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Cooperation between Shh and IGF-I in Promoting Myogenic Proliferation and Differentiation via the MAPK/ERK and PI3K/Akt Pathways Requires Smo Activity

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Abstract

Sonic hedgehog (Shh) has been shown to promote adult myoblast proliferation and differentiation and affect Akt phosphorylation via its effector Smoothened (Smo). Here, the relationship between Shh and IGF-I was examined with regard to myogenic differentiation via signaling pathways which regulate this process. Each factor enhanced Akt and MAPK/ERK (p42/44) phosphorylation and myogenic factor expression levels in a dose-responsive manner, while combinations of Shh and IGF-I showed additive effects. Blockage of the IGF-I effects by neutralizing antibody partially reduced Shh's effects on signaling pathways, suggesting that IGF-I enhances, but is not essential for Shh effects. Addition of cyclopamine, a Smo inhibitor, reduced Shh- and IGF-I-induced Akt phosphorylation in a similar manner, implying that Shh affects gain of the IGF-I signaling pathway. This implication was also examined via a genetic approach. In cultures derived from Smo^{mut} (*MCre;*$Smo^{flox/flox}$) mice lacking Smo expression specifically in hindlimb muscles, IGF-I-induced Akt and p42/44 phosphorylation was significantly reduced compared to IGF-I's effect on Smo^{cont} cells. Moreover, remarkable inhibition of the stimulatory effect of IGF-I on myogenic differentiation was observed in Smo^{mut} cultures, implying that intact Smo is required for IGF-I effects in myoblasts. Immunoprecipitation assays revealed that p-Tyr proteins, including the regulatory unit of PI3K (p85), are recruited to Smo in response to Shh. Moreover, IGF-IR was found to associate with Smo in response to Shh and to IGF-I, suggesting that Shh and IGF-I are already integrated at the receptor level, a mechanism by which their signaling pathways interact in augmenting their effects on adult myoblasts.

Keywords

myoblast; differentiation; sonic hedgehog; smoothened; Akt

Introduction

Satellite cell progeny, normally located in a quiescent state between the basal lamina and sarcolemma of the myofiber, contribute to the growth and regeneration of adult skeletal muscle. During growth or upon muscle damage, these cells are capable of activating their

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myogenic program, and either proliferating and differentiating into myofibers or undergoing self-renewal to maintain the satellite cell pool (reviewed in Bischoff, 1994; Zammit et al., 2004). The myogenesis of these muscle progenitors (also termed adult myoblasts) is governed at the transcriptional level by the paired box proteins Pax3 and Pax7 (Conboy and Rando, 2002; Halevy et al., 2004; Zammit et al., 2004) and by MyoD family members, which act in coordination with MEF2 and many other transcription factors (reviewed in Naya and Olson, 1999).

Several growth factors and hormones regulate myoblast proliferation and differentiation in autocrine/paracrine fashion. One of these factors is insulin-like growth factor I (IGF-I), which undergoes alternative splicing to give rise to several isoforms, one of which is produced locally by myoblasts (reviewed by Goldspink et al., 2008). IGF-I has been shown to induce myoblast proliferation and differentiation as well as muscle hypertrophy (Florini et al., 1996; Paul and Rosenthal, 2002). IGF-I receptor (IGF-IR) belongs to the receptor tyrosine kinase (RTK) family which, upon ligand binding, undergoes dimerization and activation by autophosphorylation of tyrosine residues located in the intracellular domain. As a result, the receptor interacts with intermediate signaling proteins, e.g., Src homology-containing proteins or the docking protein insulin receptor substrate-1 (IRS-1), leading to activation of downstream protein kinase pathways, such as the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) and the phosphoinositide-3 kinase (PI3K)/Akt pathways (reviewed in Ciampolillo et al., 2005). Whereas the MAPK/ERK pathway plays a role mainly in myoblast proliferation, the PI3K/Akt pathway is essential to their differentiation and myofiber hypertrophy (Coolican et al., 1997; Jiang et al, 1999; Rommel et al., 2001; Halevy and Cantley, 2004).

The morphogenic factor sonic hedgehog (Shh), a member of the Hedgehog (Hh) family, upregulates the formation and survival of myotomal cell lineage in the embryo (Münsterberg et al., 1995; Borycki et al., 1999; Kruger et al., 2001; reviewed in Christ and Brand-Saberi, 2002), and stimulates the proliferation and subsequent differentiation of committed myoblasts in the limb bud (Duprez et al., 1998; Christ and Brand-Saberi, 2002). In zebrafish, unequivocal evidence shows that Shh promotes terminal differentiation of myogenic cells by driving myogenic regulatory factors, *prdm1* and *cdkn1c* expression (Baxendale et al., 2004; Hinits et al., 2009; Osborn et al., 2011). Recently, Shh has been reported to be expressed in adult myoblasts (Elia et al., 2007) and to promote their proliferation and differentiation (Pola et al., 2003; Li et al., 2004; Koleva et al., 2005; Elia et al., 2007).

The binding of Shh to a receptor complex including the multipass-transmembrane protein Patched (Ptch) relieves Ptch inhibition of the G-protein-coupled membrane protein Smoothened (Smo), resulting in translocation of the latter to the primary cilium (Rohatgi et al., 2007). Once activated, Smo induces a complex series of intracellular reactions that activate the glioma-associated oncogene (Gli) protein(s) Gli-1, Gli-2 or Gli-3, for translocation to the nucleus and regulation of target gene transcription (reviewed in Ingham and McMahon, 2001; Lum and Beachy, 2004; Hooper and Scott, 2005; Ingham and Placzek, 2006; Mimeault et al., 2010). Shh has also been shown to regulate myoblast proliferation and differentiation via the MAPK/ERK and especially the PI3K/Akt signaling pathways (Elia et al., 2007). A relationship between Shh and IGF-I via the PI3K/Akt pathway has been suggested in fibroblasts (Riobo et al., 2006). In cerebellar neural precursors, IRS-1 has been reported as an effector of Shh signaling (Parathath et al., 2008). Shh and IGF-I have been reported to act synergistically to promote somite myogenesis (Pirskanen et al., 2000). In the present study, we sought to elucidate the relationship between Shh and IGF-I in activating the MAPK/ERK and PI3K/Akt pathways and regulating adult myoblast proliferation and differentiation. Our results demonstrate that Shh and IGF-I act additively on the MAPK/ERK and PI3K/Akt pathways and even synergistically in promoting myoblast

