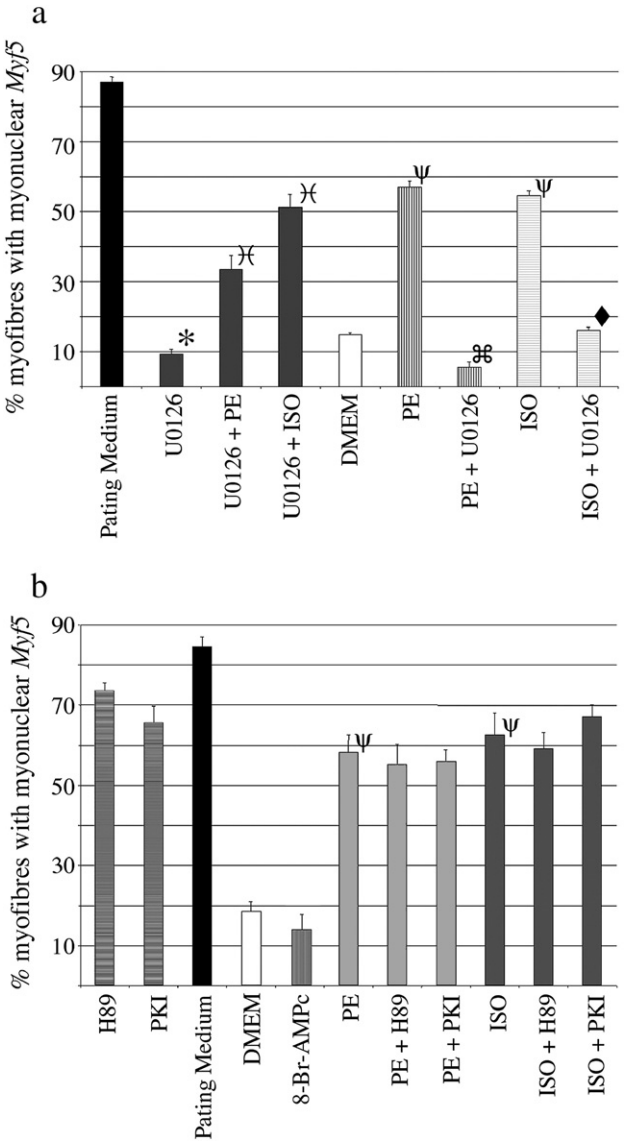

Fig. 4. The *Myf5* locus is activated by promoting ERK signalling. (a) Bar graph depicting the influence of ERK in *Myf5* activation using the MEK1/2 inhibitor U0126 (25 μM) in Plating Medium and α1- and β-adrenergic receptor agonists phenylephrine (40 μM) and isoproterenol (40 μM) in DMEM. (b) Bar graph showing that ERK is not cross-interacting with PKA signalling in *Myf5* activation using the PKA inhibitors H89 (10 μM) and PKI (10 μM) in Plating Medium, or the PKA agonist 8Br-cAMP (100 μM) in DMEM. Values are the mean±SEM of data pooled from approximately 20 myofibres from each of at least 3 $Myf5^{nlacZ/+}$ mice. ($p<0.05$, comparing each treatment to myofibres cultured in control Plating Medium (*), U0126 (⋇), DMEM (ψ), phenylephrine (PE; ⌘) or isoproterenol (ISO; ♦)).

levels by exposure to thapsigargin (1 μM) or ionomycin (1 μM) in DMEM for 24 h was sufficient to activate the *Myf5* locus (Fig. 1; $p<0.05$). A brief exposure (2, 4 or 6 h) though was not sufficient, with at least 18 h in the presence of ionomycin required to elicit myonuclear *Myf5* expression (data not shown).

Treatment with dantrolene (50 μM), which decreases cytosolic Ca^{2+} levels by blocking Ca^{2+} release from intracellular stores, resulted in an inhibition of the myonuclear *Myf5* activation normally elicited by Plating Medium (Fig. 1; $p<0.05$). Ionomycin was unable to completely reverse this effect on *Myf5* activation ($p=0.07$). Chelation of extracellular calcium with EGTA was also used, since it not only prevents extracellular Ca^{2+} influx but also depletes both cytosolic and intracellular Ca^{2+} stores. When EGTA (2 mM) was added to Plating Medium for 24 h, myonuclear reactivation was significantly reduced (Fig. 1; $p<0.05$).

3.2. Calmodulin regulates myonuclear Myf5 activation

Calmodulin is one of the main Ca^{2+}-interacting proteins in the cytosol and its Ca^{2+}-dependent activation leads to the subsequent activation of calmodulin kinase and calcineurin [30]. To further examine the role of calcium on *Myf5* transcription in myonuclei, we inhibited Ca^{2+}–calmodulin interactions by adding W7 (30 μM) in Plating Medium. This resulted in a significant decrease in activation of *Myf5* to only ~23% of control Plating Medium (Fig. 2; $p<0.05$) and raising Ca^{2+} levels

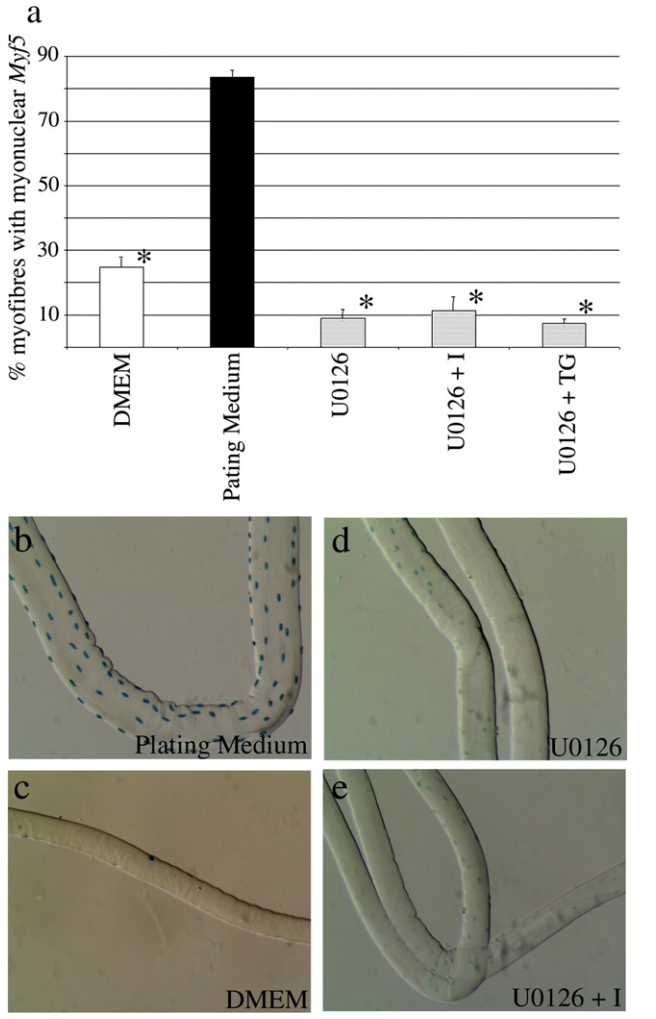

Fig. 5. Increasing calcium levels can not overcome ERK inhibition of *Myf5* activation. (a) Bar graph showing *Myf5* activation using the MEK1/2 inhibitor U0126 (25 μM) with ionomycin (1 μM) or thapsigargin (1 μM) in Plating Medium. Values are the mean±SEM of data pooled from approximately 20 myofibres from each of at least 3 $Myf5^{nlacZ/+}$ mice. ($p<0.05$, comparing each treatment to myofibres cultured in control Plating Medium (*). (b–e) Typical myofibres after exposure to the reagents indicated on the panels and incubation in X-Gal solution to reveal β-galactosidase activity in myonuclei. I, Ionomycin; TG, thapsigargin.

using ionomycin or thapsigargin could not overcome this effect (Fig. 2). To analyse the role of calmodulin kinase on this process, myofibres were incubated in Plating Medium with the calmodulin kinase inhibitor KN-93 (20 μM), which also significantly reduced the level of *Myf5* activation, to 46% of control Plating Medium (Fig. 2; $p<0.05$), though not to the extent observed with W7 (~23%). Neither ionomycin nor thapsigargin could reverse this decrease on *Myf5* activation caused by blocking calmodulin kinase with KN-93 (Fig. 2).

3.3. Calcineurin is involved in Myf5 regulation

To next examine the role of calcineurin in myonuclear *Myf5* expression, we blocked its activity by incubating myofibres in Plating Medium with FK506 (20 μM). Calcineurin inhibition caused a significant decrease in myonuclear *Myf5* activation to ~54% of Plating Medium control levels (Fig. 3; $p<0.05$) though again, not to the extent observed with W7 (~23%). Similar results were obtained by inhibiting calcineurin with Cyclosporine A (20 μM) (data not shown). Since NFAT is a known downstream target of calcineurin [31], we investigated whether it was responsible for mediating the calcineurin effect on *Myf5*. Culture of myofibres in Plating Medium with an NFAT inhibitor, Inca-6 (6.25 μM), significantly reduced activation of the *Myf5* locus in myonuclei (Fig. 3; $p<0.05$).

Since Ca^{2+} signalling works through numerous pathways, myofibres were treated with combinations of pharmacological agents to simultaneously affect Ca^{2+} levels and calcineurin/NFAT signalling. Activation of *Myf5* in myonuclei was restored to levels observed in Plating Medium alone when ionomycin was added together with the calcineurin inhibitor FK506 (Fig. 3; $p<0.05$). Crucially though, neither ionomycin nor thapsigargin in Plating Medium could overcome the block on myonuclear *Myf5* activation induced by inhibiting NFAT with Inca-6 (Fig. 3; $p<0.05$).

Together, these results suggest that incubation in mitogen-rich medium increases cytosolic Ca^{2+} levels, which regulates *Myf5* activation in myonuclei. This effect is mediated via Ca^{2+}–calmodulin interactions, which activate both calmodulin kinase and calcineurin-NFAT pathways. However, it seems that regulation of the *Myf5* locus by Ca^{2+} through calmodulin kinase is more potent than that of calcineurin signalling.

3.4. ERK signalling controls Myf5 activation

The ERK signalling pathway is known to be involved in various aspects of myogenesis and previous studies have shown that ERK signalling can be activated by Ca^{2+} [32]. Therefore, we next explored whether ERK is also part of the signalling cascade that results in *Myf5* activation in myonuclei.

Incubation of myofibres in Plating Medium with the MEK1/2 inhibitor U0126 (25 μM) resulted in an ~89% decrease in myonuclear *Myf5* activation (Fig. 4a; $p<0.05$). Treatment with U0126 together with α1- and β-adrenergic receptor agonists phenylephrine (40 μM) and isoproterenol (40 μM), that activate ERK signalling through G-protein coupled receptors, caused an increase in the percentage of myofibres with *Myf5* activation but still resulted in a significant decrease in myonuclear activation of

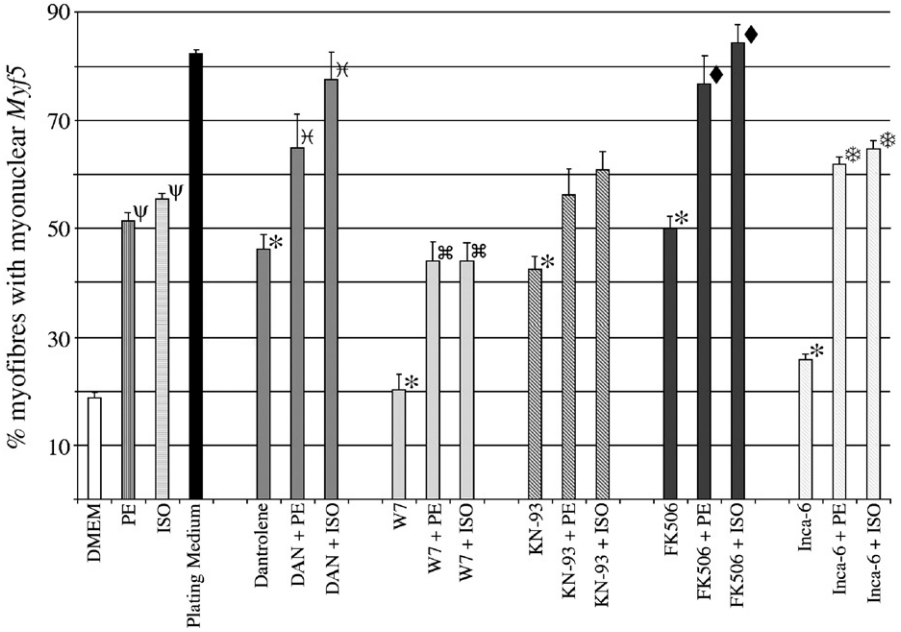

Fig. 6. Interactions between calcium and ERK signalling pathways regulate *Myf5* expression. Bar graph showing interaction of ERK signalling with calcium (dantrolene), calmodulin (W7, KN93), calcineurin (FK506) and NFAT (Inca-6). Myofibres cultured in DMEM plus phenylephrine (40 μM), isoproterenol (40 μM), DMEM alone or Plating Medium were used as controls. Dantrolene (50 μM), W7 (30 μM), KN-93 (20 μM), FK506 (20 μ) and Inca-6 (6.25 μM) were added in Plating Medium together with phenylephrine or isoproterenol. Values are the mean±SEM of data pooled from approximately 20 myofibres from each of at least 3 *Myf5*$^{nlacZ/+}$ mice. ($p<0.05$, comparing each treatment to myofibres cultured in Plating Medium (*), DMEM (ψ), dantrolene (DAN; ⋇), W7 (⌘), FK506 (♦) or Inca-6(❄)).

