

The chemokine SDF1 controls multiple steps of myogenesis through atypical PKC ζ

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Summary

Mice deficient in the SDF1-chemokine-receptor CXCR4, exhibit severe defects of secondary limb myogenesis. To further elucidate the role of SDF1 in muscle development, we have now analyzed putative effects of this chemokine on proliferation, migration and myogenic differentiation of mouse C2C12 myogenic progenitor/myoblast cells. In addition, we have characterized the signaling pathways employed by SDF1-CXCR4 to control myogenesis. We found that SDF1 stimulates proliferation and induces migration of C2C12 cells with a potency similar to that of FGF2 and HGF, which both represent prototypical extracellular regulators of myogenesis. In addition, SDF1 inhibits myogenic differentiation in both C2C12 cells and primary myoblasts, as assessed by MyoD, myosin heavy chain and/or myogenin expression. Regarding signaling pathways, C2C12 cells responded to SDF1 with activation (phosphorylation) of Erk and PKC ζ , whereas even after prolonged SDF1 treatment for up to 120 minutes, levels of activated Akt, p38 and PKC α or PKC β remained unaffected. Preventing activation of the classic MAP kinase

cascade with the Erk inhibitor UO126 abolished SDF1-induced proliferation and migration of C2C12 cells but not the inhibitory action of SDF1 on myogenic differentiation. Moreover, the effects of SDF1 on proliferation, migration and differentiation of C2C12 cells were all abrogated in the presence of myristoylated PKC ζ peptide pseudosubstrate and/or upon cellular depletion of PKC ζ by RNA interference. In conclusion, our findings unravel a previously unknown role of CXCR4-PKC ζ signaling in myogenesis. The potent inhibitory effects of SDF1 on myogenic differentiation point to a major function of CXCR4-PKC ζ signaling in the control of secondary muscle growth.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/120/22/4050/DC1>

Key words: CXCR4, Myogenin, MyoD, Myosin heavy chain, Myoblasts

Introduction

In vertebrate embryos, skeletal muscles develop from transient mesodermal structures, the somites (Christ and Ordahl, 1995). Within the somite, muscle progenitors are restricted to its dorsal part, the dermomyotome (Christ and Ordahl, 1995). The medial lip of the dermomyotome generates epaxial musculature (deep paraspinal and intercostal muscles), whereas the lateral lip gives rise to muscles of the ventral body wall, diaphragm, tongue and limbs (Buckingham et al., 2003). In contrast to muscles of the body wall, development of muscles of limbs, diaphragm and tongue requires that myogenic progenitors delaminate from the dermomyotome and take up long-range migration (Buckingham et al., 2003). Progenitor-cell delamination and migration are controlled by several genes, including *Pax3*, a paired domain transcription factor that is initially expressed throughout the segmental plate and, later on, delineates the migratory progenitor pool within the dermomyotome (Williams and Ordahl, 1994), and the tyrosine receptor *Met*, only present in delaminating premyogenic cells (Christ and Brand-Saberi, 2002). The correct migration of delaminated precursors into the limb buds further requires expression of the homeodomain transcription factor *Lbx1* (Gross et al., 2000). Migrating precursors only become committed to the myogenic lineage

and, hence, express the myogenic differentiation factors MyoD, Myf5, myogenin and MRF4 upon entering the limb bud (Tajbakhsh and Buckingham, 2000). The expression of MyoD and Myf5 mark the onset of the myogenic commitment and is succeeded by myogenin and MRF4 (Pownall et al., 2002). Myogenic commitment and progression along the myogenic differentiation cascade is again tightly controlled by a multitude of gene products, including the homeobox factors *Mox2* and *Msx1*, FGF and TGF β family members, IGF1, sonic hedgehog as well as by Notch and Wnt signaling (Francis-West et al., 2003). Committed myoblasts finally fuse to form muscle fibers. In the mouse limb, first muscle fibers are laid down between embryonic day (E)11 and E14, and are commonly referred to as primary fibers (Buckingham et al., 2003). During fetal development, subsequent muscle growth is achieved by addition of so-called secondary muscle fibers to preexisting primary fibers (Buckingham et al., 2003; Christ and Brand-Saberi, 2002). The formation of secondary muscle fibers is preceded by a 'secondary' wave of proliferation of either a previously quiescent population of myoblasts (Cusella-De Angelis et al., 1994) or, as more recently suggested, a population of Pax3-Pax7-positive progenitor cells (Relaix, 2006).

The chemokine, stromal cell-derived factor 1 (SDF1, also known as CXCL12) was originally identified as a pre-B cell stimulatory factor and was subsequently shown to control trafficking, transendothelial migration, proliferation, and differentiation of hematopoietic cells (Broxmeyer, 2001). SDF1 is the only known endogenous ligand of the chemokine receptor CXCR4. A role of SDF1-CXCR4 beyond the control of immunological processes has been implicated initially by various defects seen in animals carrying null mutations of either the *Cxcr4* or the *Sdf1* (*Cxcl12*) gene. These defects include cardiac malformation (Zou et al., 1998; Nagasawa et al., 1996), impaired morphogenesis of cerebellum and hippocampus (Zou et al., 1998; Ma et al., 1998), and reduced vascularization of the gastrointestinal tract (Tachibana et al., 1998). We and others have recently provided evidence that inactivation of CXCR4 also impairs limb myogenesis (Ödemis et al., 2005; Vasyutina et al., 2005; Yusuf et al., 2006). To further define the roles of SDF1 and CXCR4 in myogenesis, we have now assessed putative influences of SDF1 and CXCR4 on proliferation, migration, and differentiation of myogenic precursors/myoblasts.

Results

Murine C2C12 cells continue to proliferate and show features of myogenic progenitors when kept in high-serum (10%) DMEM (growth conditions). By contrast, C2C12 cells undergo myogenic differentiation as evidenced by the expression of myogenic differentiation factors, such as MyoD and myogenin, and a subsequent fusion into myotubes upon switching them to low-serum (1%) DMEM (differentiation conditions) (see also Bennett and Tonks, 1997; Rommel et al., 1999). Both total CXCR4 expression levels as well as CXCR4 cell-surface expression levels remained virtually unaffected under these different culture conditions (see supplementary material Fig. S1). Immunocytochemistry further demonstrated that CXCR4 is expressed in mononucleated C2C12 cells and in (polynucleated) myotubes (see supplementary material Fig. S2). Effects of SDF1 on C2C12 cells were routinely analyzed either under growth conditions or at the transition from growth to differentiation (immediately after switching to low-serum medium). In selected experiments, effects of SDF1 were additionally characterized under differentiation conditions (24 hours after switching to low-serum medium). As potential steps of myogenesis affected by SDF1-CXCR4, we considered myogenic progenitor/myoblast migration, proliferation and differentiation.