differentiation. Furthermore, we show that Smo activity is required for both Shh and IGF-I action and that the IGF-IR and its effector IRS-1 associate with Smo in response to these factors, suggesting that cross talk is already occurring at the receptor level.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), sera and antibiotic-antimycotic solution were purchased from Biological Industries (Beit Haemek, Israel). Human recombinant IGF-I was purchased from R&D Systems (Minneapolis, MN). Mouse recombinant N-terminally active Shh (N-Shh) was prepared according to a protocol kindly provided by David Bumcrot and Andrew McMahon (Harvard University, Cambridge, MA) (Bumcrot et al., 1995).

Transgenic mice

Transgenic mice lacking expression of Smo specifically in the limb muscles were generated by crossing $Smo^{flox/flox}$ mice (Long et al., 2001; termed here Smo^{cont}) with *MCre* mice expressing Cre recombinase driven by the proximal Pax3 promoter (Brown et al., 2005) (*MCre;*$Smo^{flox/flox}$, termed here Smo^{mut}). To test for the ability to recombine LoxP sites in limb myogenic cells, *MCre* mice were crossed with $Gt(ROSA)26Sort^{m1(EYFP)Cos}/J$ (Srinivas et al., 2001; termed here *R26R-EYFP*). Dual-carrier pups at postnatal day 1 were identified by EYFP fluorescence and analyzed for the presence of EYFP in limb muscles by dual-immunofluorescent detection with Hoechst 33258 for DNA (Sigma, XX), and the antibodies phalloidin-Alexa568 which binds actin, monoclonal antibody A4.1025 for myosin heavy chain (MyHC; Blagden et al., 1997), polyclonal rabbit anti-GFP (Torrey Pines Biolabs, East Orange, NJ) and Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) on cryosections of hind limbs. Most limb muscle fibers and muscle precursor cells underwent recombination, although other experiments have suggested that not every limb myogenic cell undergoes recombination in *MCre;*$Smo^{flox/flox}$ mice (V.C.W. and S.M.H., data not shown). Mice were housed and maintained under licence from the UK Home Office.

Cell cultures

Primary cultures of adult myoblasts were prepared from the hind limbs of 5-week-old Smo^{cont} and Smo^{mut} mice as described previously (Ben Dov et al., 1999). The primary cultures and C2 mouse myogenic cells (Yaffe and Saxel, 1977) were grown in DMEM supplemented with 20% (v/v) fetal bovine serum (FBS) containing antibiotic-antimycotic solution. For the experiments, cells were plated sparsely at 3 × 105 cells/100 mm Petri dish in growth medium for 1 day, after which the medium was changed to serum-free DMEM and cells were incubated for an additional 48 h. We have previously shown that under this type of starvation, cells can be driven back into the cell cycle in the presence of mitogens (Elia et al., 2007; Kornasio et al., 2009). Primary cultures from both Smo^{mut} and Smo^{cont} mice contained approximately 50% non-myogenic cells, as shown by the detection of desmin (data not shown). These cultures were monitored for Smo expression by western blot analysis which revealed approximately twofold less Smo protein in Smo^{mut} cultures than in Smo^{cont} cells, consistent with loss of Smo from most if not all myogenic cells, but presence of normal Smo in non-myogenic cells.

RNA preparation and RT-PCR

Total RNA was prepared using TRIzol™ Reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed into cDNA using random primers and SuperScript reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR) was then performed using *Taq* DNA polymerase (Fermentas, Glen Burnie, MD) for each set of specific primers. Primer sets and

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reaction conditions are summarized in Table 1. *GAPDH* was used as an internal control for normalization of sample mRNA amounts. PCR products were separated by electrophoresis and bands were visualized by video camera (UVltec, Cambridge, UK).

Immunoprecipitation and western blot analysis

For immunoprecipitation (IP) assays, cells were lysed in lysis buffer (Halevy and Cantley, 2004) and subjected to IP with anti-P-Tyr (Santa Cruz Biotechnology, Santa Cruz, CA), followed by western blotting analysis as described previously (Halevy and Cantley, 2004). In brief, proteins were resolved by 7.5% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). After blocking, the membranes were incubated with the following primary antibodies: polyclonal anti-Akt, anti-phospho-Akt, anti-phospho-p42/44, anti-p42/44, anti-IRS-1, anti-p85, anti phospho-p85 (Cell Signaling, Boston, MA), monoclonal anti-myogenin (F5D, Santa Cruz), anti-Pax7 and anti-MyHC (MF20; Hybridoma Bank, Iowa City, IA).

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and to all-pairs Tukey-Kramer HSD test using JMP® software (SAS, 2002).

Results

Cooperation of Shh and IGF-I on MAPK/ERK and Akt phosphorylation and on myogenic factor expression levels in C2 cells

In adult myogenic cells, IGF-I as well as Shh exert their effects on proliferation and differentiation mainly via the PI3K/Akt pathway and to a lesser extent via the MAPK/ERK pathway (Coolican et al., 1997; Rommel et al., 2001; Halevy and Cantley, 2004; Elia et al., 2007). Here, we evaluated the potential relationship between Shh and IGF-I with respect to their effects on these signaling pathways, and in relation to myoblast proliferation and differentiation. Various concentrations of Shh and IGF-I were added alone or in combination to serum-starved C2 cells for 12 min, after which cell lysates were analyzed for phosphorylation of MAPK/ERK (p42/44) and Akt. Addition of either Shh or IGF-I alone increased the expression levels of phospho-MAPK/ERK and phospho-Akt in a dose-dependent manner (Fig. 1). An additive effect was observed on Akt phosphorylation when the highest concentration of Shh (0.5 μg/ml) was added to any concentration of IGF-I and between the lower dose of Shh (0.2 μg/ml) and the highest dose of IGF-I (5 ng/ml) (Fig. 1B), both of which are sub-optimal concentrations (Halevy and Cantley, 2004, Elia et al., 2007). The additive effect was less pronounced with respect to MAPK/ERK phosphorylation and was observed only in cells treated with 0.5 μg/ml and 5 ng/ml Shh and IGF-I, respectively (Fig. 1C). Thus, Shh and IGF-I could affect the same early response pathways, in agreement with our previous studies (Elia et al., 2007).