Myf5 compared to exposure to Plating Medium (Fig. 4a; $p<0.05$). Conversely, culture in DMEM with phenylephrine (40 μM) and isoproterenol (40 μM) showed that promoting ERK signalling resulted in an ~4 fold increase in myofibres that had activated the *Myf5* locus (Fig. 4a; $p<0.05$) in the absence of mitogens. Indeed, exposure to U0126 in DMEM resulted in a complete absence of myonuclear β-galactosidase activity induced by phenylephrine or isoproterenol (Fig. 4a; $p<0.05$), indicating that the effect is ERK-specific. Western blot analysis of phosphorylated ERK levels was carried out on isolated myofibres exposed to Plating Medium or DMEM/phenylephrine for 24 h, which both increased phosphorylated ERK levels, while exposure to U0126 in Plating Medium decreased levels (data not shown).

In order to further confirm that isoproterenol and phenylephrine were activating ERK signalling, and not adenylyl cyclase [33], myofibres were cultured in Plating Medium with the PKA inhibitors H89 (10 μM) and PKI (10 μM), or in DMEM with the PKA agonist 8Br-cAMP (100 μM). No effect on myonuclear *Myf5* expression was observed, indicating that PKA, thus adenylyl cyclase, is not regulating *Myf5* in adult muscle (Fig. 4b). Moreover, neither H89 and PKI prevented isoproterenol nor phenylephrine/DMEM-induced activation of the *Myf5* locus (Fig. 4b).

3.5. Calcium and ERK signalling pathways interact to control *Myf5* activation

To investigate whether Ca^{2+} and ERK signalling pathways interact to control myonuclear *Myf5* activation, myofibres in Plating Medium were treated with U0126 together with drugs that increase cytosolic Ca^{2+}. Increasing cytosolic Ca^{2+} by adding ionomycin or thapsigargin was not enough to overcome the 9 fold suppression induced by ERK inhibition in myonuclei, indicating that ERK is acting downstream of Ca^{2+} (Fig. 5).

Conversely, decreasing cytosolic Ca^{2+} by adding dantrolene in Plating Medium in the presence of phenylephrine or isoproterenol resulted in *Myf5* activation (Fig. 6; $p<0.05$). These ERK agonists also partially overcame the effect of blocking Ca^{2+}–calmodulin interactions with W7 on *Myf5* activation (Fig. 6; $p<0.05$), but were unable to reverse the inhibition caused by suppressing calmodulin kinase activity (Fig. 6), indicating that calmodulin kinase activity is necessary for ERK signalling.

It has been proposed that ERK can act either as a downstream or upstream effector of calcineurin and that it can either activate or inhibit calcineurin–NFAT activity, depending on cell type and specific NFAT isoforms present [34]. When *Myf5* activation was suppressed by inhibiting calcineurin activity, both phenylephrine and isoproterenol were able to reverse this effect (Fig. 6; $p<0.05$). Finally, these ERK agonists overcome the effect of NFAT inhibition (Fig. 6; $p<0.05$), indicating that ERK signalling is involved in myonuclear *Myf5* activation through a calcineurin/NFAT-independent mechanism.

3.6. p38 and JNK activity block *Myf5* activation

It is known that in myocytes, p38 and JNK phosphorylate NFAT, blocking its nuclear translocation [34]. To determine whether these MAPK signalling pathways were also involved in regulating *Myf5*, they were inhibited using JNK inhibitor II (20 μM) and the p38 kinase inhibitor SB202190 (20 μM). Neither was able to suppress the myonuclear activation of the *Myf5* locus caused by exposure to Plating Medium (data not shown). However, β-galactosidase activity was found after myofibres were cultured with these MAPK inhibitors in DMEM (Fig. 7; $p<0.05$). In accordance with these observations, the JNK and p38 agonist, anisomycin (20 μM) blocked the activation of *Myf5* completely in Plating Medium ($p<0.05$). Finally, the NFAT inhibitor Inca-6 (6.25 μM) counteracted the myonuclear *Myf5* activation induced by inhibiting JNK or p38 in DMEM (Fig. 7; $p<0.05$).

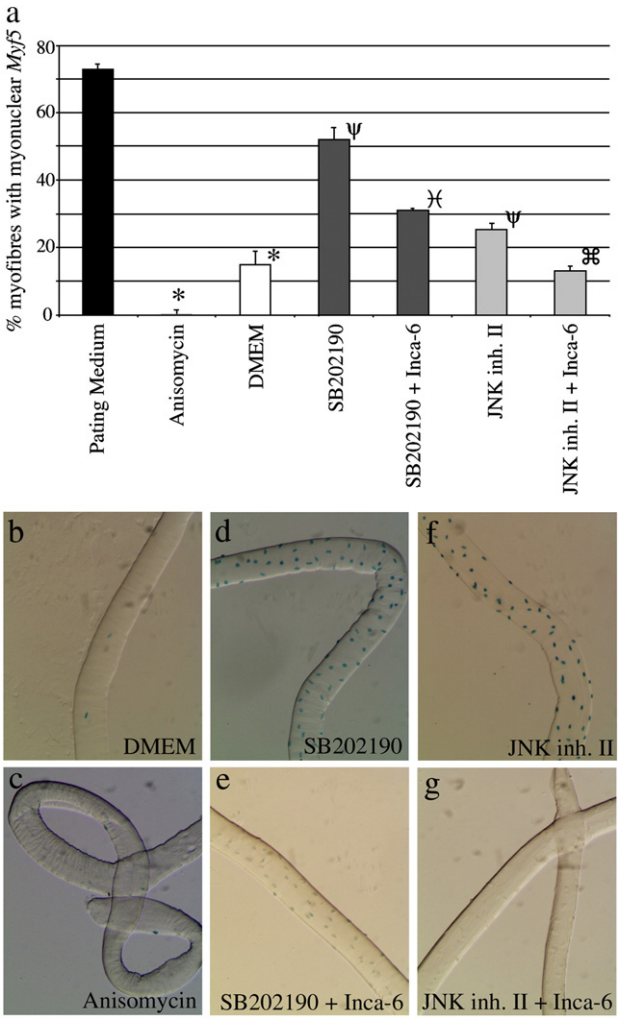

Fig. 7. JNK/p38 MAPKs block myonuclear activation of the *Myf5* locus. (a) Bar graph depicting the percentage of myofibres that had activated *Myf5* after treatment with the JNK and p38 agonist, anisomycin (20 μM) in Plating Medium, or JNK inhibitor II (20 μM) and the p38 kinase inhibitor SB202190 (20 μM) in DMEM. Interaction of JNK/p38 MAPKs with NFAT was examined using the NFAT inhibitor Inca-6 (10 μM). Approximately 20 myofibres from each of at least 3 $Myf5^{nlacZ/+}$ mice were analysed. Values are represented as mean±SEM. ($p<0.05$, comparing each treatment to myofibres cultured in control Plating Medium (*), DMEM (ψ), SB202190 (⋇) or JNK inhibitor II (⌘). (b–g) Typical myofibres after exposure to the reagents indicated on the panels for 24 h and incubation in X-Gal solution to reveal β-galactosidase activity in myonuclei.

3.7. IGF-1 can substitute for Plating Medium in activating Myf5

Plating Medium is undefined, being composed of DMEM with 10% horse serum and 0.5% CEE. To examine growth factors that could be involved in *Myf5* activation, we began with those known to be important in regulating muscle cell behaviour. 50 ng/ml Hepatocyte Growth Factor or 50 ng/ml basic Fibroblast Growth Factor in DMEM both failed to elicit *Myf5* activation in myonuclei (data not shown). In contrast, DMEM supplemented with 50 ng/ml of IGF-1 resulted in significant myonuclear activation of the *Myf5* locus after 24 h (Fig. 8a; $p<0.05$).

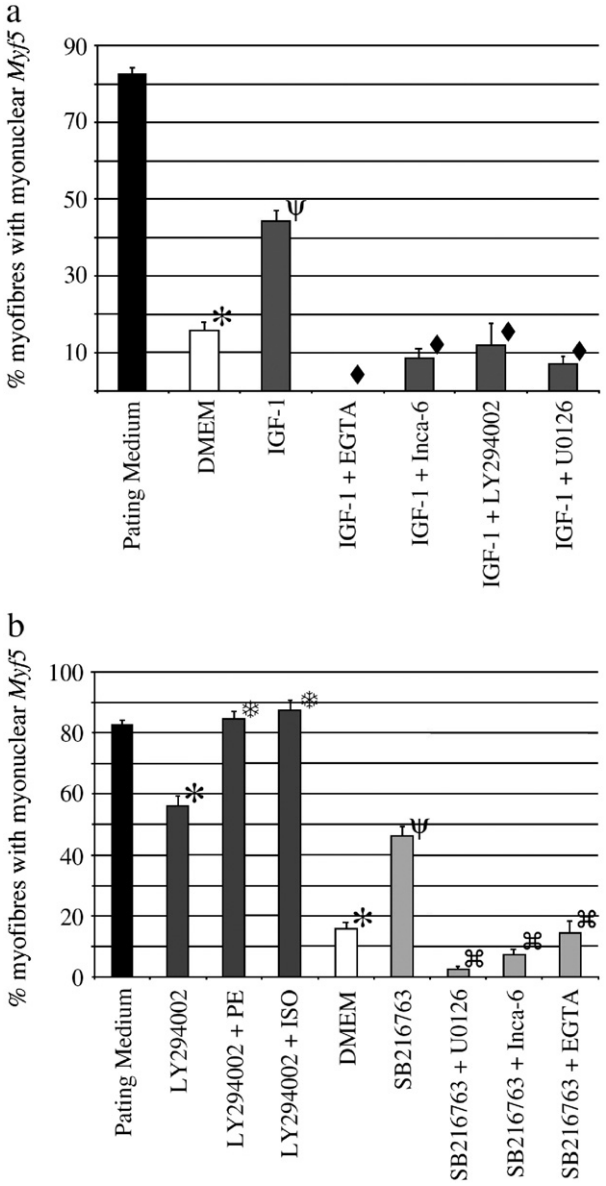

Fig. 8. IGF-1 activates the *Myf5* locus through different signalling pathways. (a) Bar graph depicting effects of IGF-1 in DMEM by inhibiting its three main modes of signalling: calcium/calcineurin (blocked with 2 mM EGTA or 6.25 μM Inca-6), PI3K (blocked with 25 μM LY294002) and ERK (inhibited with 25 μM U0126). (b) Bar graph showing the role of PI3K and its downstream target, GSK3β, in *Myf5* activation. LY294002 (25 μM), phenylephrine (40 μM) or isoproterenol (40 μM) were added in Plating Medium. GSK3β was blocked by adding SB216763 (20 μM) in DMEM and its interaction with ERK and/or calcium was analysed by adding U0126 (20 μM), Inca-6 (6.25 μM) or EGTA (2 mM). Data are means±SEM of approximately 20 myofibres from 3 $Myf5^{nlacZ/+}$ mice that were cultured for 24 h, fixed and stained in X-Gal solution. ($p<0.05$, comparing each treatment to myofibres cultured in control Plating Medium (*), DMEM (ψ), IGF-1 (♦), LY294002 (❄) or SB216763 (⌘)).