The putative chemotactic response of C2C12 cells to SDF1 was assessed in a migration assay using a modified Boyden chamber. Confirming a previous report (Ratajczak et al., 2003), SDF1 (10 ng/ml) induced migration of C2C12 cells maintained under growth conditions (Fig. 1A). Checkerboard analysis further demonstrated that this migratory response is chemotactic in nature and occurs only along a concentration gradient of SDF1 (Fig. 1B). The migratory response of C2C12 cells to SDF1 was similar to that elicited by HGF (also known as scatter factor SF) (10 ng/ml), which served as a positive control (Fig. 1A) (Lee et al., 1999). Effects on cell proliferation were evaluated by treating C2C12 cells with SDF1 for 24 hours or 48 hours, starting with the switch to low serum. Immunocytochemical staining of cultured cells with the proliferation marker Ki67 and subsequent FACS analysis

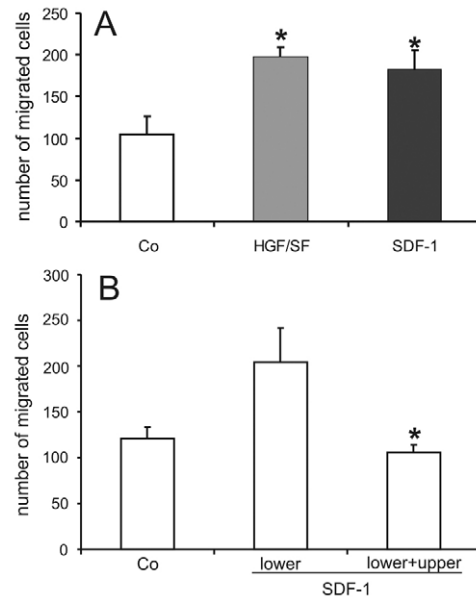


Fig. 1. SDF1 is a chemoattractant for C2C12 cells. (A) C2C12 cells were grown in 90% DMEM and 10% FCS to subconfluency and subsequently tested for SDF1-dependent (10 ng/ml) and HGF-dependent (HGF, 10 ng/ml) chemotaxis in a modified Boyden chamber assay as described in Materials and Methods. Data represent the mean \pm s.d. from three independent experiments. Note that the migratory response elicited by SDF1 was similar to that induced by HGF. * $P < 0.001$; Co, control. (B) Checkerboard analysis demonstrating a directed migratory (chemotactic) response of C2C12 cells towards SDF1. Numbers of migrated cells were determined in a Boyden chamber in which either only the lower well received SDF1 (10 ng/ml) or in which the concentration gradient was eliminated by adding identical concentrations of SDF1 to both the lower and the upper well (10 ng/ml each). Note that C2C12 cells fail to migrate through the filter when no SDF1 concentration gradient is present. * $P < 0.001$; Co, control.

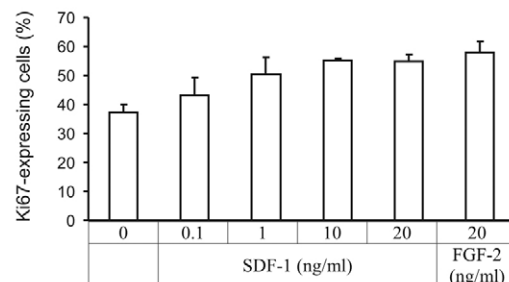


Fig. 2. SDF1 is a mitogen for C2C12 cells. C2C12 cells were maintained in DMEM containing 1% HS and varying concentrations of SDF1. FGF2 (20 ng/ml) served as a positive control. After 24 hours, cells were stained with antibodies against the cell-proliferation marker Ki67. The number of Ki67-expressing cells was quantified by FACS analysis and expressed as the percentage of total cell numbers. Data represent the mean \pm s.d. from four independent experiments. SDF1 activated C2C12 proliferation in a dose-dependent manner ($F = 10.9$, $dF = 13$, $P < 0.002$; ANOVA). Proliferative response was maximal with SDF1 at concentrations of ≥ 10 ng/ml. A comparable proliferative response was induced by FGF2 ($P < 0.002$).

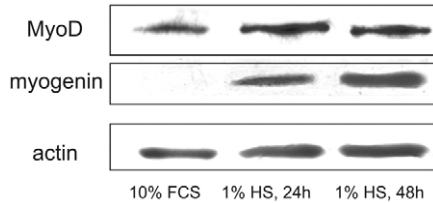


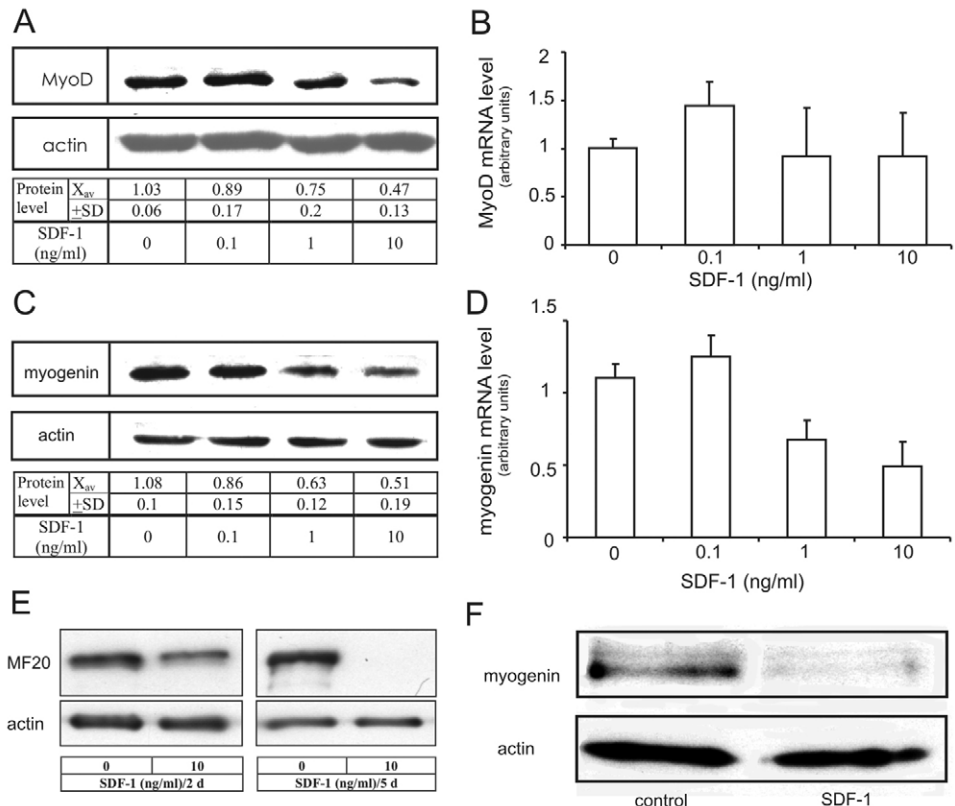
Fig. 3. Serum-deprivation induces myogenic differentiation of C2C12 cells. MyoD and myogenin protein levels were analyzed by western blotting either in C2C12 cells maintained in DMEM containing 10% FCS (growth condition), or 24 hours and 48 hours after switching cultures to DMEM supplemented with 1% HS (differentiation condition). Protein loading was controlled by staining blots with anti- β -actin antibodies. MyoD and myogenin protein levels gradually increased after switching cultures to differentiation conditions.

showed a concentration-dependent increase in the number of Ki67-expressing cells after 24 hours (Fig. 2). This increase was maximal with SDF1 at concentrations of ≥ 10 ng/ml. A similar increase in the number of Ki67-positive cells occurred in cultures maintained with FGF2 (20 ng/ml; 24 hours) used as a positive control (Fig. 2) (Tortorella et al., 2001). After 48 hours, the numbers of Ki67-positive cells in cultures treated

with either SDF1 or FGF2 were indistinguishable from those of controls (data not shown). Likewise, numbers of Ki67-positive cells remained unaffected when SDF1 (10 ng/ml) was added to C2C12 cells that had been maintained for 24 hours under differentiation conditions (data not shown). Collectively, these data establish that SDF1 controls both myogenic progenitor/myoblast migration and proliferation. The absence of mitogenic effects after prolonged treatment with SDF1 or FGF2 suggests further that SDF1, like FGF2 (Tortorella et al., 2001), only allows few rounds of cell division.