To investigate the consequence of exposure to each signal, similar combinations of Shh and IGF-I were added to serum-starved C2 cells for 24 h and expression levels of Pax7, a marker of both undifferentiated myogenic precursor and proliferating adult myoblasts (Zammit et al., 2004; Halevy et al., 2004; Gros et al., 2005; Relaix et al., 2005; Piestun et al., 2009), and myogenin, a marker for the onset of myogenic differentiation (Naya and Olson, 1999), were analyzed by western blot (Fig. 2A). Addition of IGF-I alone increased Pax7 levels in a dose-responsive manner, whereas the effect of Shh was bell-shaped with a maximal effect at 0.2 μg/ml (Fig. 2A,B). Combinations of 2 ng/ml IGF-I with either 0.2 or 0.5 μg/ml Shh did not have any significant effect on Pax7 expression levels compared to the effect of IGF-I alone. However, 5 ng/ml IGF-I combined with all concentrations of Shh produced an additive effect on Pax7 levels (Fig. 2B). Myogenin levels increased dose-responsively in response to

either Shh or IGF-I alone (Fig. 2C). At 0.2 μg/ml Shh, a synergistic effect on myogenin levels was observed when combined with any of the IGF-I concentrations, whereas a higher concentration of Shh showed an additive effect with 2 ng/ml IGF-I and but not with 5 ng/ml IGF-I (Fig. 2C). These data confirmed the ability of Shh and IGF-I to elicit similar responses from mouse myogenic cells.

IGF-I enhances the effect of Shh on signaling pathways

The additive effects of Shh and IGF-I on the MAPK/ERK and PI3K/Akt pathways and additive and synergistic effects on markers for muscle-cell proliferation and differentiation raised the question of these factors' interdependence with respect to their actions in muscle cells. We first asked whether the effect of Shh depends on IGF-I secretion by the myogenic cells themselves. Addition of various concentrations of Shh to C2 cells for 24 h increased IGF-I mRNA expression levels nearly twofold relative to control, untreated cells (Fig. 3A). To test whether IGF-I is essential for Shh's effects on C2 cells, a neutralizing antibody to IGF-I was added to serum-starved C2 cells 2 h prior to the experiment to neutralize any residual or secreted IGF-I in the medium. Shh and IGF-I were added separately for 12 min to cells which were pre-incubated with or without the neutralizing antibody, and MAPK/ERK and Akt phosphorylation levels were analyzed by western blot. In the absence of the antibody, IGF-I induced Akt phosphorylation up to 6.3-fold compared to control cells (Fig. 3B). Addition of the neutralizing antibody completely abrogated the IGF-I-induced Akt phosphorylation. In contrast, addition of the antibody did not block the IGF-I-induced effect on phospho-p42/44 levels, probably due to the very rapid phosphorylation of p42/44, which is maximal after 5 min (Halevy and Cantley, 2004). We therefore focused on the mechanism by which Shh affects Akt. Shh caused a 3.7-fold increase in phospho-Akt in the absence of the IGF-I neutralizing antibody. However, although reduced, the effect of Shh on antibody-treated cells was not fully ablated: a twofold induction was still observed. This implied that IGF-I is important for achieving an enhanced effect, but is not necessary for Akt phosphorylation following Shh administration in C2 cells.

Shh enhances the effects of IGF-I on myogenic differentiation

Next, we tested whether the Shh pathway is required for the effects of IGF-I on the signaling pathways involved in myoblast proliferation and differentiation. Addition of various concentrations of IGF-I to serum-starved C2 cells for 24 h had no effect on Shh mRNA levels, which remained undetectable even after 40 cycles of PCR, whereas a clear signal for Shh mRNA was detected in control E9 mouse embryonic tissue (Fig. 4A). This showed that IGF-I does not act through Shh secretion. We next blocked the Shh-signaling pathway with cyclopamine, a Smo inhibitor, and evaluated the effects of IGF-I or Shh on Akt and p42/44 phosphorylation in serum-starved C2 cells. As expected, addition of each factor alone at its optimal level (Halevy and Cantley, 2004; Elia et al., 2007) induced the phosphorylation of both Akt and p42/44 in control experiments, the effect of IGF-I being greater than that of Shh (Fig. 4B-D). Addition of cyclopamine to control non-treated cells had no effect on phosphorylation. Although cyclopamine treatment was unable to entirely block the promotive effect of Shh on Akt and p42/44 phosphorylation, this reagent decreased this effect significantly, by approximately twofold (Fig. 4C). Despite the partial effectiveness of cyclopamine, this drug also reduced the response elicited by IGF-I to a similar extent (twofold). Thus, although Shh itself does not mediate IGF-I action, Smo activity appears to be required for the IGF-I signaling effect.