3.8. IGF-1 activates the Myf5 locus through different signalling pathways

As IGF-1 induces Ca^{2+} transients in cultured cells [35] and operates through calcium/calcineurin in muscle [63], we analysed whether IGF-1-induced activation of *Myf5* requires Ca^{2+} signalling. We found that chelation of extracellular calcium (2 mM EGTA) blocked myonuclear *Myf5* reactivation elicited by IGF-1 (Fig. 8a; $p<0.05$). Indeed, the NFAT inhibitor (Inca-6; 6.25 μM) had the same effect (Fig. 8a; $p<0.05$), suggesting that IGF-1 requires calcium/NFAT signalling to reactivate *Myf5*.

The two major routes for IGF-1 signalling are the PI3K and ERK pathways [36,37]. GSK3β is a PI3K downstream target, that is inactivated by phosphorylation, allowing nuclear translocation of NFAT [38]. A cross-interaction between PI3K/GSK3β and ERK signalling has been previously described [38–40]. To first determine whether myonuclear *Myf5* reactivation induced by IGF-1 involves PI3K and/or ERK, we added the PI3K inhibitor (LY294002; 20 μM) or ERK inhibitor (U0126; 20 μM) to myofibres cultured in DMEM plus IGF-1. Both inhibitors blocked the activation of the *Myf5* locus (Fig. 8a; $p<0.05$), confirming that IGF-1 acts through these pathways.

To show that Plating Medium also elicits the IGF-1/PI3K signalling pathway, we added LY294002 to myofibres cultured in Plating Medium, and found a significant decrease in *Myf5* activation (Fig. 8b; $p<0.05$). This effect however, was abolished by adding phenylephrine or isoproterenol, indicating that ERK is downstream of PI3K. To further analyse the PI3K/GSK3β pathway, we added GSK3β inhibitor (SB216763; 20 μM) to DMEM. The *Myf5* locus was activated ($p<0.05$), and either U0126, Inca-6 or EGTA blocked this effect completely (Fig. 8b; $p<0.05$), suggesting that GSK3β is cross-interacting with ERK and calcium/NFAT signalling in activating *Myf5*.

Together, these results show that the IGF-1 can substitute for Plating Medium and induce myonuclear activation of *Myf5*, acting through multiple signalling pathways, including calcium, PI3K/GSK3β and ERK.

4. Discussion

Myf5 plays a central role in muscle myogenesis and we are only just beginning to understand the signalling pathways that control its activation. Using our model of myonuclear activation of the *Myf5* locus, we show that mitogens in Plating Medium (specifically IGF-1) initiate a cascade of signalling that results in *Myf5* activation (Fig. 9). Ca^{2+} binds calmodulin and activates both calmodulin kinase and calcineurin, which then act through separate, though cross-talking, pathways. Calcineurin promotes NFAT, leading to myonuclear *Myf5* expression. Calmodulin kinase is necessary for ERK signalling which in turn, also promotes *Myf5* activation, but through a calcineurin/NFAT-

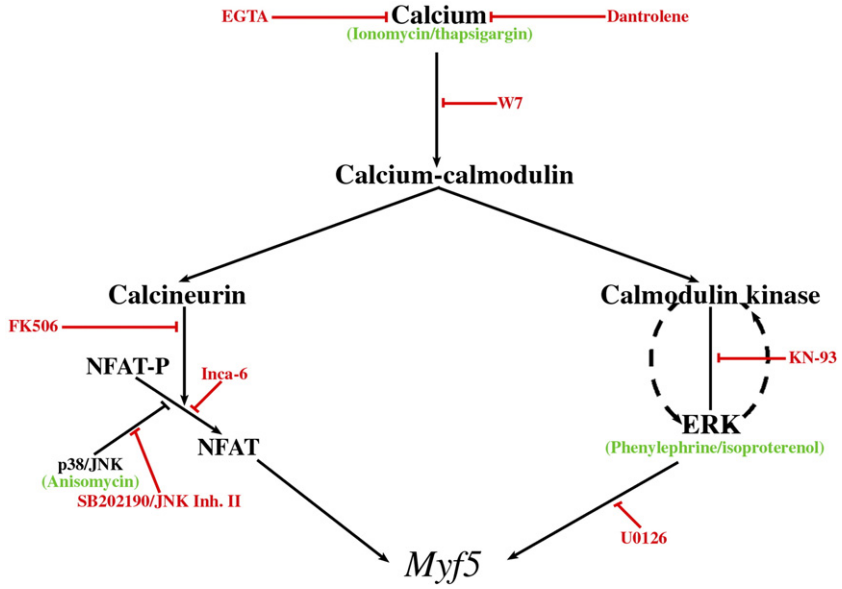

Fig. 9. Model of the pathways controlling myonuclear *Myf5* activation. When calcium cytosolic levels increases, calcium binds to calmodulin and this complex activates two independent, yet interconnected, signalling pathways. Calcineurin promotes myonuclear *Myf5* activation through NFAT activity. This process can be modified by p38/JNK activities. The second signalling pathway is through calmodulin kinase activity, which is necessary for ERK signalling. A positive feed-back between calmodulin kinase and ERK promotes myonuclear *Myf5* activation. That neither calcium nor ERK agonists could overcome the lack of *Myf5* activation suggest that calmodulin kinase activity might interact with calcineurin to promote NFAT, thus increasing *Myf5* activation. IGF-1 is responsible for a significant part of the *Myf5* activation caused by Plating Medium, acting through different signalling pathways, including PI3K, ERK and calcium. Agonists are shown in green and inhibitors in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

independent mechanism (Fig. 9). IGF-1 alone can also regulate *Myf5* activation via ERK, but also through PI3K.

While myonuclei of innervated adult $Myf5^{nlacZ/+}$ muscle do not contain β-galactosidase, muscle denervation results in myonuclear activation of the *Myf5* locus [18], in accord with previous studies that described an accumulation of *Myf5* transcript following denervation [22]. Muscle denervation produces a state of hyperexcitability where calcium homeostasis is de-regulated [29] and EDL myonuclei will activate expression of *nlacZ* when exposed to serum in culture [27]. Amongst other effects, serum induces depletion of internal calcium stores so increasing cytosolic calcium levels [41]. Chemically increasing intracellular Ca^{2+} levels also resulted in myonuclear *Myf5* activation whereas reducing levels using chelation of extracellular Ca^{2+} produced the opposite effect.

Increased cytosolic Ca^{2+} levels acting through Ca^{2+}-binding proteins (e.g. calmodulin) mediate many cellular events [42]. Ca^{2+}–calmodulin complex binds to and activates key proteins, including calcineurin [31] and calmodulin kinase [30], which we show here act through separate, but not independent pathways, to activate *Myf5* (Fig. 9).

Calcineurin regulates *Myf5* activation through NFAT activity. It has been postulated that muscle intracellular calcium transients evoked by fast nerve activity are insufficient to activate calcineurin, whereas sustained calcium elevation such as those occurring in slow muscle fibres can, thus promoting NFAT nuclear translocation [43]. However, recent studies have shown that this enzyme is not completely refractory to the activity of fast motor units and muscle denervation induces the activation of calcineurin in fast but not in slow muscles [44]. Denervation produces a state of hyperexcitability that is associated with higher intracellular Ca^{2+} [45], which would activate calcineurin independently of nerve activity [44]. Indeed, the catalytic subunit of calcineurin is expressed at higher levels in fast twitch muscles [46] while expression of proteins that block calcineurin activity are lower [47]. Thus when exposed to high Ca^{2+} levels such as when denervated, fast twitch fibres could respond more rapidly than slow fibres in promoting NFAT nuclear translocation. This may explain why the *Myf5* locus is rapidly activated in culture in the myonuclei of EDL myofibres but takes several days to be activated in the myonuclei of slow soleus myofibres [27].

Certainly this pathway has been shown to be important in skeletal muscle, suggesting that NFAT has distinct functions in multiple steps of skeletal myogenesis and may in turn be regulated by various signalling cascades. Whereas NFAT3 plays a pivotal role during embryonic development of primary myofibres, NFAT2 is essential for postnatal growth of skeletal muscle. Particularly relevant to our data is the observation that calcineurin/NFAT also regulates *Myf5* in reserve cells, a model of myogenic quiescence [48,49].

p38 kinase and JNK signalling operates in skeletal myogenesis [50,51] and can phosphorylate NFAT so blocking its nuclear translocation [34]. These MAPK are clearly involved in regulation of the *Myf5* locus, since their induction with anisomycin prevents *Myf5* activation while their inhibition promotes it. Indeed, the combined effects of NFAT inhibition with MAPK modulation suggests that p38 and JNK prevent myonuclear *Myf5* activation via inhibiting NFAT. This p38/JNK negative regulation of NFAT activity we see here is consistent with observations made in cardiomyocytes [34].

The other Ca^{2+} dependent pathway controlling *Myf5* activation signals through calmodulin kinase/ERK in a calcineurin/NFAT-independent mechanism. When either calcineurin or NFAT are inhibited, ERK agonists can still activate *Myf5* but not when calmodulin kinase is blocked. The role of ERK signalling has been characterised in cardiomyocytes and vascular smooth muscle cells [52] but little was known of its role in skeletal muscle [53]. There is increasing evidence of interactions between ERK and calcium signalling. In neurons and PC12 cells, ERK signalling is activated by Ca^{2+} increases through calmodulin kinase activity, however, in fibroblasts and keratinocytes, either Ca^{2+} or calmodulin kinase have a negative effect on ERK activation [32,54]. ERK can promote NFAT nuclear translocation in cardiomyocytes [34], but can also act directly to phosphorylate NFAT though, thus inhibiting its nuclear translocation [55].

The calcineurin and calmodulin kinase pathways do not act completely independently in *Myf5* activation though. These two pathways have been observed to cross-talk in several systems: calcineurin and calmodulin kinase induce cardiac hypertrophy [56], promote slow genes in skeletal muscle [57] and both act synergistically to activate cytokine genes in T cells [58]. While calmodulin kinase acts preferentially through MEF activation [56], it has been suggested that it cooperates in a synergistic manner with calcineurin to activate NFAT [44]. In cardiomyocytes a reciprocal but reinforcing signalling relationship between calcineurin and ERK has been shown, suggesting that calcineurin activation promotes ERK activation and that Ras–MEK–ERK activation promotes NFAT activation through an unknown mechanism [59,60]. The involvement of calcineurin and calmodulin kinase in regulating different aspects of skeletal myogenesis is still being unravelled [30,43] but *Myf5* can now be added to the list of genes regulated by this pathway (Fig. 9).

To identify the factor in Plating Medium that is responsible for *Myf5* activation, we screened several growth factors known to be important in muscle biology. Of those tested, only IGF-1 elicited myonuclear *Myf5* activation when added to myofibres in DMEM. Growth factors such as IGF-1 are produced by skeletal non-muscle cells or by myogenic cells in response to mechanical stress, and through specific cell surface receptors and intracellular signalling cascades, regulate myogenesis. Interestingly, despite the two main routes for IGF-1 signalling being PI3K and ERK [36,37], IGF-1 can also act through a calcium-dependent mechanism [61]. Our data suggest that IGF-1 activates the *Myf5* locus through all these pathways, including Ca^{2+}, PI3K/GSK3-β and ERK. Control of *Myf5* by IGF-I in adult muscle is consistent with observations that specific expression of IGF-I in muscle fibres results in significant muscle hypertrophy [62], working through calcineurin [63]. Thus, IGF-I could also be inducing *Myf5* activation of target muscle genes to contribute to hypertrophy.

5. Conclusions

The expression of *Myf5* in adult muscle is controlled by a complex, cross-talking signalling pathway, where calcium acts through calcineurin and calmodulin-mediated mechanisms. Calcineurin is necessary for NFAT activation, while calmodulin kinase is required for ERK signalling, to promote *Myf5* activation in the myonuclei of adult fast myofibres (Fig. 9). Interestingly, IGF-1 can substitute for Plating Medium and elicits myonuclear *Myf5* activation through a complex mechanism that includes calcium, ERK and PI3K/GSK3-β signalling pathways. These results may also provide useful insights to understand the mechanism by which *Myf5* is activated during development to determine the skeletal myogenic lineage.