Myogenic differentiation is characterized by the sequential induction of various members of the MyoD basic helix-loop-helix transcription factor family (Pownall et al., 2002). Committed myoblasts express Myf5 and MyoD. Expression of myogenin and (re-expression) of MRF4 occurs at later stages of myogenic differentiation and is assumed to regulate, among others, expression of contractile proteins (Berkes and Tapscott, 2005). To analyze how SDF1 interferes with myogenic differentiation, we assessed MyoD and myogenin protein levels by western blotting in C2C12 cells cultured in the presence or absence of SDF1. C2C12 cells maintained under growth conditions in the absence of SDF1 showed low expression of MyoD and undetectable levels of myogenin (Fig. 3). Upon switching cultures to differentiation conditions (1% serum), MyoD and myogenin protein levels gradually

Fig. 4. SDF1 attenuates myogenic differentiation of C2C12 cells and primary myoblasts. (A,C) At the switch from growth to differentiation conditions, C2C12 cells were treated with the indicated concentrations of SDF1 for 24 hours or 48 hours and then analyzed for (A) MyoD or (C) myogenin expression, respectively, by western blotting. Numbers represent average MyoD or myogenin protein levels (arbitrary units), corrected for β -actin levels, as determined by densitometric analysis of blots from five independent experiments. SDF1 significantly attenuates myogenic differentiation of C2C12 cells, maintained under differentiation conditions (MyoD, $F=7.9$, $dF=15$, $P<0.005$; myogenin, $F=8.6$, $dF=15$, $P<0.005$, ANOVA). (B,D) Real time (RT)-PCR analysis of C2C12 cells, maintained for 24 hours under differentiation conditions and the additional presence of SDF1 at different concentrations, for (B) MyoD and (D) myogenin mRNA levels. Data represent the mean \pm s.d. from four independent experiments. Note that SDF1 induces a significant loss in myogenin mRNA levels ($F=13.7$, $dF=12$, $P<0.001$; ANOVA) but does not affect MyoD mRNA levels. (E) C2C12 cells were treated at the switch from growth to differentiation conditions with SDF1 (10 ng/ml) and analyzed for MHC expression after 2 days and 5 days by western blotting using the MF20 antibody. Staining for β -actin served as a loading control. MHC was undetectable in C2C12 cells maintained with SDF1 for 5 days. (F) Cultures of primary myoblasts and myogenic precursors were established from rodent hindlimb musculature as described in Materials and Methods. Cells were maintained for 4 days in the presence or absence of SDF1 (10 ng/ml) and subsequently analyzed for myogenin expression levels by western blotting. As found for C2C12 cells, SDF1 inhibits differentiation of primary myoblasts.



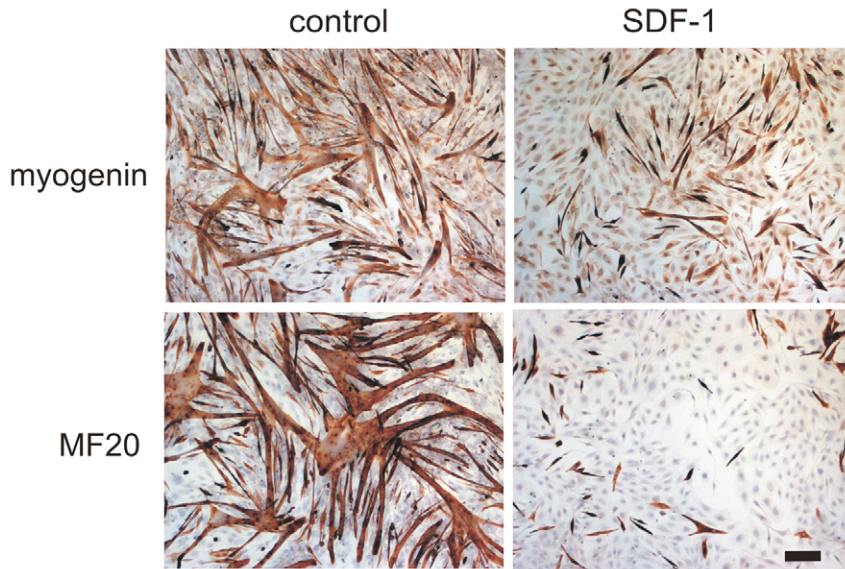


Fig. 5. Immunocytochemical analysis of C2C12 cells for myogenin and MHC expression. C2C12 cells were maintained for 72 hours under differentiation conditions in the presence or absence of SDF1 (10 ng/ml) and subsequently stained with antibodies against myogenin and MHC (MF20) as indicated. Trypan Blue was used for counterstaining. Note that SDF1 clearly reduces the number of myotubes that express myogenin or MHC. Bar, 100 μ m.

increased within the following 48 hours (Fig. 3). This increase was partially abrogated upon immediate treatment of C2C12 cells at the switch from proliferation to differentiation conditions with SDF1 at concentrations of >1 ng/ml for 24 hours or 48 hours (Fig. 4A,C). Likewise, immunocytochemistry demonstrated the presence of much smaller numbers of mononucleated C2C12 cells as well as polynucleated myotubes expressing myogenin in cultures maintained with SDF1 as compared with untreated controls (Fig. 5). The decrease in total MyoD and myogenin protein levels seen after immediately treating C2C12 cells with SDF1 at the switch to differentiation conditions was fully recapitulated in cultures exposed to SDF1 (48 hours) with a delay of 24 hours after the switch from proliferation to differentiation conditions (data not shown). Myogenin expression was almost completely abolished in C2C12 cells overexpressing CXCR4 (see supplementary material Fig. S3). Complementary real time (RT)-PCR analysis further demonstrated that the SDF1-induced loss in myogenin protein is accompanied by a decline in mRNA levels encoding myogenin, whereas a similar decline was not detectable for mRNA encoding MyoD (Fig. 4B,D). These findings point to the possibility that SDF1 inhibits myogenin expression at the transcriptional level and interferes with MyoD expression at the translational level. We can, however, not dismiss at present that SDF1 differentially affects the degradation of myogenin and MyoD mRNAs. In an additional set of experiments, we assessed myogenic differentiation by the use of MF20 antibody that recognizes sarcomeric myosin heavy chains (MHC). Western blot analysis of C2C12 cells that had been maintained with SDF1 (10 ng/ml) under differentiation conditions showed a decline of MHC expression after 2 days and a complete loss of MF20-immunoreactive protein bands after 5 days (Fig. 4E).

On the cellular level, this SDF1-dependent decline in MHC protein level was again reflected by smaller numbers of both MF20-immunopositive cells and myotubes (Fig. 5). Finally, applying SDF1 to cultures of primary limb myoblasts resulted in a decrease of myogenin protein levels, comparable to those seen in C2C12 cells (Fig. 4F). Collectively, these data establish that SDF1 inhibits myogenic differentiation.