The requirement of Smo activity for IGF-I action was also analyzed using a genetic approach. In *MCre* mice, the hypaxial muscle promoter of *Pax3* drives Cre expression in early limb myogenic cells. To show the specific recombination of Pax3 in myogenic cells, these mice were crossed with mice expressing *R26R-EYFP* reporter. As expected, non-

muscle cells showed no recombination and did not express myogenin or phalloidin, which specifically binds actin (Fig. 5A). Therefore, the *MCre* mice were crossed to $Smo^{flox/flox}$ mice to ablate Smo specifically from cells of the limb myogenic lineage (Smo^{mut} mice). Primary myoblasts derived from transgenic mice lacking Smo expression in their limb muscle cells were used for this purpose. Primary cultures of myoblasts were prepared from the hind limbs of 5-week-old Smo^{cont} and Smo^{mut} mice and serum-starved for 48 h, after which they were exposed to Shh for 12 min and tested for Akt and p42/44 phosphorylation. Shh mildly but significantly increased Akt phosphorylation levels in cells derived from Smo^{cont} mice, whereas this effect was abolished in cells derived from Smo^{mut} mice (Fig. 5B,C), showing that primary limb myoblasts can respond to Shh in a manner similar to C2 cells, and that this effect depends on Smo activity, as expected.

The effect of IGF-I on Smo^{cont} cells was much more robust than that of Shh, consistent with previous results (Figs. 1, 4; Elia et al., 2007). However, in cells derived from Smo^{mut}, IGF-I-induced Akt phosphorylation reached only one-third the levels observed in the Smo^{cont} cells. Similar results were observed for p42/44 phosphorylation levels, except that in Smo^{mut} cells treated with Shh, these levels were significantly lower than those in untreated cells (Fig. 5B,D). We concluded that Smo activity is required for maximal IGF-I effect.

Smo mutation caused a similar inhibition of the effect of IGF-I on p42/44 phosphorylation level. However, Smo^{mut} cells treated with Shh showed suppressed p42/44 phosphorylation (Fig. 5B,D). These lower levels could be due to some inhibitory effect of Shh on the MAPK/ERK pathway in non-myogenic cells which still contain intact Smo and are present in the cultures (Fig. 5A). Taken together, these findings indicated that Smo activity upregulates both Akt and p42/44 phosphorylation in primary satellite cell-derived muscle cells by affecting gain of the IGF-I signaling pathway.

Smo is required for IGF-I's effect on myogenic differentiation

Levels of the myogenic proteins involved in the proliferation and differentiation of myoblasts were evaluated in the absence and presence of Shh or IGF-I in cultures of cells derived from Smo^{cont} and Smo^{mut} mice (Fig. 6). Pax7 protein was induced to comparable levels by Shh and IGF-I in Smo^{cont} cells. However, this induction was blocked in Smo^{mut} cells (Fig. 6A,B). The levels of myogenin and MyHC, markers for myoblast differentiation, in Smo^{cont} cells were induced in response to IGF-I and Shh treatment by two- and threefold, respectively, relative to control untreated cells (Fig. 6A,C,D). In contrast, in cells derived from Smo^{mut} mice, a reduction in the levels of these proteins was observed in response to either Shh or IGF-I compared to control untreated cells. Taken together, these results suggested a requirement for Smo in IGF-I's effects on myogenic differentiation.

Smo interacts with PI3K pathway molecules downstream of IGF-IR in response to Shh

In light of the above findings, we sought a possible mechanism by which Smo affects IGF-I signaling, underlying the cooperation of Shh and IGF-I with respect to their signaling pathways. The effect of Shh on signaling molecules downstream of IGF-IR was analyzed in a dose-response experiment in C2 cells using an IP assay with an antibody for tyrosine-phosphorylated (P-Tyr) proteins, followed by western blot analysis for the p85 subunit of PI3K and IRS-1, the docking proteins downstream of IGF-IR. Three- and fourfold induction in the recruitment of p85 and IRS-1, respectively, to P-Tyr proteins was observed in response to 0.5 μg/ml Shh (Fig. 7A). No association between these proteins was observed in a parallel negative control (-IP) which was not treated with the P-Tyr antibody. In addition, in a dose-response experiment, induction in the levels of phospho-p85, the activated form of the PI3K regulatory subunit, was observed with a maximal response at 0.5 μg/ml Shh after

12 min of incubation. At higher concentrations of Shh, phospho-p85 declined to close to control levels (Fig. 7A).

To determine the putative mechanism by which Shh affects IGF-I signaling, the possibility that p85 and IRS-1 are found in association with Smo in response to Shh was analyzed in serum-starved C2 cells. An IP assay using an antibody against Smo followed by western blot analysis for p85 revealed a robust elevation in the association of these proteins after 12 min incubation in response to Shh (0.5 μg/ml), reaching a maximum at 20 min, followed by a decline after 60 min (Fig. 7C). Western blot analysis for IRS-1 following IP for Smo revealed an association between these proteins in controls, that increased dose-dependently in response to Shh (Fig. 7D). An association between Smo and IGF-IR was observed in response to Shh (1 μg/ml) and to a greater extent in response to IGF-I (7.5 ng/ml; Fig. 7E), implying a direct association between signaling molecules downstream of Shh and IGF-I. These data suggested that Smo, IGF-IR, p85 and IRS-1 associate upon Shh stimulation of muscle cells.

Discussion

In this study, we demonstrated the interaction between Shh and IGF-I signaling pathways in adult myogenic cells. Both factors have been reported to promote the proliferation and differentiation of adult myoblasts via the MAPK/ERK and PI3K/Akt pathways (Coolican et al., 1997; Halevy and Cantley, 2004; Elia et al., 2007). Co-incubation of C2 cells with both factors (at sub-maximal levels) additively induced MAPK/ERK and Akt phosphorylation. The data suggest a mechanism by which Shh and IGF-I interact to affect myoblast signaling pathways. First, recruitment of IRS-1 to p-Tyr proteins, indicating activation of IGF-IR, was demonstrated in response to Shh. Second, we observed dose-responsive interactions between Smo and IRS-1 and between Smo and IGF-IR in response to either IGF-I or Shh, suggesting either a direct association between Smo and IGF-IR or an indirect association via a third component. In previous studies, cross-talk between Shh and IGF-I has been shown via downstream effectors such as Akt (Riobó et al., 2006). In cancer cells, EGF-receptor-induced MAPK/ERK can also promote the nuclear import of Gli, thereby explaining the synergistic effect of Hh and EGF signaling pathways in cell transformation (Schnidar et al., 2009; Mimeault et al., 2010). We do not rule out the notion that cross-talk between Shh and IGF-I pathways also occurs at the level of Gli during myoblast proliferation and differentiation. However, our findings imply that cross-talk between Shh and IGF-I is already occurring at the receptor level or with its immediate associates in the primary cilium, the membrane region to which Smo is translocated in response to Shh (Rohatgi et al., 2007). This is supported by a recent report that during adipocyte differentiation, IGF-IR located within the primary cilium is more sensitive to insulin stimulation than receptors that are not located in the cilium (Zhu et al., 2009).