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Figure 1

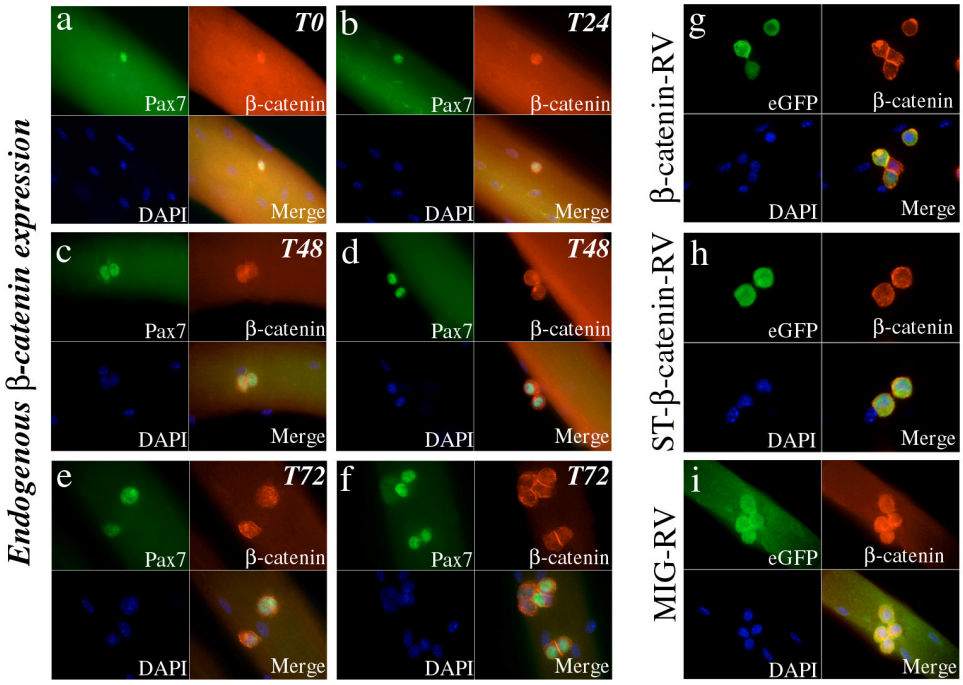

Endogenous β-catenin expression
a
T0
Pax7
β-catenin
DAPI
Merge
b
T24
c
T48
d
e
T72
f
g
β-catenin-RV
eGFP
h
ST-β-catenin-RV
i
MIG-RV

Figure 2

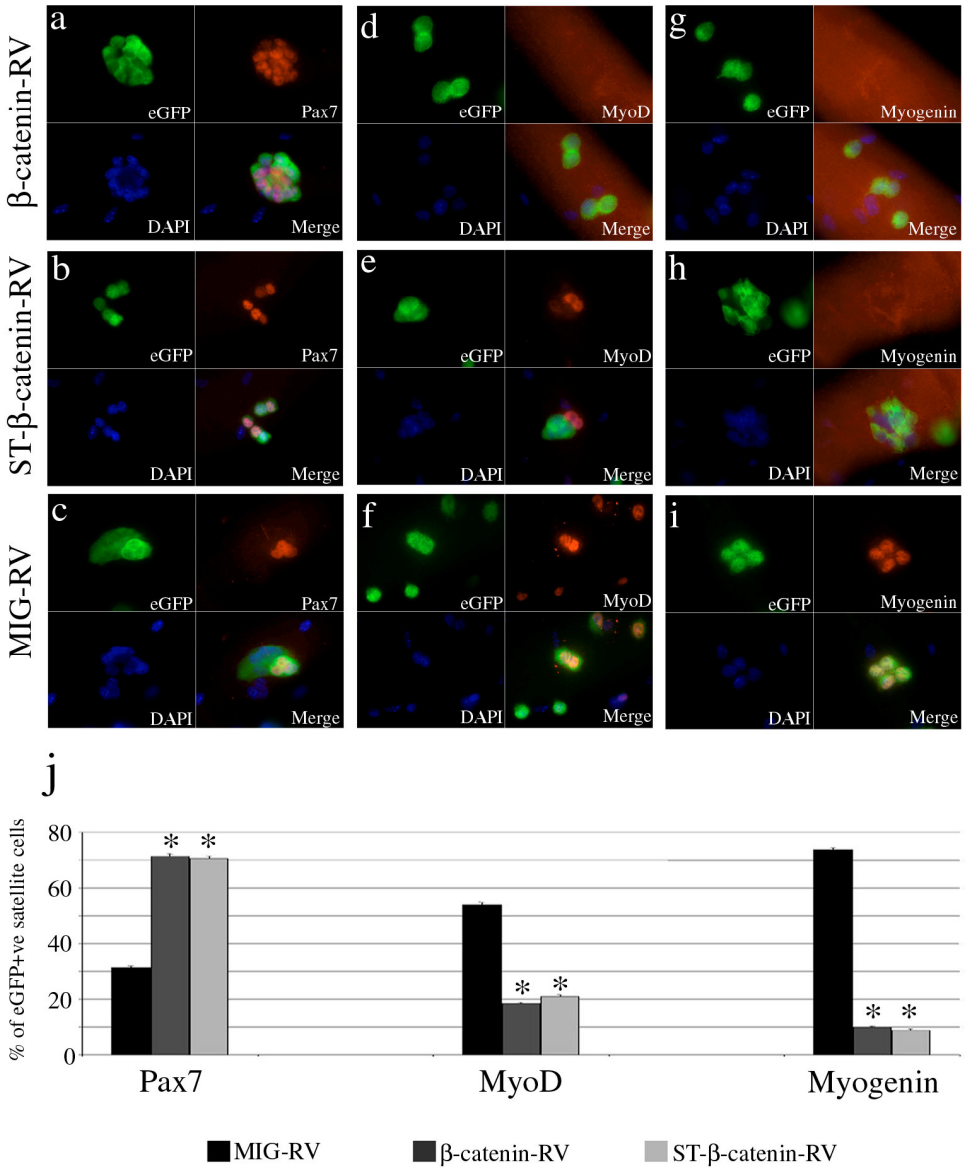

β-catenin-RV
ST-β-catenin-RV
MIG-RV
a
b
c
d
e
f
g
h
i
eGFP
Pax7
MyoD
Myogenin
DAPI
Merge
j
% of eGFP+ve satellite cells
80
60
40
20
0
Pax7
MyoD
Myogenin
MIG-RV
β-catenin-RV
ST-β-catenin-RV

Figure 3

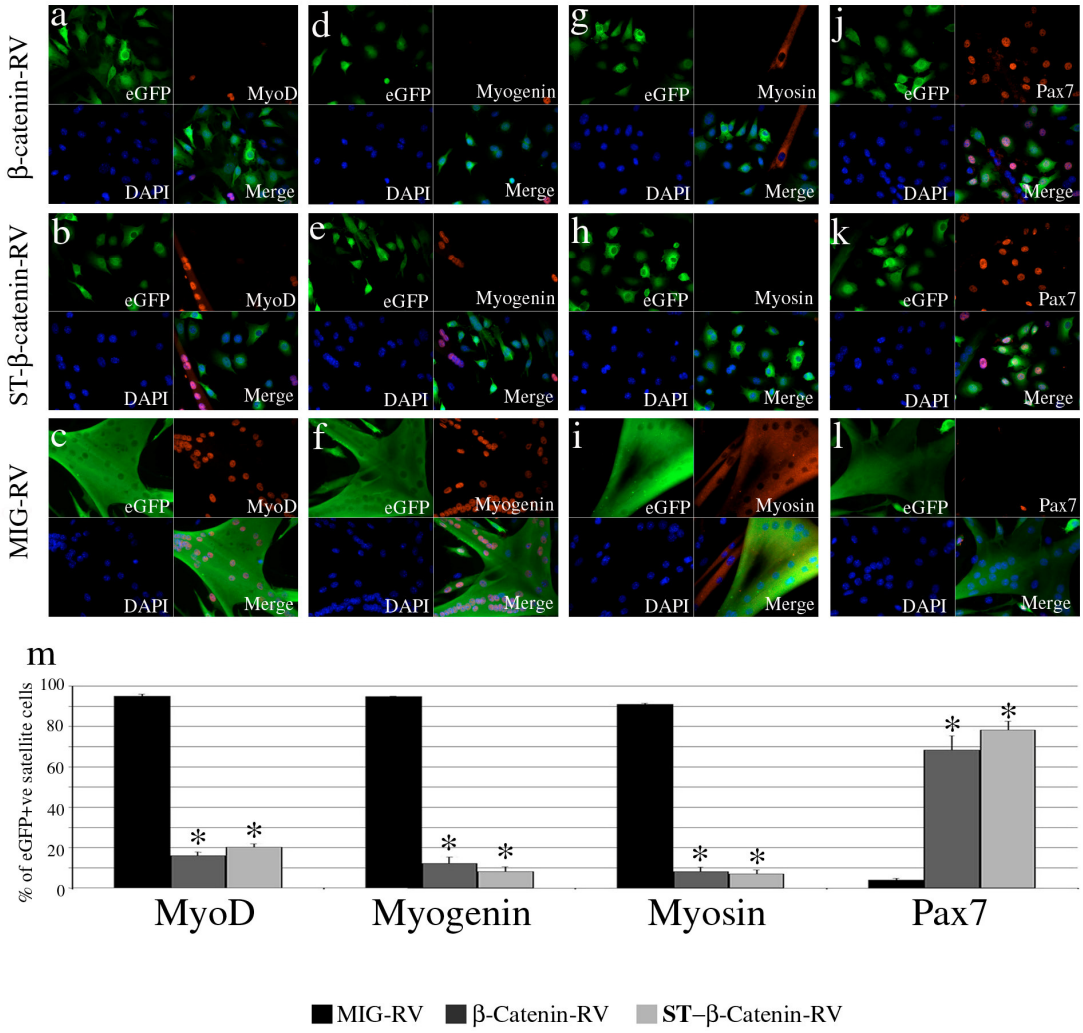

β-catenin-RV
ST-β-catenin-RV
MIG-RV
a
b
c
d
e
f
g
h
i
j
k
l
eGFP
MyoD
Myogenin
Myosin
Pax7
DAPI
Merge
m
% of eGFP+ve satellite cells
100
80
60
40
20
0
MyoD
Myogenin
Myosin
Pax7
MIG-RV
β-Catenin-RV
ST–β-Catenin-RV

Figure 4

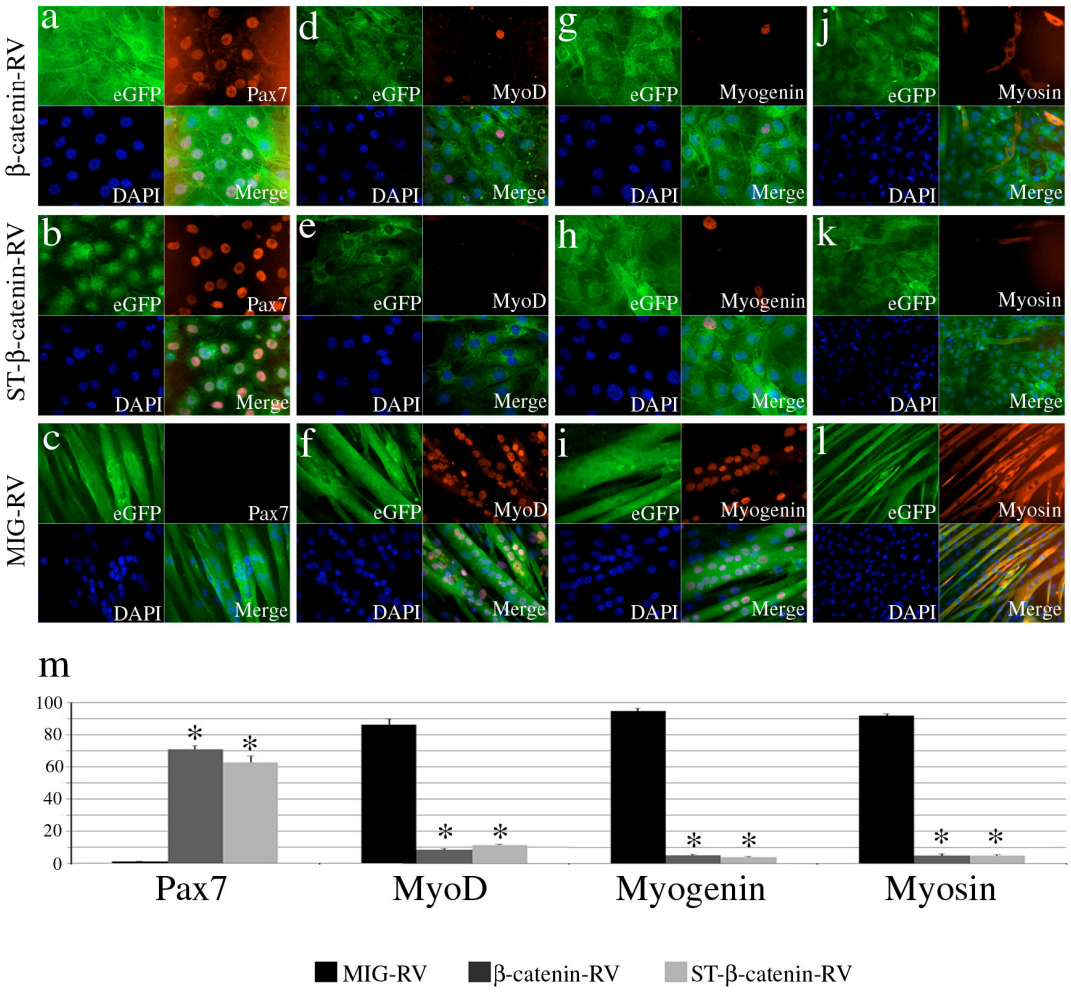

β-catenin-RV
ST-β-catenin-RV
MIG-RV
a
b
c
d
e
f
g
h
i
j
k
l
eGFP
Pax7
MyoD
Myogenin
Myosin
DAPI
Merge
m
100
80
60
40
20
0
*
Pax7
MyoD
Myogenin
Myosin
MIG-RV
β-catenin-RV
ST-β-catenin-RV