Prototypical extracellular factors controlling myogenic differentiation are FGF2 or IGF1, which respectively inhibit or promote myogenic progenitor/myoblast differentiation (e.g. Adi et al., 2002; Milasincic et al., 1996; Kontaridis et al., 2002). A co-treatment paradigm was chosen to analyze how SDF1 interacts with these factors during myogenic differentiation. Confirming previous reports (Adi et al., 2002; Milasincic et al., 1996; Kontaridis et al., 2002), treatment of C2C12 cells with FGF2 (20 ng/ml, 48 hours) decreased myogenin protein levels by roughly 50%, whereas myogenin expression increased twofold with IGF1 (10 ng/ml; 48 hours; Fig. 6). Supplementing the culture medium additionally with SDF1 (10 ng/ml) did not

further modify the inhibitory action of FGF2 on myogenin expression, but completely prevented the IGF1-induced increase in myogenin protein levels (Fig. 6). These findings indicate that SDF1 counteracts stimulatory influences on differentiation of myogenic precursors/myoblasts.

There is ample evidence that myogenic differentiation occurs upon activation of the phosphoinositide 3 kinase (PI3-kinase)-Akt pathway and/or the MAPK-p38 cascade, whereas

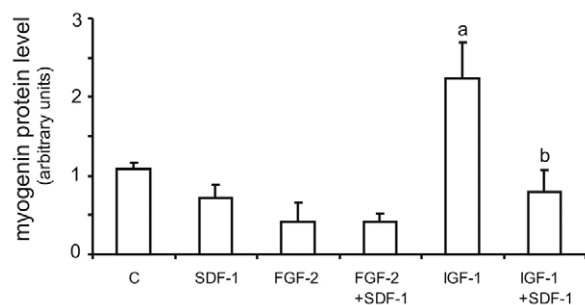


Fig. 6. Interaction of SDF1 with other extracellular factors controlling myogenesis. C2C12 cells were treated at the switch from growth to differentiation conditions with either SDF1 (10 ng/ml), FGF2 (10 ng/ml) or IGF1 (10 ng/ml) alone or in combination as indicated. After 48 hours, myogenin protein levels were analyzed by western blotting. Immunoreactive protein bands were measured by densitometry and corrected for β -actin. Data represent average myogenin protein levels \pm s.d. as determined in four independent experiments. SDF1 did not further modify the known inhibitory influences of FGF2 on myogenic differentiation. By apparent contrast, SDF1 completely prevented the established stimulatory influences of IGF1 on myogenesis. C, control; ^a $P < 0.05$, IGF1 vs control; ^b $P < 0.05$, IGF1+SDF1 vs IGF1.

proliferation of myogenic progenitors/myoblasts requires activation of the classic MAP kinase (Erk) cascade (Bennett and Tonks, 1997; Tortorella et al., 2001; Milasincic et al., 1996; Coolican et al., 1997; Chun et al., 2000; Li et al., 2000; Wu et al., 2000; Tiffin et al., 2004). It is, however, of note that the classic MAP kinase cascade exerts dual effects on myogenesis and can also promote myogenic differentiation (Gredinger et al., 1998; Sarbassov et al., 1997). To determine whether SDF1-CXCR4 control myogenic differentiation either through or by interfering with these classic signaling proteins and related pathways, we tested whether SDF1 affects levels of activated (phosphorylated) Erk1 and Erk2, p38 and Akt by western blotting using phosphorylation-specific antibodies. Exposure of C2C12 cells to SDF1 for 10 minutes induced a concentration-dependent phosphorylation (activation) of Erk1 and Erk2 (Fig. 7). Activation was maximal with SDF1 at concentrations of ≥ 1 ng/ml. By contrast, SDF1 at concentrations of up to 10 ng/ml did not lead to obvious changes in the levels of activated Akt or p38 after 10 minutes (Fig. 7). Levels of these phosphorylated signal proteins also remained unchanged when cells were analyzed after a 30-minute, 60-minute or 120-minute stimulation period with SDF1 (data not shown). A recent report suggested that SDF1 controls the development of human CD34⁺ progenitor cells through PKC ζ (Petit et al., 2005). Western blot analysis and the use of an antibody specific against phosphorylated PKC ζ and PKC λ revealed a concentration-dependent activation of PKC ζ and PKC λ in C2C12 cells exposed to SDF1 for 10 minutes (Fig. 7). By contrast, SDF1 did not affect phosphorylation of PKC α or PKC β . Together, these findings suggest that SDF1-CXCR4 control myogenesis and myogenic differentiation through Erk1 and Erk2, and/or PKC ζ and PKC λ .

In a subsequent series of experiments, the Erk inhibitor UO126 as well as myristoylated PKC ζ pseudosubstrate inhibitor were used to define the role of both signaling

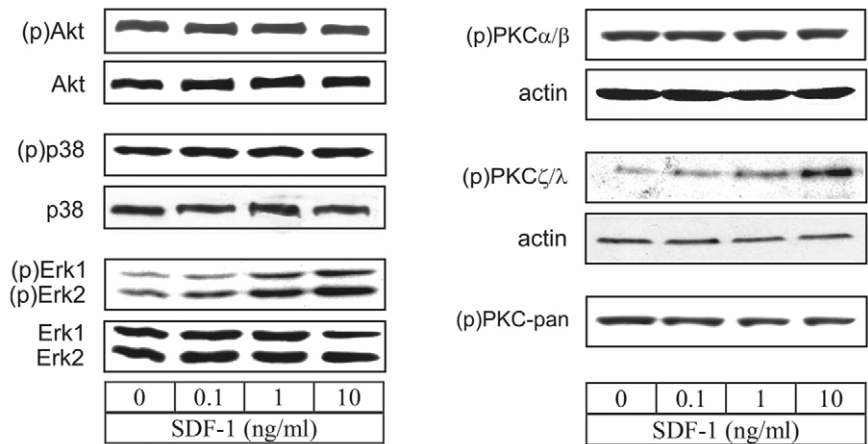


Fig. 7. Characterization of signaling proteins or pathways activated by SDF1 in C2C12 cells. C2C12 cells were stimulated for 10 minutes with SDF1 at the indicated concentrations and subsequently analyzed for activation (phosphorylation) of Akt, p38, Erk and different PKC isoforms by western blotting using phosphospecific antibodies. Protein loading was controlled by staining blots with (pan) antibodies that recognize the non-phosphorylated forms of the respective signaling molecules. In case of PKC isoforms, β -actin staining in combination with staining for all phosphorylated PKC isoforms using *P*-PKC-pan served as a loading control. SDF1 induced a dose-dependent increase in levels of phosphorylated Erk1, Erk2 or PKC ζ , PKC λ [(p)-Erk1, (p)-Erk2 or (p)-PKC ζ , (p)-PKC λ , respectively], but showed no effects on phosphorylated levels of Akt, p38 or pPKC α , PKC β [(p)-Akt, (p)-p38 or (p)-PKC α , (p)-PKC β , respectively].

molecules in the SDF1-dependent control of myogenesis. UO126 (1 μ M) prevented the SDF1-induced proliferation of C2C12 cells (Table 1). Moreover, corroborating previous studies (e.g. Adi et al., 2002), UO126 induced a clear increase of myogenin expression in C2C12 cells maintained under differentiation conditions (Table 1). This increase in myogenin was completely abolished in the additional presence of SDF1 (10 ng/ml). Finally, UO126 abrogated SDF1-induced migration of C2C12 cells (Table 1). Together, these findings establish that CXCR4 recruits Erk1 and Erk2 to control proliferation and migration but not differentiation of C2C12 cells.

The myristoylated PKC ζ pseudosubstrate inhibitor (2 μ M) completely abolished both the proliferative and the chemotactical response of C2C12 cells to SDF1 (Table 2).