The cooperation of Shh and IGF-I on signaling pathways is pronounced in their effects on expression of myogenic regulatory factors. Co-incubation of Shh and IGF-I for 24 h revealed an additive effect on increasing Pax7 and myogenin protein levels, and even a synergistic effect in C2 cells. A previous study has reported a synergistic effect of Shh and IGF-I in promoting MyHC expression in chick somite explants (Pirskanen et al., 2000). The synergy between Shh and IGF-I found in our data, in particular with respect to myogenin-expression levels, suggests that it also occurs in adult myogenic cells. Our view is that this synergistic interaction between Shh and IGF-I signaling serves as a critical step in adult myoblast differentiation.

In addition to the cooperative effects of Shh and IGF-I on signaling pathways and myogenic differentiation, the data suggest that they have effects on each other. On the one hand,

exposure of muscle cells to Shh led to a dose-dependent increase in IGF-I mRNA expression, which in turn led to enhancement of myogenic differentiation in an autocrine fashion. On the other, blocking IGF-I-induced Akt phosphorylation by IGF-I-neutralizing antibody caused a reduction, but not complete inhibition of Akt phosphorylation in response to Shh. We rule out the possibility that the effect of Shh on Akt phosphorylation is due only to the residual presence of IGF-I, since Shh alone was able to induce Akt phosphorylation in myoblasts (Fig. 1 and Elia et al., 2007). Therefore, the data suggest that IGF-I augments Shh signaling but is not essential for it.

In cultures derived from Smo^{mut} mice specifically lacking Smo expression in their hind limb muscles, the IGF-I-induced Akt and p42/44 phosphorylation was significantly reduced compared to the IGF-I effect on Smo^{cont} cells, yet some phosphorylation was still observed. The residual phospho-Akt and p42/44 could be attributed to IGF-I's effects on non-myogenic cells that were present in the primary cultures (data not shown) and expressed intact Smo. This view is supported by the complete inhibition of IGF-I-induced Pax7, myogenin and MHC levels found in Smo^{mut} myoblasts. Taken together, the data show that Smo activity affects gain of the IGF-I signaling pathway.

Notably, the expression levels of myogenin and MyHC were lower in Smo^{mut} than in Smo^{cont} cells in response to either Shh or IGF-I. This marked effect is not likely to be due to lesser myoblast numbers in the primary cultures, because Pax7 expression levels (Fig. 6A), as well as the number of desmin-expressing cells (data not shown) in the Smo^{mut} culture were comparable to those in the Smo^{cont} culture. Another possibility is that the action of Shh on non-muscle cells causes release of an inhibitor of muscle differentiation that may affect muscle cells and becomes apparent when they do not express Smo. We speculate that blocking Smo expression in vivo causes a potential negative feedback effect of Shh and IGF-I on myoblast differentiation, which in turn causes a further decrease in this process. This idea is supported by our finding of transcriptional upregulation of IGF-I by Shh.

Our data do not indicate that IGF-I affects the Shh signaling pathway *per se*. Instead, IGF-I acts additively with Shh to promote myogenesis. This is supported by evidence that Shh can elicit IGF-I synthesis in muscle cells which, through autocrine action, enhances myogenesis. Our findings are in agreement with another study, in fibroblasts, which reported that the Smo signaling pathway, although requiring Akt phosphorylation, is not induced by IGF-I alone (Riobó et al., 2006). Collectively, these findings show that Shh and IGF-I collaborate in promoting myoblast differentiation.

The remarkable effect of loss of Smo function inhibiting the stimulatory effect of IGF-I on myogenic differentiation implies that Shh promotes IGF-I's effects. However, the fact that each factor alone induced signaling pathways and myogenic factors in serum-free medium (Figs. 1, 2; Halevy and Cantley, 2004; Elia et al., 2007) suggests that the presence of Shh *per se* is not required for IGF-I's effects. Three lines of evidence suggest that maximal IGF-I signaling requires the activity of intact Smo in muscle cells: (1) in myoblasts lacking Smo expression, the IGF-I-induced increase in myogenic marker was completely abolished; (2) cyclopamine, which is known to inhibit the translocation of Smo to the cilium, thereby inhibiting Shh activity (Corbit et al., 2005), inhibited the phosphorylation of Akt and p42/44 in response to IGF-I; (3) IGF-IR was found to associate with Smo in response to IGF-I. Nevertheless, IGF-I alone could elicit Akt and p42/44 activity despite the absence of Shh in the serum-free medium (Fig. 3A and data not shown). Because activation of Smo is dependent on its presence in the primary cilium (Rohatgi et al., 2007), we speculate that some of the Smo molecules are present in the cilium allowing their association to IGF-IR and IGF-I signaling pathway activation. Recent reports have suggested that Smo activity might be modulated by endogenous sterol-like small molecules that can mimic Shh activity

(Corcoran and Scott, 2006; Dwyer et al., 2007). We hypothesize that without Hh ligand, there is a small basal level of Smo in the cilium which is enough to allow the IGF-IR to transduce IGF-I's effects on myogenic differentiation. In the presence of Shh, additional Smo is recruited to the cilium and can interact with IGF-IR, thereby enhancing IGF-I signaling.