Figure 5

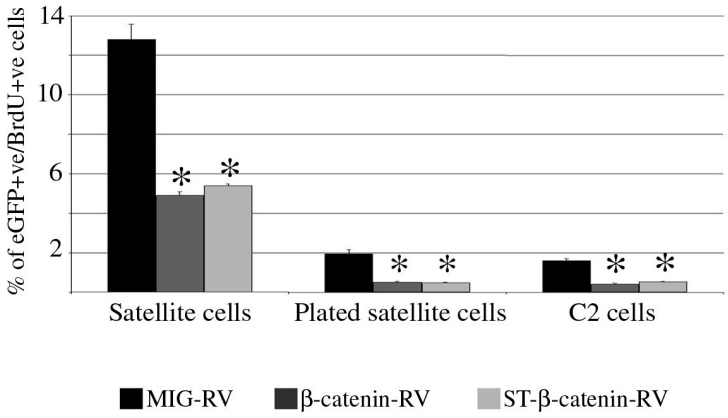

% of eGFP+ve/BrdU+ve cells
14
10
6
2
*
*
*
*
*
*
Satellite cells
Plated satellite cells
C2 cells
MIG-RV
β-catenin-RV
ST-β-catenin-RV

Figure 6

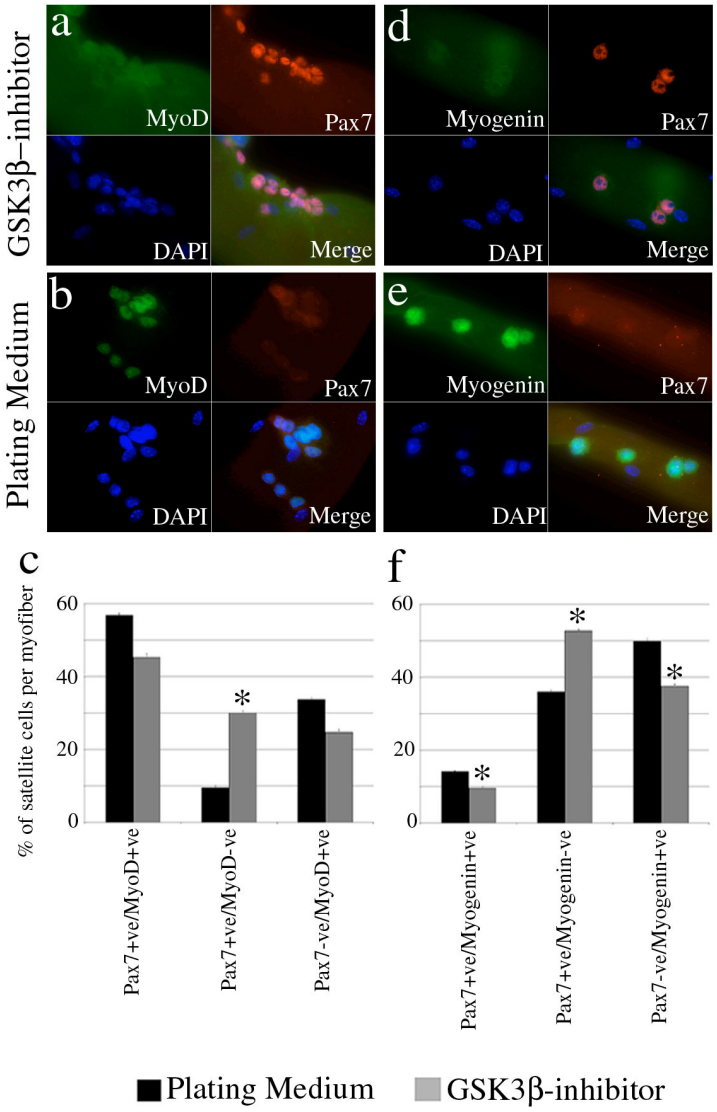

GSK3β–inhibitor
Plating Medium
a
MyoD
Pax7
DAPI
Merge
d
Myogenin
Pax7
DAPI
Merge
b
MyoD
Pax7
DAPI
Merge
e
Myogenin
Pax7
DAPI
Merge
c
% of satellite cells per myofiber
60
40
20
0
Pax7+ve/MyoD+ve
Pax7+ve/MyoD-ve
Pax7-ve/MyoD+ve
f
60
40
20
0
Pax7+ve/Myogenin+ve
Pax7+ve/Myogenin-ve
Pax7-ve/Myogenin+ve
Plating Medium
GSK3β-inhibitor

Figure 7

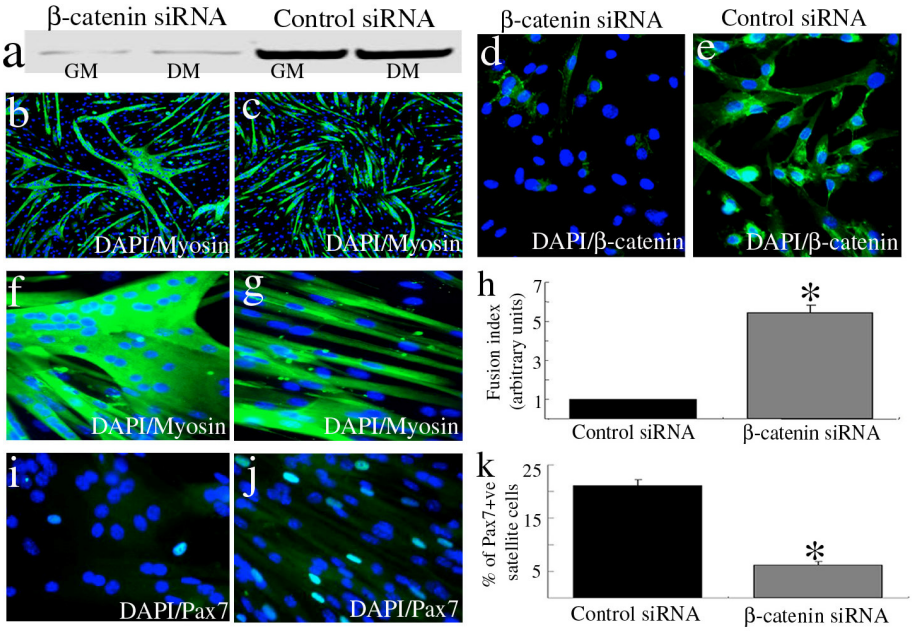
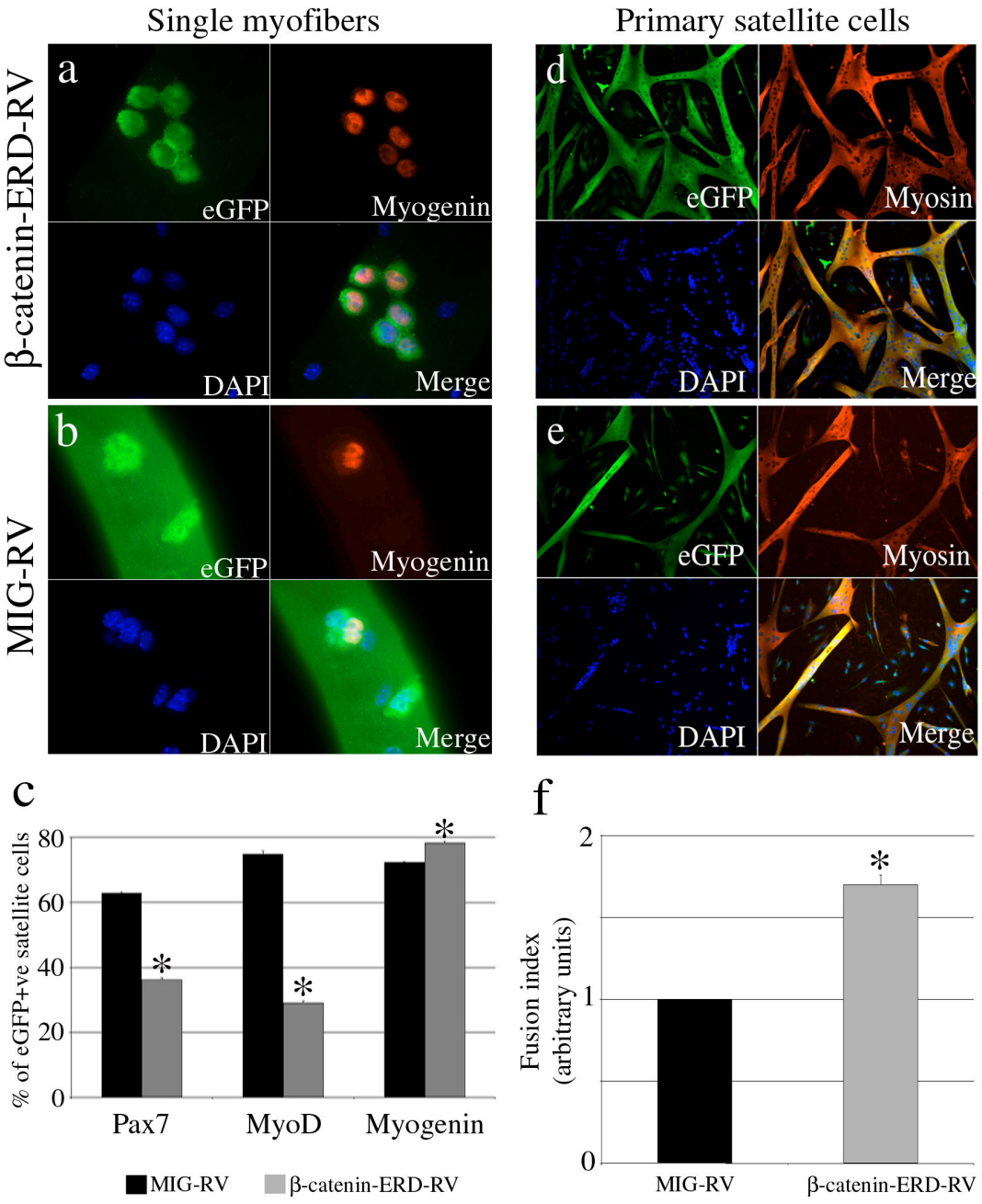

β-catenin siRNA
Control siRNA
a
GM
DM
GM
DM
b
DAPI/Myosin
c
DAPI/Myosin
β-catenin siRNA
Control siRNA
d
DAPI/β-catenin
e
DAPI/β-catenin
f
DAPI/Myosin
g
DAPI/Myosin
h
Fusion index (arbitrary units)
7
5
3
1
*
Control siRNA
β-catenin siRNA
i
DAPI/Pax7
j
DAPI/Pax7
k
% of Pax7+ve satellite cells
25
15
5
*
Control siRNA
β-catenin siRNA

Figure 8


Single myofibers
Primary satellite cells
β-catenin-ERD-RV
MIG-RV
a
b
d
e
eGFP
Myogenin
DAPI
Merge
Myosin
c
% of eGFP+ve satellite cells
0
20
40
60
80
Pax7
MyoD
Myogenin
MIG-RV
β-catenin-ERD-RV
f
Fusion index (arbitrary units)
0
1
2
MIG-RV
β-catenin-ERD-RV

Muscle satellite cell fate choice is influenced by β-catenin

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Running title: β-catenin influences satellite cell fate

Key words: satellite cell, stem cell, skeletal muscle, β-catenin, Pax7, MyoD, cell fate, self-renewal

Abstract

Satellite cells are the resident stem cells of adult skeletal muscle. As with all stem cells, how the choice between self-renewal or differentiation is controlled is central to understanding function. Here we have explored the role played by β-catenin in satellite cell fate choice. Satellite cells express β-catenin, which is maintained as they activate and undergo proliferation. Constitutive expression of wild-type or stabilised β-catenin results in more satellite cells expressing Pax7 without MyoD and so adopting the self-renewal pathway, with fewer satellite cell progeny undergoing myogenic differentiation. Similarly, preventing degradation of endogenous β-catenin by inhibiting GSK3β activity also results in more satellite cell progeny expressing Pax7 and not differentiating. Consistent with these observations, it was found that using *siRNA* to down-regulate β-catenin or a dominant-negative version to repress β-catenin transcriptional targets in satellite cells, both augmented differentiation. Together, these observations show that the role of β-catenin in satellite cells is bimodal: directing satellite cells to the self-renewal pathway and away from immediate myogenic differentiation, in addition to its acknowledged role in myoblast fusion.