Table 1. SDF1 controls proliferation and migration of C2C12 cells through Erk

	Control	SDF1	UO126	SDF1 +UO126
Ki67-expressing cells (%)	30 \pm 1.6	38 \pm 0.8	28 \pm 2.1	25 \pm 2.8*
Myogenin protein levels (arbitrary units)	1.1 \pm 0.1	0.7 \pm 0.2	2.1 \pm 0.2**	0.8 \pm 0.3***
Number of migrated cells	413 \pm 22	500 \pm 37	305 \pm 12	295 \pm 7****

Ki67 expression and myogenin protein levels were determined in C2C12 cells maintained for 24 hours and 48 hours, respectively, with either SDF1 (10 ng/ml) alone or in the additional presence of the Erk-inhibitor UO126 (1 μ M), as described in Figs 2 and 4. Migration assay was performed with C2C12 cells prior a 1-hour-incubation with UO126 (1 μ M), $n=4$. * $P<0.001$, UO126+SDF1 vs SDF1; ** $P<0.05$, UO126 vs control; *** $P<0.01$, SDF1+UO126 vs UO126; **** $P<0.001$. SDF1+UO126 vs SDF1.

Table 2. The effects of SDF-1 on myogenesis require activation of atypical PKC ζ

	Control	SDF1	myrPKC ζ PI	SDF1 +myrPKC ζ PI
Ki67-expressing cells (%)	27 \pm 1.5	34 \pm 0.8	25 \pm 0.9	22 \pm 0.8*
Myogenin protein level (arbitrary units)	1.2 \pm 0.1	0.7 \pm 0.1	2.2 \pm 0.2**	2.0 \pm 0.2***
Number of migrated cells	400 \pm 89	600 \pm 100	240 \pm 38	281 \pm 90****

Ki67 expression and myogenin protein levels were determined in C2C12 cells maintained for 24 h and 48 h, respectively, with either SDF-1 (10 ng/ml) alone or in the additional presence of myristoylated PKC ζ peptide inhibitor (myrPKC ζ PI; 2 μ M), as described in Figs 2 and 4. Migration assay was performed with C2C12 cells prior a 1 hour-incubation with myrPKC ζ PI (2 μ M), $n=4$. * $P<0.005$, SDF1+myrPKC ζ PI vs SDF1; ** $P<0.02$, myrPKC ζ PI vs control; *** $P<0.001$, SDF1+myrPKC ζ PI vs SDF1; **** $P<0.02$, SDF1+myrPKC ζ PI vs SDF1.

Moreover, maintaining C2C12 cells in differentiation medium with the cell-membrane-permeable PKC ζ pseudosubstrate peptide (2 μ M) for 48 hours promoted myogenin expression (Table 2, Fig. 8A). This inhibitor-induced increase in myogenin was not affected by the additional presence of SDF1 (Table 2, Fig. 8A). Likewise, SDF1 failed to inhibit myogenin expression in C2C12 cells when PKC ζ expression was selectively depleted by RNA interference (Fig. 8B,C). Collectively these findings establish that SDF1-CXCR4 control C2C12 differentiation through PKC ζ , and further demonstrate that – in addition to Erk – PKC ζ is an essential downstream event in both the mitogenic and migratory response of myogenic progenitors to SDF1.

To distinguish whether SDF1 activates Erk and PKC ζ in parallel or in an upstream-downstream manner, we additionally determined levels of activated Erk and PKC ζ in C2C12 cells that had been stimulated with SDF1 for 10 minutes in the presence of UO126 (1 μ M) or myristoylated PKC ζ pseudosubstrate inhibitor (2 μ M). We found that UO126 fails to affect basal or SDF1-induced levels of activated PKC ζ and PKC λ in C2C12 cells (see supplementary material Fig. S4). Likewise, basal and SDF1-induced levels of phosphorylated Erk did not significantly decline in the presence of the PKC ζ pseudosubstrate peptide, hence, suggesting that SDF1-CXCR4 activate both signaling molecules or pathways in parallel.

Recent studies demonstrated that SDF1 also binds to the orphan receptor RDC1 (also referred to as CXCR7) (Burns et

al., 2006; Balabanian et al., 2005). Maintaining C2C12 cells in the presence of the CXCR4-receptor antagonist AMD3100, prevented SDF1-induced inhibition of myogenin expression and even allowed for a small, although statistically not significant, increase in myogenin protein levels (see supplementary material Fig. S5). These findings imply that SDF1 controls myogenesis through CXCR4.

Discussion

Our studies attempted to further define the role of SDF1-CXCR4 in limb myogenesis. By using C2C12 myogenic progenitors/myoblasts derived from mouse limb musculature, we demonstrate that SDF1-CXCR4 not only induce the migration of myogenic progenitors/myoblasts as previously suggested (Vasyutina et al., 2005; Ratajczak et al., 2003), but also promote their proliferation and, in addition, inhibit their myogenic differentiation – as assessed by expression of myogenin, MyoD and MHC. Moreover, we provide evidence that SDF1-CXCR4 affect migration, proliferation and differentiation of myogenic precursors and myoblasts through the atypical PKC isoform PKC ζ . The present findings, together with our previous observations of a massive impairment of fetal but not embryonic limb-muscle development in CXCR4-deficient animals (Ödemis et al., 2005), point to a prime role of CXCR4-PKC ζ signaling in the (secondary) expansion of muscle masses.

Confirming the validity of C2C12 cells as a model system, we observed that SDF1 also inhibits expression of myogenin in primary myoblasts. Moreover, SDF1 failed to affect myogenic differentiation in the presence of the CXCR4 receptor antagonist AMD3100, suggesting that SDF1 controls myogenesis through CXCR4. The effects exerted by SDF1-CXCR4 on proliferation and migration of C2C12 cells were equally potent than those elicited by FGF2 and HGF, and,