In summary, our findings suggest that Shh and IGF-I affect, in an additive manner, adult myoblast proliferation and differentiation via the MAPK/ERK and PI3K/Akt pathways. While each factor acts alone, the findings suggest that Smo is required for IGF-I action, at least with respect to myogenic differentiation. The results suggest that the molecular mechanism by which Shh and IGF-I integrate their effect on myoblast proliferation and differentiation can already be found at the receptor level via an interaction of Smo with IGF-IR and IRS-1.

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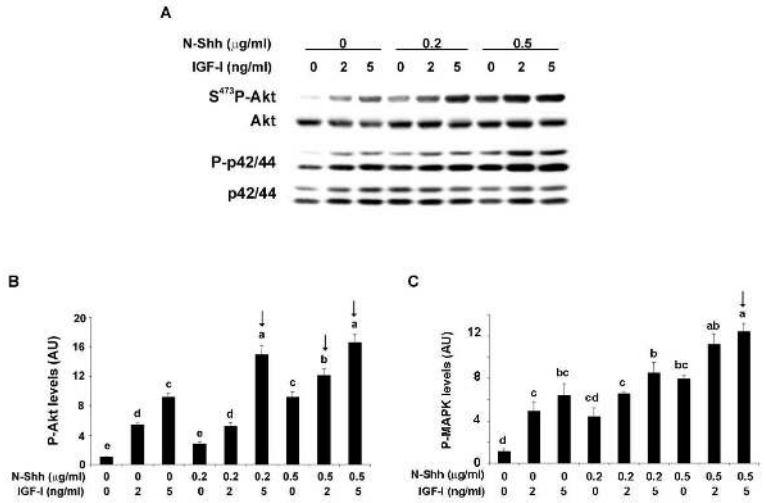

Fig. 1.

Effects of Shh and IGF-I on MAPK/ERK and Akt phosphorylation. C2 cells were serum-starved for 48 h, then DMEM was replaced with fresh DMEM containing various combinations of Shh and IGF-I for an additional 12 min. Cells were lysed with lysis buffer and equal amounts of protein were subjected to SDS-PAGE followed by western blot analysis with antibodies against phospho-S^{473}Akt and phospho-p42/p44. Parallel gels were reacted with anti-total Akt and anti-total p42/p44 antibodies. (A) Representative western blot analysis. Bands were quantified by densitometry and the phospho-S^{473}Akt (B) and phospho-p42/p44 (C) protein levels were normalized to total proteins. Results represent means ± SE of three independent experiments. Data with different letters differ significantly ($P < 0.05$). AU, arbitrary units.

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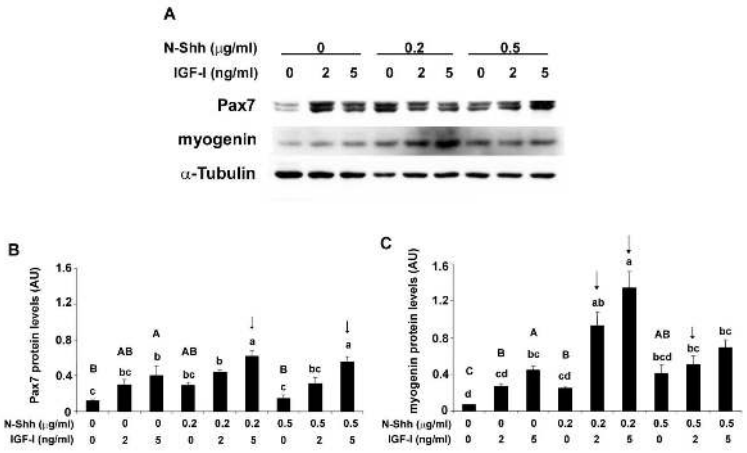

Fig. 2.

Relationship between Shh and IGF-I in their effects on myogenic protein levels. C2 cells were treated as described in Fig. 1 except that incubation with the various factors was for 24 h. Pax7 and myogenin protein levels were analyzed by western blot (A). Densitometry analysis for Pax7 (B) and myogenin (C) protein levels normalized to α-tubulin. Results represent means ± SE of three independent experiments. Different letters indicate significant difference at $P < 0.05$. AU, arbitrary units.

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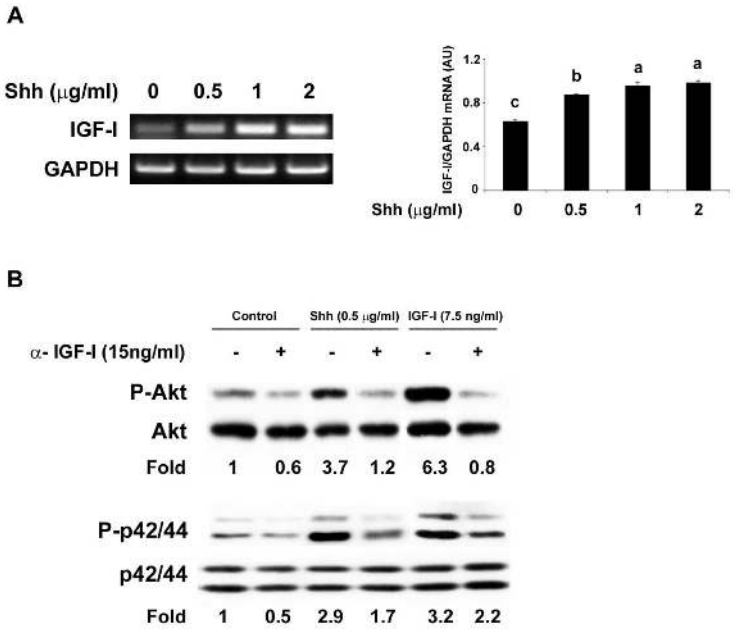

Fig. 3.