Introduction

Homeostasis, hypertrophy and repair of adult skeletal muscle are carried out by resident stem cells called satellite cells, located on the surface of the myofibre, below the surrounding basal lamina (Mauro, 1961; reviewed in Zammit, et al., 2006a). Normally mitotically quiescent, satellite cells must first be activated to undergo extensive proliferation to generate myoblasts that eventually differentiate to repair/replace myofibres. We have recently shown that satellite cell self-renewal is the primary mechanism responsible for maintaining a viable satellite cell pool (Zammit et al., 2004; Collins et al., 2005). When transplanted into muscle in association with a myofibre, satellite cells proliferate extensively and give rise to both much donor-derived muscle and many new viable satellite cells (Collins et al., 2005). This process can be modelled in culture where satellite cell progeny adopt divergent fates. Quiescent satellite cells express the paired box transcription factor family member Pax7 (Seale et al., 2000). When activated, they co-express Pax7 with MyoD (Grounds et al., 1992; Yablonka-Reuveni and Rivera, 1994), a member of the myogenic regulatory factor family (MyoD, Myf5, myogenin and Mrf4). After proliferation, most then down-regulate Pax7 and differentiate. In contrast, other satellite cell progeny maintain Pax7 but lose MyoD and withdraw from both cell cycle and immediate differentiation, returning to a quiescent-like state (Zammit et al., 2004). A similar mechanism has also been described during muscle growth in chicken (Halevy et al., 2004) and rat (Schultz et al., 2006).

What controls whether a satellite cell either self-renews or differentiates is only beginning to be understood. Pax7 is transcriptionally active in quiescent satellite cells and remains so in those that adopt the self-renewal pathway, with constitutive Pax7 expression delaying myogenic differentiation (Zammit et al., 2006b). The Notch pathway too has been implicated in this fate choice (Conboy and Rando, 2002) and recently it has been shown that inhibition of Notch signalling leads to less satellite cell self-renewal and increased commitment to differentiation (Kuang et al., 2007).

Wnt signalling also plays a vital role in myogenesis, particularly during development (reviewed in Parker et al., 2003). It has been shown to contribute to induction of the myogenic lineage (Munsterberg et al., 1995; Tajbakhsh et al., 1998), and may be actively suppressed initially during adult muscle regeneration (Zhao and Hoffman, 2004). Wnts act through distinct canonical and non-canonical signalling pathways. The canonical pathway involves the stabilisation of β-catenin that can then translocate to the nucleus and control transcription via the T cell factor/lymphocyte enhancement factor (TCF/LEF) family of transcription factors (reviewed in Willert and Jones, 2006). β-catenin however, not only influences cellular events as a necessary transcriptional co-activator, but also has an important role in cell adhesion complexes. It binds members of the

cadherin family of adhesion molecules at the cell membrane (Kuch et al., 1997; Ozawa et al., 1989) that is involved in myogenic differentiation, particularly myoblasts fusion into multinucleated myotubes (Goichberg et al., 2001). Indeed, the importance of β-catenin to adult muscle function is demonstrated by the recent observation that it is essential for muscle hypertophy in response to overload (Armstrong et al., 2006).

Here, we sought to determine whether β-catenin was involved in satellite cell fate choice. We found that β-catenin was expressed in satellite cells and their progeny. Constitutive β-catenin expression or blocking degradation of endogenous β-catenin in satellite cells affects cell fate choice, with more satellite cells remaining Pax7+ve and less undergoing myogenic differentiation. Similarly, β-catenin inhibited differentiation but increased the proportion of reserve cells in C2 cultures. In comparison, reducing levels of β-catenin using *siRNA* or repressing its transcriptional targets with a dominant-negative version, both lead to enhanced myogenic differentiation of satellite cell progeny. Together these observations show that the role of β-catenin is bimodal: directing satellite cells to the self-renewal pathway and away from immediate myogenic differentiation, in addition to its acknowledged role in myoblast fusion.

Results

Satellite cells express β-catenin

Immunostaining was first used to determine if satellite cells express β-catenin during myogenic progression. Freshly isolated satellite cells weakly expressed β-catenin with most exhibiting a perinuclear/cytoplasmic staining at this time before MyoD is detectable (Fig. 1a). β-catenin expression is up-regulated in activated satellite cells (Fig. 1b) after 24 hours of culture in their niche on the myofibre. When analysed after the first division (48h of culture), many cells had nuclear located β-catenin (Fig. 1c-d). Satellite cells adopt divergent fates after ~72 hours in culture (Zammit et al., 2004) and at this time, β-catenin expression was clearly evident in both Pax7+ve and Pax7-ve cells (Fig. 1e-f). Nuclear β-catenin levels were decreased as satellite cells differentiated or underwent self-renewal, with β-catenin located at the cell surface or peri-nuclearly in most cells (Fig. 1d and e). Therefore, mouse satellite cells in culture express β-catenin during myogenic progression, as has been shown for rat adult myogenic cells (Ishido et al., 2006; Wrobel et al., 2007).

Constitutive β-catenin maintains Pax7 expression in satellite cells

Having shown that β-catenin is present in satellite cells, we next examined the effects of consitutive β-catenin expression on satellite cell fate choice. Retroviral constructs encoding wild-type β-catenin (*pMSCV-β-catenin-IRES-eGFP*) or a stabilised version (*pMSCV-ST-β-catenin-IRES-eGFP*) with amino acid substitutions to prevent phosphorylation and subsequent degradation (Barth et al., 1999) were made. Myofibre-associated satellite cell progeny infected with *pMSCV-β-catenin-IRES-eGFP*, *pMSCV-ST-β-catenin-IRES-eGFP* or control *pMSCV-IRES-eGFP* (*MIG)* were identified by the presence of eGFP (Fig. 1g-i). Co-immunostaining for eGFP and β-catenin showed that the two immunosignals co-localised in infected satellite cells (Fig. 1g-i).

When infected satellite cell progeny were co-immunostained for eGFP and Pax7, it was found that the percentage of cells infected with either wild-type (Fig. 2a) or stabilised β-catenin retroviral construct (Fig. 2b) that were expressing Pax7 was significantly higher ($p<0.05$) than those cells infected with control retrovirus (Figure 2c, quantified in j - ~71% versus ~31% respectively).

Constitutive β-catenin prevents satellite cells entering myogenic differentiation

Since more satellite cell progeny expressing either wild-type or stabilised β-catenin contained Pax7, we next examined MyoD and myogenin expression to determine whether myogenic differentiation was compromised (Zammit et al., 2004). Co-immunostaining for eGFP and MyoD

showed that there were significantly less infected satellite cell progeny (*p<0.05*) expressing MyoD in the presence of constitutively expressed wild-type or stabilised β-catenin (Fig 2d and e) than control retrovirus (Figure 2f, quantified in j). The predominance of the Pax7+ve/MyoD-ve phenotype in cells with constitutively expressed β-catenin indicates that β-catenin drives satellite cells towards the self-renewal pathway (Zammit et al., 2004). Co-immunostaining for eGFP and myogenin demonstrated that there were significantly fewer (*p<0.05*) eGFP+ve satellite cell progeny co-expressing myogenin in cultures of *pMSCV-β-catenin-IRES-eGFP* (Fig. 2g) or *pMSCV-ST-β-catenin-IRES-eGFP* (Fig. 2h) infected cells compared to parallel cultures infected with control retrovirus (Fig. 2i and quantified in j - ~10% versus ~74% respectively), showing that fewer satellite cells were entering myogenic differentiation in the presence of constitutively expressed β-catenin.

Constitutive β-catenin inhibits fusion of satellite cell-derived myoblasts

Culture of satellite cells associated with a myofibre is a useful model to examine the early events of myogenic progression in satellite cells but is less suited for studying the later events of differentiation, such as myoblast fusion. Culture of myofibres on Matrigel allows satellite cells to migrate from the myofibre onto the tissue culture substrate and proliferate, before differentiating and fusing into multi-nucleated myotubes. Such satellite cell-derived myoblast cultures were infected with *pMSCV-β-catenin-IRES-eGFP*, *pMSCV-ST-β-catenin-IRES-eGFP* or control *pMSCV-IRES-eGFP*, followed later by culture in mitogen-poor medium to trigger differentiation. Myogenic differentiation and fusion were clearly compromised, as shown by immunostaining for MyoD (Fig. 3a-c), myogenin (Fig 3d-f) and myosin heavy chain (MyHC) (Fig. 3g-i, quantified in m).

Constitutive β-catenin promotes self-renewal in adult myogenic cells

While the majority of plated primary myogenic cells challenged with serum withdrawal respond by differentiating, others maintain Pax7, down regulate MyoD and exit the cell cycle, entering a quiescent-like state (Kitzmann et al., 1998; Collins et al., 2007). The presence of constitutive wild-type or stabilised β-catenin increased the proportion of Pax7 expressing cells in mitogen-poor medium compared to controls (Fig. 3j-l, quantified in m). Since the percentage of cells expressing MyoD was reduced and myogenin failed to be robustly induced by mitogen withdrawal, these observations show that β-catenin induces myogenic quiescence.

We also tested the effects of β-catenin using the C2 reserve cell model of myogenic quiescence (Yoshida et al., 1998). Levels of Pax7 vary between C2 clones, with some completely lacking Pax7 expression as proliferating myoblasts, but this does not affect their myogenicity (Olguin and Olwin, 2004; Zammit et al., 2006b). The C2 clone used here had a mean of 17±5% proliferating

cells expressing Pax7, which was not significantly changed by infection with retroviruses encoding wild-type or stabilised β-catenin (data not shown). When C2 cells infected with control *pMSCV-IRES-eGFP* were switched to mitogen-poor medium to force a fate choice, the vast majority differentiated, while rare cells became Pax7 expressing reserve cells (Fig 4c, quantified in m), consistent with previous observations (Olguin and Olwin, 2004; Zammit et al., 2006b). Crucially, retroviral infection with wild-type or stabilised β-catenin, followed later by culture in mitogen-poor medium, resulted in a considerable increase in the number of reserve cells (Pax7+ve/MyoD-ve), compared to control cultures (Fig 4a-b, quantified in m). Consistent with observations in primary satellite cells, the presence of wild-type or stabilised β-catenin effectively inhibited myogenic differentiation (Fig 4 d-l, quantified in m). In both satellite cells and a myogenic cell line therefore, the presence of wild-type or stabilised β-catenin is pro self-renewal.

Constitutive β-catenin slows cell cycle progression

To determine whether constitutive β-catenin perturbed the cell cycle, we infected satellite cell progeny with either *pMSCV-β-catenin-IRES-eGFP*, *pMSCV-ST-β-catenin-IRES-eGFP* or control *pMSCV-IRES-eGFP* and then after 72 hours, pulsed with BrdU for 3 hours and fixed. Co-immunostaining for eGFP and BrdU showed that approximately 60% less satellite cell progeny containing wild-type or stabilised β-catenin incorporated BrdU compared to control infected cells when maintained on the myofibre (Fig. 5). Satellite cell-derived myoblasts and C2 cells were also infected as above and then cultured in differentiation medium for several days to determine whether the presence of wild-type or stabilised β-catenin allowed the cells to continue to proliferate, but it did not (Fig. 5).