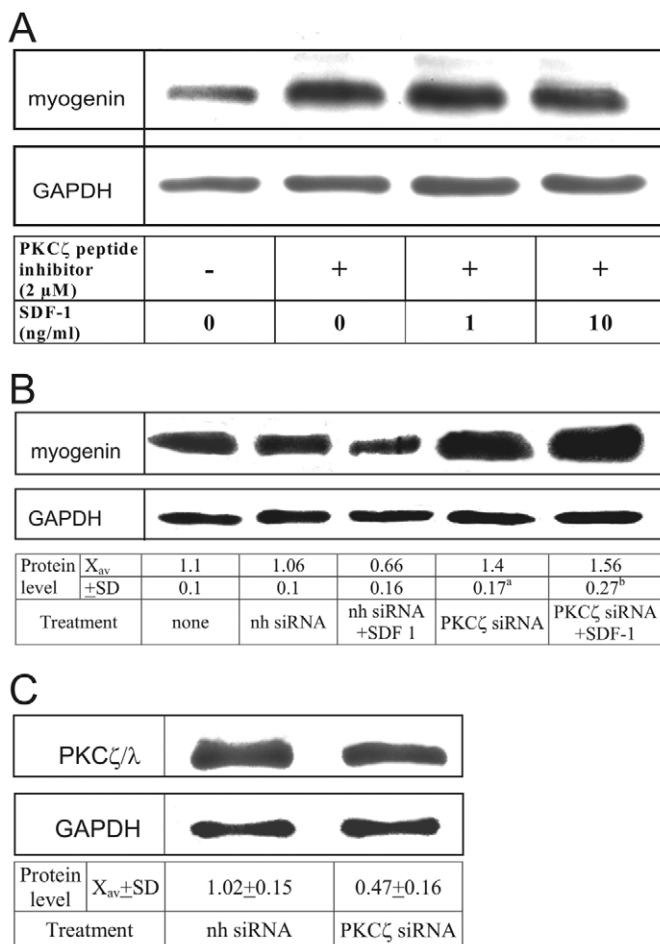


Fig. 8. PKC ζ mediates the inhibitory influences of SDF1 on myogenic differentiation. (A) C2C12 cells were maintained for 48 hours with either myristoylated PKC ζ peptide inhibitor (2 μ M) alone or in combination with the indicated concentrations of SDF1, and subsequently analyzed for myogenin expression by western blotting. Protein loading was controlled by GAPDH staining. Myogenin levels increased with myristoylated PKC ζ peptide inhibitor and remained unaffected by SDF1. Data are given in Table 2. (B) C2C12 cells were transfected overnight with PKC ζ siRNA or non-homologous (nh) siRNA, and maintained further in DMEM containing 1% HS and SDF1 (10 ng/ml). After 48 hours, cells were lysed and myogenin expression was determined by western blotting. Immunoreactive protein bands were measured by densitometry and corrected for GAPDH. Numbers represent average protein level \pm s.d. (arbitrary units) as determined in three independent experiments. Myogenin protein levels increased in C2C12 cells transfected with PKC ζ siRNA. This increase was not affected by the additional presence of SDF1. By contrast, C2C12 cells transfected with nh siRNA showed unchanged expression levels of myogenin which declined in the presence of SDF1. ^a $P < 0.05$, PKC ζ siRNA vs control; ^b $P < 0.05$, PKC ζ siRNA+SDF1 vs control. (C) Assessment of PKC ζ expression in cells transfected with PKC ζ siRNA. C2C12 cells were transfected with PKC ζ siRNA or non-homologous (nh) siRNA. Levels of PKC ζ were determined by western blotting. Numbers indicate average levels of PKC ζ \pm s.d. (arbitrary units) as determined in five independent experiments. Note that PKC ζ levels significantly decline ($P < 0.001$) in C2C12 cells transfected with PKC ζ siRNA as compared with cells transfected with nh siRNA.

hence, have to be considered as physiologically relevant. FGF receptor signaling is regarded as a key event regulating myogenic progenitor/myoblast proliferation (e.g. Itoh et al., 1996). HGF and its receptor Met, are essential for migration of delaminated myogenic progenitors (Christ and Brand-Saberi, 2002). In contrast to the complete loss of MHC expression, SDF1 only partially attenuated myogenin and MyoD expression in C2C12 cells. Interestingly, myogenin levels further declined in C2C12 cells that overexpressed CXCR4. We consider this finding as an indication that, like previously reported for several other cell types (Ödemis et al., 2002; Porecha et al., 2006; Ehtesham et al., 2006), the response of myogenic progenitors/myoblasts to SDF1 is additionally controlled by CXCR4 (cell surface) expression levels. Although data on SDF1 concentrations present in the limb bud are at present unavailable, SDF1 concentrations of ≥ 1 ng/ml, which were found by us to affect myogenesis, could – during development – well be reached locally. In fact, in adult mice SDF1 plasma concentrations are in the range of 0.5–1 ng/ml. In addition, the (limb) bone marrow even contains slightly higher SDF1 concentrations of about 1.5 ng/ml (De Falco et al., 2004).

First evidence for the recruitment of PKC ζ in SDF1-CXCR4 signaling emerged from recent studies on SDF1-dependent migration, adherence and polarization of human CD34⁺ progenitor cells (Petit et al., 2005). Several of our findings now identify PKC ζ also as a central downstream molecule in the observed effects SDF1 has on proliferation, migration and differentiation of myogenic precursors and myoblasts. Exposure of C2C12 cells to SDF1 resulted in a concentration-dependent activation of PKC ζ but not of PKC α and PKC β . Moreover, C2C12 cells failed to respond to SDF1 with migration or proliferation when the PKC ζ pseudosubstrate peptide inhibitor was also present. Likewise, PKC ζ pseudosubstrate inhibitor or the cellular depletion of PKC ζ by RNA interference prevented the SDF1-induced inhibition of myogenesis, as assessed by MyoD and myogenin expression. In addition to PKC ζ pseudosubstrate inhibitor, the effects of SDF1 on proliferation and migration of C2C12 cells were sensitive to the Erk inhibitor UO126. These findings further unravel that PKC ζ seems to be sufficient to affect myogenic differentiation but additionally requires Erk to control myogenic progenitor cell proliferation and migration. A similar requirement of both PKC ζ and Erk has been recently demonstrated for the mitogenic effects of SDF1 on CD34⁺ progenitors (Petit et al., 2005). It is of note that, SDF1 when applied to C2C12 cells that had been maintained under differentiation conditions for 24 hours still inhibited myogenic differentiation but failed to induce cell proliferation. We consider these findings as an indication that the SDF1-dependent block of myogenesis is not necessarily related to enhanced cell proliferation.

Interestingly, SDF1 seems to activate both PKC ζ and Erk signaling pathways in parallel, as judged from our observations that UO126 did not affect levels of activated PKC ζ in C2C12 cells and, vice versa, PKC ζ pseudosubstrate peptide inhibitor remained without effects on levels of activated Erk. The exact mechanism of cooperation between both signaling pathways that eventually allows for cell proliferation remains to be identified. Likewise, it remains to be seen whether SDF1-dependent proliferation of myoblasts/myogenic progenitors involves a block of apoptosis. Anti-apoptotic effects of SDF1

have been previously demonstrated for cancer cells as well as non-transformed cells (e.g. Jaleel et al., 2004; Burger et al., 2005). Additional downstream signaling constituents recruited by PKC ζ and Erk to control cell migration might be MLCK, calpain, paxillin, FAK or Pyk2 (Hauck et al., 2000; Huang et al., 2004). In this respect, it is interesting to note that in CD34⁺ progenitors, Pyk2 is activated by PKC ζ but not other PKC isoforms (Petit et al., 2005).

All data currently available dismiss the possibility that CXCR4-PKC ζ signaling inhibits myogenic differentiation by a direct cross-talk with signaling events that have previously shown to control myogenic differentiation, such as the PI3K-Akt pathway, the p38-MAP kinase cascade or the classic MAP kinase cascade (Chun et al., 2000; Li et al., 2000; Wu et al., 2000; Tamir and Bengal, 2000; Conejo et al., 2001). In fact, SDF1 did not affect levels of phosphorylated Akt and p38 in C2C12 cells. Moreover, although SDF1 stimulated Erk phosphorylation in C2C12 cells, the inhibitory action of SDF1 on myogenic differentiation persisted in the presence of the Erk inhibitor UO126. Interestingly, we further found that SDF1 abolishes the stimulatory influences of IGF1 on myogenic differentiation, which are believed to be mediated through the PI3-kinase-Akt and/or p38-MAP kinase pathway (Coolican et al., 1997; Conejo et al., 2001). In addition, SDF1 did not cooperate with FGF2, an extracellular factor known to prevent myogenic differentiation through the MAP kinase cascade (Tortorella et al., 2001). These findings favor the possibility that SDF1, IGF and FGF signaling pathways involved in the control of myogenic differentiation converge on a common, as-yet-unknown, effector molecule.