IGF-I enhances the effects of Shh on Akt and MAPK/ERK phosphorylation. (A) Shh was added at the specified levels to serum-starved C2 cells for 24 h, after which total RNA was extracted and subjected to semi-quantitative RT-PCR analysis for IGF-I mRNA expression levels. Left panel: equal samples were analyzed and electrophoresed as demonstrated by GAPDH mRNA levels. Right panel: densitometry analysis for IGF-I mRNA levels normalized to GAPDH. Data are means ± SE of three independent experiments. Data with different letters differ significantly ($P < 0.05$). AU, arbitrary units. (B) Neutralizing antibody against IGF-I was added to serum-starved C2 cells 2 h prior to the addition of Shh and IGF-I for an additional 12 min. Levels of phosphorylated and total proteins of Akt and p42/44 were analyzed by western blot. Densitometry analysis for levels of phosphorylated proteins normalized to total Akt or total p42/44; data are presented as fold induction relative to control untreated cells for each treatment, representing one out of three individual experiments.

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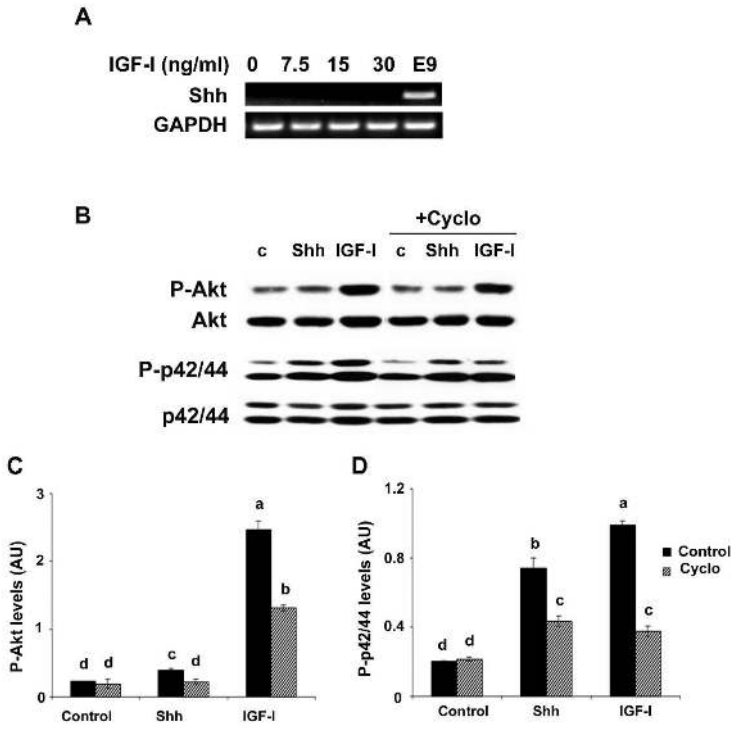

Fig. 4.

Effect of IGF-I on Shh gene expression in adult myoblasts. (A) IGF-I was added at the specified levels to serum-starved C2 cells for 24 h, after which total RNA was extracted and subjected to semi-quantitative RT-PCR analysis for Shh mRNA expression levels. E9, control embryonic tissue from E9 mouse. (B-D) Effect of cyclopamine on IGF-I-dependent MAPK/ERK and Akt phosphorylation. Cyclopamine (1 μg/ml) was added to serum-starved C2 cells for 30 min after which Shh (1 μg/ml) or IGF-I (7.5 ng/ml) was added for an additional 12 min. Phosphorylation of Akt and p42/44 in cell lysates was analyzed by western blot (B) and band intensity was quantified by densitometry and normalized to total Akt (C) or total p42/44 (D). Results represent means ± SE of three independent experiments. Different letters indicate significant difference at $P < 0.05$. AU, arbitrary units.

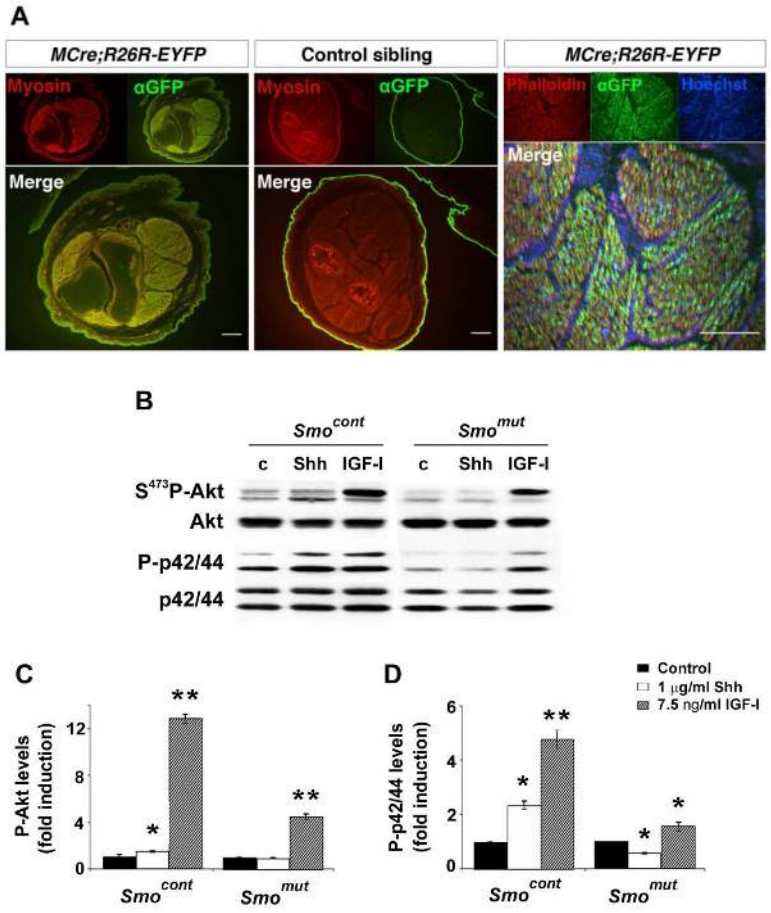

Fig. 5.