Inhibition of β-catenin phosphorylation promotes Pax7 expression

β-catenin is phosphorylated by GSK3β at its NH_2 terminus and tagged for ubiquitination and subsequent proteosomal degradation (Aberle et al., 1997). Therefore, when GSK3β is inhibited, β-catenin is stabilised, leading to accumulation in the cytoplasm, translocation to the nucleus and activation of transcriptional targets. Satellite cells associated with a myofibre were exposed to 10 μM SB216763 (Coghlan et al., 2000) in plating medium for 3 days to inhibit GSK3 and so stabilize endogenous β-catenin. It was found that the number of Pax7+ve/MyoD-ve satellite cell progeny was significantly increased ($p<0.05$) compared to myofibres cultured in plating medium alone (Fig. 6a and b, quantified in c), demonstrating that the stabilized endogenous β-catenin was driving satellite cells towards self-renewal. Immunostaining for Pax7 and myogenin (Fig 6d and e, quantified in f) also showed more satellite cell progeny were Pax7+ve/myogenin-ve when GSK3β was inhibited, so less were committing to differentiation (Pax7-ve/Myogenin+ve) ($p<0.05$, Fig. 6f). Thus these observations on pharmacological inhibition of GSK3β to raise endogenous β-catenin

levels are consistent with observations using constitutive retroviral expression of wild-type or stabilised β-catenin (Fig. 2).

Down-regulation of β-catenin promotes myogenic differentiation

Since increasing the levels of β-catenin promotes Pax7 expression and inhibits differentiation, we next asked whether decreasing levels of β-catenin would have the opposite effect. β-catenin levels were reduced using *siRNA*-mediated gene down regulation. Transfection with *β-catenin-siRNA* caused a significant decrease in β-catenin levels in both proliferating (GM) and differentiated (DM) C2 cells, as shown by Western blot analysis (Fig 7a). Dropping levels of β-catenin with *β-catenin-siRNA* resulted in an increased number and size of C2-derived myotubes (Fig. 7b and c). Having established that the *β-catenin-siRNA* was effective, primary plated satellite cell-derived myoblasts were transfected and analysed by immunostaining. As expected, in the presence of *β-catenin-siRNA*, few satellite cell-derived myoblasts were found with significant β-catenin immunosignal compared to those transfected with control *siRNA* (Fig. 7d and e). Reducing β-catenin levels using *β-catenin-siRNA* and then inducing differentiation caused enhanced fusion compared to satellite cell-derived myoblasts transfected with control *siRNA* (Fig 7f and g, quantified in h). Significantly fewer satellite cell-derived Pax7+ve cells were observed in differentiated cultures after transfection with *β-catenin-siRNA* than control transfected (Fig 7i and j, quantified in k).

Repressing β-catenin transcriptional targets stimulates differentiation

To examine whether the enhanced differentiation observed with decreased β-catenin levels was due to transcriptional activity, we employed a dominant-negative version of β-catenin (Montross et al., 2000). In the β-catenin-ERD construct, the activation domain of β-catenin is replaced by the engrailed repressor domain (ERD), which blocks the activation of target gene transcription by β-catenin (Han and Manley, 1993). Importantly, β-catenin-ERD is still able to associate and function with the cadherin complex, so allowing examination of its direct role in transcriptional regulation without perturbing its role in cadherin-mediated events (Montross et al., 2000). Myofibres were infected with *pMSCV-β-catenin-ERD-IRES-eGFP* and 48 hours later were fixed and immunostained (Fig. 8). The presence of β-catenin-ERD, as shown by *eGFP* expression, significantly increased the number of satellite cells containing myogenin (Fig. 8a) compared to control infected (Fig. 8b, quantified in c), while less satellite cells expressing β-catenin-ERD (eGFP+ve) contained Pax7 and MyoD (quantified in Fig. 8c). Plated satellite cell-derived myoblasts infected with β-catenin-ERD encoding retrovirus and allowed to differentiate (Fig. 8d), had a higher fusion index compared to control *pMSCV-IRES-eGFP* infected (Fig 8e, quantified in

f). These experiments show that transcriptional repression of β-catenin targets is pro-differentiation.

Discussion

One of the fundamental questions in stem cell biology is what drives the choice between self-renewal or differentiation? We previously showed that satellite cells adopt alternative fates in culture: while most differentiate, others maintain Pax7 but lose MyoD and escape immediate differentiation (Zammit et al., 2004). This provides an accessible model with which to examine factors that influence satellite cell fate.

Hitherto, control of muscle satellite cell fate choice has mainly centred on Pax and Notch genes. We have found that Pax7 is transcriptionally active in satellite cells that adopt the self-renewal pathway and that constitutive Pax7 can delay myogenic differentiation (Zammit et al., 2006b). Recently Pax7 has been shown to inhibit MyoD function, while myogenin can inhibit Pax7, providing possible mechanistic insight (Olguin et al., 2007). In the case of Notch signalling, activated Notch is cleaved by γ-secretase to allow the intracellular domain to translocate to the nucleus to control transcription. Satellite cell activation is accompanied by activation of Notch1 that leads to proliferation (Conboy and Rando, 2002). Treatment of proliferating satellite cells with an inhibitor of γ-secretase results in a shift to a Pax7-ve/MyoD+ve phenotype, indicating that Notch signalling may also be involved in adoption of the self-renewal pathway (Kuang et al., 2007). Inhibition of Notch signalling may be achieved by Numb, which is proposed to direct self-renewal in one daughter after an asymmetric division in satellite cells (Conboy and Rando, 2002; Kuang et al., 2007; Shinin et al., 2006). Recently, it has been suggested that the satellite cell pool contains a sub-population that is more stem cell-like, with the remainder acting more like transit-amplifying cells (Kuang et al., 2007; Shinin et al., 2006).

β-catenin is also able to control stem cell fate; promoting self-renewal of haematopoietic stem cells (Reya et al., 2003) and neuronal precursor cells in early development (Hirabayashi et al., 2004). In myogenesis, it has been shown that β-catenin can activate *Myf5* in somites, as part of the commitment of multi-potent stem cells to the myogenic lineage (Borello et al., 2006) and induce myogenesis in pluripotent embryonal carcinoma P19 cells (Petropoulos and Skerjanc, 2002).

α-catenin and β-catenin are present in skeletal muscle cells (Kuch et al., 1997) and here we show that mouse satellite cells in culture express β-catenin during myogenic progression, as has been shown for adult rat-derived myogenic cells (Ishido et al., 2006; Wrobel et al., 2007) and C2 cells (e.g. Goichberg et al., 2001). We found that constitutive expression of wild-type or stabilised β-catenin, or prevention of degradation of endogenous β-catenin, result in MyoD down-regulation and no induction of myogenin in satellite cells, consistent with observations in C2 cells

(Goichberg et al., 2001). These experimental interventions actually directed satellite cell-derived myoblasts towards the self-renewal pathway, with Pax7 expression maintained and less cell division. Indeed, constitutive β-catenin actually increased the number of C2 reserve cells expressing Pax7 after induction of myogenic differentiation by mitogen withdrawal, consistent with a role in promoting self-renewal (Collins et al., 2007). β-catenin has been shown to also induce Pax gene expression during development (Capdevila et al., 1998) and in P19 cells (Petropoulos and Skerjanc, 2002). Conversely, when β-catenin is down-regulated with *siRNA* or has its transcriptional targets repressed using a dominant-negative β-catenin-ERD, satellite cells tend towards differentiation, producing myotubes with a higher fusion index, and fewer reserve cells; again compatible with β-catenin silencing having a stimulatory effect on myogenin in C2 cells (Gavard et al., 2004).

β-catenin can affect cell function as part of canonical Wnt signalling. Wnts comprise a large family of secreted signalling molecules, which bind different frizzled receptors of the serpentine family and low-density lipoprotein receptor-related protein family and act through distinct canonical and non-canonical signalling pathways. The canonical pathway involves the stabilisation of β-catenin that can then translocate to the nucleus and control transcription as a necessary coactivator of TCF/LEF transcription factors (reviewed in Willert and Jones, 2006).

The role of Wnts in developmental myogenesis is well established. Wnt1 is expressed in the neural tube and has been shown to activate expression of *Myf5* in epaxial myotome, while Wnt7a is expressed in dorsal ectoderm and activates expression of *MyoD* in hypaxial myotome (Munsterberg et al., 1995; Tajbakhsh et al., 1998) with Wnt3a also able to activate *MyoD* (reviewed in Cossu et al., 1996; Parker et al., 2003). Recently, it has been shown that Wnt activation of Myf5 in somites involves this canonical β–catenin pathway (Borello et al., 2006). Members of the Wnt family are also implicated in regenerative myogenesis in adult, where there is evidence that Wnt signalling may be involved in myogenic activation in C2 reserve cells (Rochat et al., 2004) and recruiting non-satellite cells to the myogenic lineage (Polesskaya et al., 2003). Interestingly global trends in gene expression have been reported over the course of regeneration using entire muscle tissue, showing that secreted frizzled-related *mRNA* increases initially after induction of muscle regeneration, indicating that Wnt signalling may be actively suppressed, although this study did not reveal changes in gene expression specifically in satellite cells (Zhao and Hoffman, 2004). Speculatively, this may allow sufficient satellite cell proliferation to occur for effective muscle repair, before fate decisions need be made using Wnt/β-catenin signalling. In our hands, co-culture of satellite cells in their niche on an isolated myofibre with Wnt1 producing cells does promote self-renewal (unpublished observations), consistent with our findings on β-catenin function.

β-catenin also functions in association with the cadherin complex at the cell membrane. The C-terminus of cadherin associates with β-catenin, which in turn can recruit the actin-binding α-catenin, thus linking adherence junctions to the actin cytoskeleton. In muscle cells, β-catenin associates with both N-cadherin and M-cadherin (Kuch et al., 1997). β-catenin levels rise rapidly when differentiation is induced and it is clearly located at the cell membrane. This interaction between cadherins and β-catenin is involved in formation of functional adherens junctions, central to cell fusion (e.g. Goichberg et al., 2001). In proliferating myoblasts though, β-catenin is often found in the nucleus (Goichberg et al., 2001) and we show here that constitutive expression of wild type or stabilized β-catenin at this time prevents myogenic differentiation. However, since β-catenin-ERD is able to associate and function normally with the cadherin complex (Montross et al., 2000), yet β-catenin and β-catenin-ERD show opposite effects on satellite cell fate choice, it indicates that repression of transcriptional targets elicited by β-catenin-ERD, rather than through cadherin interaction, is more likely the mode of action.

β-catenin-TCF/LEF and β-catenin-cadherin interactions might actually be antagonist. Stimulation of cadherin-mediated adhesion in proliferating myoblasts induces cell cycle arrest and myogenic differentiation (Gavard et al., 2004; Goichberg and Geiger, 1998). Both nuclear localization and LEF-1-responsive reporter activation in cells expressing high levels of β-catenin can be inhibited by over-expressing N-cadherin or α-catenin (Sadot et al., 1998; Simcha et al., 1998). Our results suggest that cytosolic accumulation of β-catenin inhibits myogenic differentiation through direct transcriptional control, rather than an N-cadherin adhesion independent mechanism.

Together, these results indicate that the fate that a satellite cell adopts is influenced by β-catenin. Our study suggests a dual role for β-catenin: activating transcription in proliferating satellite cell progeny to influence cell fate choice in favour of self-renewal, and then later, functioning in complexes with cadherins to facilitate events involved in cell fusion into myotubes.

Materials and Methods

Myofibre isolation and cell culture

Mice were killed by cervical dislocation and the extensor digitorum longus (EDL) muscle was carefully removed and myofibres isolated as described in detail elsewhere (Rosenblatt et al., 1995). For suspension culture, myofibres were incubated in plating medium (DMEM with 10% (v/v) horse serum (PAA Laboratories) and 0.5% (v/v) chick embryo extract (ICN Flow)) at 37°C in 5% CO_2. For adherent cultures, myofibres were placed in LAB-TEK® 8-well chamber slides (Nunc) coated with 1 mg/ml Matrigel (Collaborative Research Inc.) in plating medium and maintained at 37°C in 5% CO_2. Satellite cells were then passaged and re-plated at high density to examine myogenic differentiation and fusion. C2 myogenic cells (Yaffe and Saxel, 1977) were cultured using standard techniques. Where used, BrdU was added to the medium at a final concentration of 10 μM. GSK3β was inhibited by exposing cells to 10μm SB216763 (Coghlan et al., 2000) obtained from TOCRIS bioscience. Cultures were fixed in 4% paraformaldehyde/PBS for 12 minutes and then rinsed several times in PBS.