According to their mode of activation, the PKC superfamily has been subdivided into three subgroups: the conventional PKC isoforms, including PKC α , PKC β and PKC γ that are activated by diacylglycerol (DAG) and Ca²⁺; the novel PKC isoforms (PKC δ , ϵ , η , θ) that are only sensitive to DAG but not to Ca²⁺, and the atypical PKC isoforms, such as PKC ζ and PKC λ that are neither sensitive to DAG nor Ca²⁺ (Parker and Murray-Rust, 2004). In CD34⁺ progenitors, SDF1 activates PKC ζ through PI3-kinase (Petit et al., 2005). However, a different activation mode seems to apply for C2C12 cells because, as mentioned above, SDF1 remains without effects on the activity of the PI3-kinase-Akt signaling pathway in these cells.

In vivo, CXCR4 is first expressed by delaminated Lbx1-positive myogenic progenitors (Vasyutina et al., 2005), whereas SDF1 expression is only detectable in surrounding mesenchyme or connective tissue (Ödemis et al., 2005). Despite our demonstration of potent effects of SDF1-CXCR4 on myogenic precursor cell migration, proliferation and differentiation, it is intriguing to note that inactivation of CXCR4 has only minor effects on primary myogenesis. At E10.75, CXCR4-deficient mice exhibit a moderate (30%) reduction in the number of Lbx-expressing progenitors within the limb bud (Vasyutina et al., 2005). An equally moderate loss in the number of Lbx1-expressing as well as Pax3-expressing cells was noticed in the chick limb bud upon inactivation of CXCR4 with the selective CXCR4 antagonists T140 or T14003 (Yusuf et al., 2006). Moreover, in CXCR4-deficient mice these initial subtle defects seem to be fully compensated by subsequent developmental processes, as implied by comparable numbers of MyoD- or myogenin-expressing myoblasts present in the limbs of CXCR4^{-/-} and wild-type

mice between E13.5 and E14 (Ödemis et al., 2005; Vasyutina et al., 2005). Hence, SDF1 rather seems to supplement the regulatory network controlling primary myogenesis than acting as a key regulator of these early-development events. This conclusion would also account for the massive defects in muscle development present in mice deficient for established major regulators of primary myogenesis, such as HGF or its receptor Met. In respective knockout animals, myogenic progenitors completely fail to take up long-range migration into the limbs, as well as into diaphragm and tongue, and, eventually skeletal muscles do not form within these parts of the body (Bladt et al., 1995; Dietrich et al., 1999). In sharp contrast to primary limb myogenesis, CXCR4 signaling, however, seems to be indispensable for secondary limb myogenesis. In a previous study, we noted a massive reduction of limb muscle masses in CXCR4-deficient animals starting around E16 (Ödemis et al., 2005). In light of our present identification of SDF1-CXCR4 as potent inhibitors of myogenic differentiation, we consequently suggest that the prime role of SDF1-CXCR4 during secondary myogenesis is to keep myogenic precursors/myoblasts in an undifferentiated and, hence, proliferative state. Knocking out CXCR4 would accordingly lead to premature determination or differentiation of myogenic precursors and myoblasts, and subsequently reduced muscle masses.

It has long been assumed that muscle growth involves division of myoblasts within the muscle masses. Only recently, it became evident that myoblasts quickly exit the cell cycle and, hence, have only a limited contribution to muscle growth (Gros et al., 2005; Relaix et al., 2005). Subsequent studies led to the identification of a novel Pax3-Pax7-expressing population of cells within the myotome which is maintained in embryonic and fetal muscle masses. It is currently assumed that this cell population contributes to muscle growth throughout development and, later on, forms the satellite cells of postnatal muscles (Relaix, 2006). Although direct experimental evidence is presently unavailable, it is thus tempting to speculate that CXCR4-PKC ζ signaling defines muscle masses or induces muscle regeneration by controlling the expansion of Pax3-Pax7-expressing progenitors or satellite cells, respectively.

Materials and Methods

C2C12 cells and treatments

The murine C2C12 myoblast cell line, a subclone of C3H cells established from mouse leg muscle (Yaffe and Saxel, 1977; Blau et al., 1983) (obtained from ATCC), was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Gibco). In indicated experiments, subconfluent cultures were switched to DMEM containing 1% horse serum (HS; Gibco). Media were additionally supplemented with either SDF1 (Upstate, Charlottesville, VA), fibroblast growth factor 2 (FGF2; Peprotech, Rocky Hill, NJ), hepatocyte growth factor (HGF; Peprotech), insulin-like growth factor 1 (IGF1; Peprotech), AMD3100 (Sigma-Aldrich), UO126 (Calbiochem; Schwalbach, Germany) or myristoylated PKC ζ peptide inhibitor (Biomol; Hamburg, Germany) alone or in combination as indicated.

Primary myoblasts

Cultures of primary myoblasts were established from the proximal hindlimb of postnatal rats. Tissue was chopped and incubated with papain (0.15 mg/ml)-DNase (0.1 mg/ml)-solution (Roche, Mannheim, Germany) at 37°C for 30 minutes and for another 30 minutes with antipain (0.05 mg/ml; Roche) solution. Tissue pieces were subsequently dissociated by trituration through plastic pipettes and cells were seeded into culture dishes previously coated with poly-L-ornithine (0.1 mg/ml; Sigma) and fibronectin (4 μ g/ml; Chemicon), and maintained in DMEM supplemented with 10% FCS and 1% penicillin-streptavidin (PAA; Cölbe, Germany) until cultures reached confluency (usually after 7–10 days). For treatment with SDF1 (10 ng/ml), cultures were switched to serum-free N2-medium.

Western blot analysis

Cells were lysed by ultrasonication in 62.5 mM Tris-HCl, containing 2% SDS and 10% sucrose. Proteins were denatured at 95°C for 5 minutes and further diluted in sample buffer (250 mM Tris-HCl pH 6.8 containing 4% SDS, 10% glycerol and 2% β -mercaptoethanol). For detection of phosphorylated proteins, sample buffer was additionally supplemented with sodium orthovanadate (100 mM). Protein content of cell lysates was determined using the BCA protein estimation kit (Pierce; Rockford, IL) and bovine serum albumin as a standard. Proteins (15 μ g/lane) were separated by SDS (8%, 10% or 12%) polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Upon blocking non-specific binding sites with 5% non-fat milk for 60 minutes, blots were incubated overnight at 4°C with one of the following antibodies: anti-MyoD (1:500) and anti-myogenin (1:1000; both Santa Cruz Biotechnology, Santa Cruz, CA); anti-MF20 (1:2000; DSHB, IL), anti-CXCR4 (1:1000; ProSci, Poway, CA), phosphospecific anti-Erk (1:1000; Biosource, Camarillo, CA), phosphospecific anti-p38 (1:1000; Cell Signaling Technology, Danvers, MA), phosphospecific anti-Akt (1:1000; Biosource), phosphospecific anti-PKC ζ/λ (1:500; Cell Signaling Technology), phosphospecific anti-PKC α/β (1:1000; Cell Signaling Technology), anti-PKC ζ (1:1000; Santa Cruz Biotechnology), phosphospecific PKC-pan (1:1000; Cell Signaling Technology). Antibody labeling was detected by incubating cultures for 2 hours at room temperature with appropriate horseradish-peroxidase-labeled secondary antibodies (Dianova, Hamburg, Germany) and visualized with the enhanced chemiluminescence kit (Amersham Pharmacia, Freiburg, Germany). To control for protein loading, blots were additionally stained with either anti- β -actin (1:6000; BD Transduction Laboratories, San Jose, CA), anti-GAPDH antibodies (1:6000; Research Diagnostics, Concord MA) or (pan) antibodies against the following non-phosphorylated signaling molecules: p38 (1:1000, Cell Signaling); Erk (1:6000, Biosource); Akt (1:2000, Biosource). In case of PKC isoforms, protein loading was controlled by labeling blots with β -actin as well as antibodies recognizing phosphorylated PKC isoforms (P-PKC-pan). Integrated optical densities of immunoreactive protein bands were measured using the Image Master VDS software (Pharmacia).