Shh is required for IGF-I-dependent effects on MAPK/ERK and Akt phosphorylation. (A) Fluorescent detection of myogenin (left and middle panels) and phalloidin (right panel) on *MCre;R26R-EYFP* mouse cryosections reveals extensive *MCre*-driven recombination in lower hind limb muscle. Bar = 200 μm. (B-D) Primary myoblasts were cultured from hind limb muscles of Smo^{cont} and Smo^{mut} mice and rendered quiescent for 48 h with serum-free DMEM. Then, medium was replaced with fresh DMEM containing Shh (1 μg/ml), IGF-I (7.5 ng/ml) or no addition for an additional 12 min. (B) Representative western blot for Akt and MAPK/ERK phosphorylation. Densitometry analysis for levels of phosphorylated Akt (C) and phosphorylated p42/44 (D), normalized to total Akt and total p42/44, respectively; levels are presented as fold induction relative to control for each treatment. Results are means ± SE of three individual experiments. Data with asterisks differ significantly within the same group (*$P < 0.05$; **$P < 0.01$).

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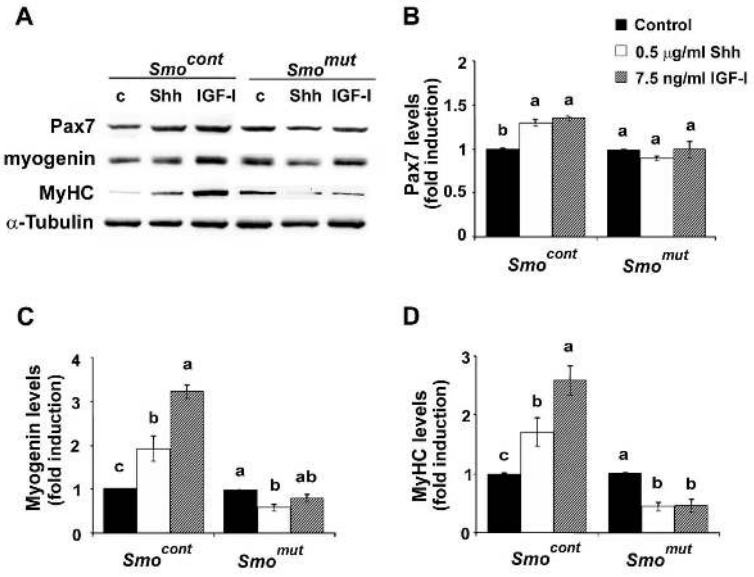

Fig. 6.

Shh is essential for IGF-I-dependent effects on myoblast proliferation and differentiation. The experiment was performed as described in Fig. 5 but cells were incubated with Shh or IGF-I for 24 h. (A) Representative western blot analysis for Pax7, myogenin and MyHC protein expression. Densitometry analysis for levels of Pax7 (B), myogenin (C) and MyHC (D) normalized to α-tubulin; levels are presented as fold induction relative to control for each treatment. Results are means ± SE of three individual experiments. Different letters within a group indicate significant difference at $P < 0.05$.

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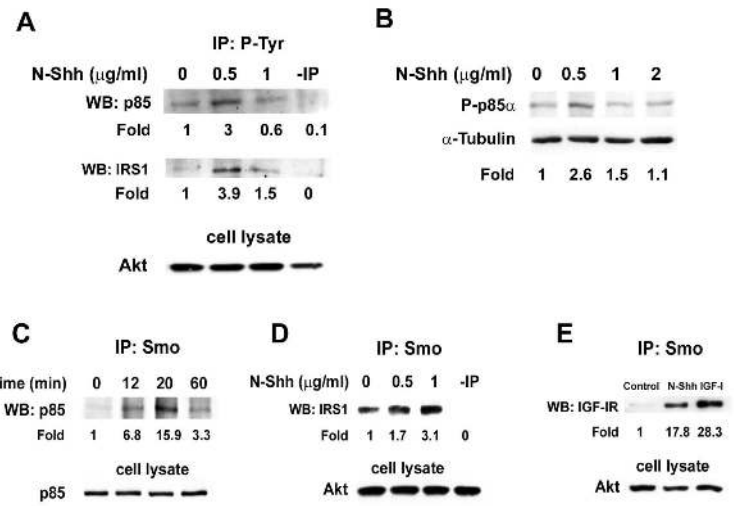

Fig. 7.

Interaction of Smo with PI3K pathway molecules downstream of IGF-IR. (A, B) IRS-1 and p85 are recruited to P-Tyr proteins and p85 is phosphorylated in response to Shh. Serum-starved C2 cells were treated with various concentrations of Shh for 12 min. (A) Cell lysates were subjected to IP with anti-P-Tyr antibody followed by western blot analysis for p85 and IRS-1. Levels of phosphorylated Akt and total Akt were evaluated in aliquots from the cell lysates before IP. Total Akt represents equal amounts of proteins taken for the IP analysis. -IP, a negative control that was not reacted with P-Tyr antibody. (B) Western blot analysis for protein expression of phosphorylated p85. Time-dependent (C) and dose-dependent (D) experiments for Shh effects on the association of Smo with p85 and IRS-1. Cell lysates were subjected to IP with anti-Smo antibody followed by western analysis for p85 (C) or IRS-1 (D). (E) Smo interacts with IGF-IR in response to Shh (0.5 μg/ml) or IGF-I (7.5 ng/ml) after 12 min in C2 cells. IP with anti-Smo antibody followed by western blot analysis for IGF-IR. Total p85 and total Akt represent equal amounts of proteins taken for the IP analysis in C and D, E, respectively.

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Table 1

Primer sets used for RT-PCR

Gene	Primers for RT-PCR	Tm	Size (bp)
GAPDH	Fwd: 5′-ACCACAGTCCATGCCATCAC-3′ Rev: 5′-TCCACCACCCTGTTGCTGTA-3′	50°C	451
Shh	Fwd: 5’- GGCAGATATGAAGGGAAGAT-3’ Rev: 5’- ACTGCTCGACCCTCATAGTG-3’	60°C	260
IGF-I	Fwd: 5′-GCTGAGCTGGTGGATGCTCTTCAGTTC-3′ Rev: 5′-CTTCTGAGTCTTGGGCATGTCAGTGTG-3′	75°C	215