Immunostaining

Fixed myofibres were permeabilised with 0.5% (v/v) Triton X-100/PBS and non- specific antibody binding blocked using 20% (v/v) goat serum/PBS (Beauchamp et al., 2000). Primary antibodies were applied overnight at 4ºC and included monoclonal anti-BrdU (clone BU1/75: Abcam), anti-β–catenin (Cell Signalling), anti-myogenin (clone F5D: DakoCytomation or DSHB), anti-MyoD1 (clone 5.8a: DakoCytomation), anti-Pax7 (DSHB), anti-myosin heavy chain (MF20), rabbit polyclonal anti-MyoD (Santa-Cruz), anti-myogenin (Santa-Cruz), anti-eGFP (Molecular Probes) and anti-β–catenin (Abcam). Primary antibodies were visualised with fluorochrome-conjugated secondary antibodies (Molecular Probes) before mounting in DakoCytomation Faramount fluorescent mounting medium containing 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI).

Retroviral expression vectors

The retroviral backbone *pMSCV-puro* (Clontech) was modified to replace the puromycin selection gene with *eGFP*, to create *pMSCV-IRES-eGFP* (*MIG-RV*), which served as the control vector (Zammit et al., 2006b). Wild-type β-catenin cDNA was then cloned in *pMSCV-IRES-eGFP* to produce *pMSCV-β-catenin-IRES-eGFP*, producing *β-catenin* as a bicistronic message with *eGFP*. We also used a stabilised version of β-catenin that contains introduced mutations to amino acids Ser-33, Ser-37, Thr-41, and Ser-45, which were all changed to Ala, to prevent phosphorylation and degradation (Barth et al., 1999) to produce *pMSCV-ST-β-catenin-IRES-eGFP*. The β-catenin dominant-negative construct has the C-terminal transactivation domain of β-catenin replaced with the active repression domain of *Drosophila* Engrailed (ERD) (Montross et al., 2000). *β-catenin-*

ERD was cloned in *pMSCV-IRES-eGFP*, generating *pMSCV-β-catenin ERD-IRES-eGFP*, used to repress β-catenin transcriptional targets. Retroviruses were then packaged in 293T cells using standard methods.

Retroviral infection

$5x10^3$ satellite cells or $2x10^3$ C2 cells were plated in each well of LAB-TEK® 8-well chamber slides (Nunc). The following day the media was replaced with undiluted 293T supernatant with 4 μg/ml polybrene and left at 37°C for 3 hours before the cells were rinsed and placed in fresh medium. Calculation of the infection rate in C2 cells showed that it was usually in the order of 80%. (control MIG - 91,8 ± 0.76%, wild-type β-catenin - 76,3 ± 1.03% and stabilised β-catenin - 82,5 ± 1,96% - percent of eGFP+ve cells/total cells (DAPI+ve) derived from 5 random fields per condition (total ~300 cells) from each of 3 independent experiments). To infect satellite cells associated with myofibres, 1/10 dilution of the retroviral containing supernatant was used but without polybrene.

RNA interference

Transfection with *siRNA* was performed in 6-well plates, when the cells were 30% confluent. *siRNA* duplexes (Stealth *siRNA*; Invitrogen) were diluted in OptiMEM (Invitrogen) to 20 pmol/well and incubated with Lipofectamine 2000 (Invitrogen) diluted in OptiMEM, according to the manufacturer's instructions. A second transfection was then carried out 24 hours after the first. To induce myogenic differentiation, transfected cells were transferred to 8-well chamber slides (Lab-Tek, Napervill, IL) and incubated in differentiation medium (DMEM plus 2% horse serum) for 72 hours. The sequence for β-catenin *siRNA* was 5'-GGACGTTCACAACCGGATTGTAAT-3' with a control *siRNA* selected by Invitrogen.

Image capture

Immunostained myofibres and plated cells were viewed on a Zeiss Axiophot 200M microscope and digital images acquired with a Charge-Coupled Device (Zeiss AxioCam HRm) using AxioVision software version 4.4 (Zeiss). Images were optimized globally and assembled into figures using Adobe Photoshop.

Data Analysis

Where satellite cells associated with an isolated myofibre were infected and examined, then eGFP+ve cells were analysed from multiple fibres until n was ~400-500 cells/mouse, at least three mice were analysed. For plated satellite cells, 10 random fields were examined, which normally equated to 400-500 eGFP+ve cells in total per mouse, and at least three mice were analysed. For C2 cells, again in the order of 400-500 cells were examined from each experiment. In all cases, data were pooled and expressed as population means±SEM and significant

differences between conditions tested using Student's *t*-test, where $p<0.05$ was considered significant.

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Figure legends

Figure 1. Satellite cells express β-catenin. Immunostaining of EDL myofibers immediately after isolation (a) showed that some Pax7+ve satellite cells (green) expressed nuclear β-catenin (red). Similarly, once activated (T24), β-catenin could be found in the nucleus of satellite cells (b). After the first division (T48) β-catenin persisted in the nucleus (c) and at cell-cell contacts (d) of satellite cell progeny. When satellite cells began to differentiate or underwent self-renewal (T72), β-catenin staining generally decreased in the nucleus, being localised in the cytoplasm or perinuclearly (e and f). To test the retroviral vectors, satellite cells were infected with either pMSCV-β-catenin-IRES-eGFP (g), pMSCV-ST-β-catenin-IRES-eGFP (h) or control pMSCV-IRES-eGFP (MIG) (i). Immunostaining for eGFP (green) and β-catenin (red) revealed that infected satellite cells co-expressed these proteins. Counterstaining with DAPI was used to identify all nuclei present.

Figure 2. β-catenin is pro-self-renewal in satellite cells. Cultured myofibers were exposed to retrovirus and immunostained 72 hours later. Most proliferating satellite cells infected with pMSCV-β-catenin-IRES-eGFP (a) or pMSCV-ST-β-catenin-IRES-eGFP (b) co-expressed eGFP (green) and Pax7 (red) in contrast to control pMSCV-IRES-eGFP (MIG) infected cells (c, quantified in j). In contrast, there were less satellite cell progeny co-expressing wild-type or stabilised β-catenin (eGFP - green) with MyoD (red in d-e) and myogenin (red in g-h) compared to controls (f and i, quantified in j). Counterstaining with DAPI was used to identify all nuclei present. Values are population means±SEM from three mice, where * denotes significant difference ($p<0.05$) from control cultures using Student's *t*-test.

Figure 3. Constitutive β-catenin prevents myogenic differentiation of satellite cell-derived myoblasts. To determine the effects of β-catenin on the later events of differentiation, such as cell fusion, plated satellite cell-derived myoblasts were infected and after one week of culture in mitogen-poor medium, fixed and immunostained. Few satellite cells containing retrovirally-encoded wild-type or stabilised β-catenin (eGFP+ve, green) contained MyoD (a and b, red), myogenin (d and e, red) or MyHC (g and h, red) in contrast to control-infected cultures, which comprised many multinucleated myotubes, expressing MyoD (c), myogenin (f) and myosin (i). By contrast, Pax7 protein (red) was present in practically all satellite cells infected with pMSCV-β-catenin-IRES-eGFP and pMSCV-ST-β-catenin-IRES-eGFP (j-k) while control pMSCV-IRES-eGFP (MIG) infected satellite cells only had occasional unfused cells with Pax7 protein (l). Counterstaining with DAPI was used to identify all nuclei present. Quantified in m, where values

are population mean±SEM from at least 10 random fields and * denotes significant difference (*p<0.05*) from control cultures using Student's *t*-test.

Figure 4. β-catenin increases the proportion of reserve cells. When C2 cells are induced to differentiate by culture in low-mitogen medium, most respond by differentiating, but some exit the cell cycle and express Pax7, entering a quiescent-like state to become "reserve cells" (Yoshida et al., 1998; Olguin and Olwin, 2004). Infection of proliferating C2 cells with retrovirus encoding wild-type (a) or stabilised β-catenin (b) and subsequent culture in low-mitogen medium resulted in many infected eGFP+ve (green) cells expressing Pax7 (red). In cells infected with control MIG-RV however, most responded by differentiating and fusing into large multi-nucleated myotubes, with Pax7 expression restricted to the occasional reserve cell (c). As with primary satellite cells, most C2 cells with constitutive β-catenin (eGFP, green) did not contain MyoD (d-e, red), myogenin (g-h, red) or myosin protein (j-k, red), while control infected cells fused well to form myotubes with robust MyoD (f), myogenin (i) and MyHC (l) expression. Counterstaining with DAPI was used to identify all nuclei present. Quantification in m where values are mean±SEM of eGFP+ve cells counted from at least 10 random fields and * denotes significant difference ($p<0.05$) from control cultures using Student´s *t*-test.

Figure 5. β-catenin perturbs cell cycle progression in myogenic cells. Myofibre-associated satellite cells, plated satellite cell-derived myoblasts or C2 cells were infected with pMSCV-β-catenin-IRES-eGFP, pMSCV-ST-β-catenin-IRES-eGFP or control pMSCV-IRES-eGFP (MIG). In myofibre-associated satellite cell cultures, BrdU was added for 3 hours, the cells fixed and immunostained. It was found that BrdU incorporation was significantly reduced by constitutive β-catenin expression. For plated satellite cells and C2 cells, infection was followed by culture in mitogen-poor medium for a week before the BrdU pulse. The presence of β-catenin further reduced the number of cells able to incorporate BrdU. Data represent the mean±SEM of infected cells per myofiber (satellite cells) or from at least 10 random field (plated satellite cells or C2 cells), all from three independent experiments. * indicates that data are statistically significant (*p<0.05*) from controls using Student's *t*-test.

Figure 6. Inhibition of degradation of endogenous β-catenin promotes satellite cell self-renewal. Myofibres were cultured in plating medium containing the GSK3β inhibitor SB216763 for 3 days, then fixed and immunostained. Inhibition of β-catenin phosphorylation, and so degradation, increased significantly the number of Pax7+ve/MyoD-ve cells (a) compared to control cells (b, quantified in c). Accordingly, stabilisation of β-catenin significantly reduced the number of myogenin+ve cells, whilst increasing Pax7+ve/myogenin-ve cells (d) compared to control cells (e,

quantified in f). Counterstaining with DAPI was used to identify all nuclei present. Data is the population mean±SEM from three independent experiments. * denotes significant statistical difference (*p<0.05*) from control using Student's *t*-test.

Figure 7. Knockdown of endogenous β-catenin levels enhances differentiation. Western blotting shows that transfection of *β-catenin-siRNA* significantly reduced β-catenin levels in proliferating (GM) C2 cells compared to transfection with control *siRNA*, which remained reduced for at least 3 days following induction of differentiation (DM) (a). C2 cells transfected with *β-catenin-siRNA* to knockdown β-catenin levels showed enhanced differentiation (b) compared to cells transfected with control *siRNA* (c). Similarly, immunostaining showed that satellite cell-derived myoblasts also exhibited reduced β-catenin levels when transfected with *β-catenin-siRNA* (d) compared to control *siRNA* (e). Lower β-catenin levels promoted fusion into large myotubes (f) compared to control cells (g, quantified in h). Reduced β-catenin levels also significantly decreased the number of Pax7+ve reserve cells (i) compared to control (j, quantified in k). Counterstaining with DAPI was used to identify all nuclei present. Random fields were selected from three independent cultures and values expressed as mean±SEM, where * denotes significant statistical difference (*p<0.05*) from control using Student's *t*-test.

Figure 8. Repressing β-catenin transcriptional activity enhances myogenic differentiation. Myofibre-associated satellite cells infected with pMSCV-β-catenin-ERD-IRES-eGFP (a) had significantly more eGFP+ve (green) cells expressing myogenin (red) compared to control infected (MIG) (b, quantified in c). Co-immunostaining for eGFP and Pax7 or MyoD showed that less satellite cell progeny expressing β-catenin-ERD contained either Pax7 or MyoD (quantified in c). Transcriptional repression of β-catenin target genes by β-catenin-ERD enhanced myogenic differentiation (d), with myotubes with a higher fusion index than control infected myotubes (e, quantified in f). Counterstaining with DAPI was used to identify all nuclei present. Values are population mean±SEM. Statistically significant difference (*p<0.05*) were denoted by * using Student´s *t*-test.