Immunocytochemistry

For immunocytochemical analysis, cultured C2C12 cells were fixed with paraformaldehyde (4%, w/v) in PBS for 15 minutes and permeabilized with 0.05% saponin for 30 minutes. Upon blocking non-specific binding sites with 2% (v/v) normal goat serum in PBS for 30 minutes, cultures were incubated overnight at 4°C with one of the following antibodies: anti-CXCR4 (1:250; Abcam, Cambridge, UK), anti-myogenin (1:500) or anti-MF20 (1:1000). In case of CXCR4, antibody labeling was detected by incubating cultures for 2 hours at room temperature with Cy3-labeled secondary antibodies (1:400; Jackson Immunolabs, West Grove, PA). In case of myogenin and MF20 staining, cultures were subsequently incubated for 2 hours with appropriate biotinylated secondary antibodies (1:400; Vector Laboratories, Burlingame, CA) and immunostaining was visualized with the ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (10 mg/ml in Tris-HCl pH 7.6) as a chromogen. Specificity of immunostaining was controlled by omitting the respective primary antibody. Cultures were additionally stained with either DAPI or Trypan Blue as indicated.

RNA isolation and RT-PCR analysis

Total RNA was isolated from C2C12 cells using the PeqGold isolation kit (Peqlab, Schwalbach, Germany) according to the manufacturer's instructions. Total RNA concentration was measured by spectrophotometric absorbance at 260 nm. A total of 5 μ g of RNA was reverse transcribed using 200 units/ μ l of moloney murine leukemia virus reverse transcriptase (Sigma, St Louis, MI) and 2 μ g of random hexamer primers (Thermo Hybaid, Ulm, Germany). Obtained templates were amplified in a final volume of 50 μ l. MyoD and myogenin mRNA levels were quantified by real time PCR using the following primers: MyoD (accession number, BC006018); sense, 5'-GGT CTG GGT TCC CTG TTC TGT GT-3', antisense 5'-CCC CGG CGG CAG AAT GGC TACG-3'; product size, 220 bp. Myogenin (accession number, BC068019); sense, 5'-TGG AGC TGT ATG AGA CAT CCC-3'; antisense, 5'-TGG ACC ATG CTC AGG GGT CCC-3'; product size, 171 bp. Actin (accession number, NM_031144), sense, 5'-CAG GTC CAG ACG CAG GAT GGC-3'; antisense, (5'-CTA CAA TGA GCT GCG TGT GGC-3'; product size, 271 bp (all from Thermo Electron, Ulm, Germany). Thermocycling for each reaction was done in a final volume of 10 μ l containing 1 μ l of cDNA sample or standard, 5 μ l of QuantiTect™ SYBR®Green PCR Kit (Qiagen; Hilden, Germany), 3.5 μ l water and 2 μ l of sense and antisense primers (10 pmol each). The thermal cycling conditions were 95°C for 15 minutes followed by 35 cycles of 64°C (myogenin) or 55°C (MyoD) for 20 seconds and 72°C for 10 seconds. Data were collected using the LightCycler instrument (Roche Diagnostics). To confirm the specificity of the amplified products, melting curves were performed at the end of the amplification by cooling samples at 20°C/second to 65°C for 15 seconds and then increasing the temperature to 95°C at 0.1°C/second with continuous fluorescence measurement. Each sample was tested in duplicate. For the generation of standard curves, PCR products (MyoD, myogenin, β -actin) were purified using QIAquick (Qiagen) spin columns and serially diluted in double-distilled water. The second derivative

maximum method as provided by the manufacturer's software was applied to determine the maximum acceleration of the amplification process, and the respective cycle number was used as a crossing point value. The concentrations of unknown samples were determined by setting their crossing points to the standard curve and were normalized to β -actin.

Chemotaxis

The chemotactic response of C2C12 cells to extracellular factors was evaluated using a modified 48-well Boyden chamber (Neuro Probe, Cabin John, MD), as previously described (Ödemis et al., 2002). For cell harvest, cultures were rinsed three times with N2 medium and cells were resuspended in fresh DMEM containing 1% HS. Fifty microliters of the cell suspension, containing 25,000 cells, were added to the upper well of the chamber. The lower well received 28 μ l of N2 medium supplemented with the indicated concentrations of either SDF1 or HGF. The upper and lower wells were separated by polyornithin-coated Nucleopore[®] PVP-free polycarbonate filters (Corning, Acton, MA) with 5- μ m pore size, and the chamber was incubated for 4 hours at 37°C in a water-saturated atmosphere of 95% air and 5% CO₂. After incubation, non-migrating cells were scraped off from the upper surface of the filter. Migrating cells, attached to the lower surface, were fixed in ethanol for 10 minutes, stained with hematoxylin/eosin and counted under a microscope (\times 100) within a total area of 2 mm². Checkerboard analysis was performed according to Zigmond and Hirsch (Zigmond and Hirsch, 1973) to distinguish between directed cell migration (chemotaxis) or random cell migration (chemokinesis). To this end, number of migrated cells was determined upon adding identical concentrations of SDF1 (10 ng/ml) to the upper and the lower well of the Boyden chamber.

Proliferation assay

Proliferating cells were identified by Ki67 expression and quantified by flow cytometric analysis. C2C12 cells were fixed with paraformaldehyde (2%, w/v) in PBS for 15 minutes, permeabilized with 0.05% saponin and incubated for 45 minutes at 4°C with anti-Ki67 antibodies (1:200; Dako), and for another 30 minutes with fluorescein-isothiocyanate-labeled secondary antibodies (1:500; Jackson Laboratories, West Grove, PA). Flow cytometric analysis was performed with a FACScan (Becton Dickinson). The forward narrow-angle light scatter was used to exclude dead and aggregated cells.

RNA interference

Mouse PKC ζ (accession number, NM_001039079) small interfering RNA (siRNA) (5'-AAC TGC AAG CTG CTG GTC CAT-3') and non-homologous (nh) siRNA (5'-AAT TCT CCG AAC GTG TCA CGT-3') were designed using the Protein Lounge database (www.proteinlounge.com/sirna_home.asp), and double-stranded RNAs were custom synthesized by Qiagen (Hilden, Germany). One hour prior to transfection, culture medium was replaced by 500 μ l DMEM containing 1% HS. Transfection was performed with the siPORT[™] Amine Transfection Agent (Ambion, Huntingdon, UK) according to the manufacturer's recommendations. For transfection, cultures additionally received 100 μ l of serum-free DMEM medium containing 100 nM of PKC ζ siRNA or nh siRNA and 3.5 μ l of transfection reagent, and were incubated overnight. Transfected cultures were further maintained in DMEM containing 1% HS and SDF1 at the indicated concentrations.

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