The zebrafish slow-muscle-omitted gene product is required for Hedgehog signal transduction and the development of slow muscle identity

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SUMMARY

Hedgehog proteins mediate many of the inductive interactions that determine cell fate during embryonic development. Hedgehog signaling has been shown to regulate slow muscle fiber type development. We report here that mutations in the zebrafish slow-muscle-omitted (smu) gene disrupt many developmental processes involving Hedgehog signaling. smu−/− embryos have a 99% reduction in the number of slow muscle fibers and a complete loss of Engrailed-expressing muscle pioneers. In addition, mutant embryos have partial cyclopia, and defects in jaw cartilage, circulation and fin growth. The smu−/− phenotype is phenocopied by treatment of wild-type embryos with forskolin, which inhibits the response of cells to Hedgehog signaling by indirect activation of cAMP-dependent protein kinase (PKA). Overexpression of Sonic hedgehog (Shh) or dominant negative PKA (dnPKA) in wild-type embryos causes all somitic cells to develop into slow muscle fibers. Overexpression of Shh does not rescue slow muscle fiber development in smu−/− embryos, whereas overexpression of dnPKA does. Cell transplantation experiments confirm that smu function is required cell-autonomously within the muscle precursors: wild-type muscle cells rescue slow muscle fiber development in smu−/− embryos, whereas mutant muscle cells cannot develop into slow muscle fibers in wild-type embryos. Slow muscle fiber development in smu mutant embryos is also rescued by expression of rat Smoothened. Therefore, Hedgehog signaling through Slow-muscle-omitted is necessary for slow muscle fiber type development. We propose that smu encodes a vital component in the Hedgehog response pathway.

Key words: Slow muscle, Muscle fiber type, Zebrafish, Muscle pioneer, Axial muscle, Hedgehog signaling, Slow-muscle-omitted, Smoothened, Patched, Protein kinase A, Forskolin.

INTRODUCTION

Vertebrate skeletal muscle fibers can be subdivided into multiple fiber types based on contraction speeds, innervation, metabolism, morphology and the expression of specific contractile proteins. Muscle cells become committed to specific fiber type identities very early in development (for reviews, see Hughes and Salinas, 1999; Stockdale, 1992). However, the signals that regulate embryonic fiber type development in amniotes are unknown. Recent work in zebrafish suggests that some of the positive and negative signals from surrounding tissues that are known to influence myogenesis may also influence the development of muscle fiber type identity (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997; for a recent review, see Currie and Ingham, 1998).

Zebrafish have three distinct embryonic muscle fiber types: muscle pioneer slow muscle fibers, non-pioneer slow muscle fibers and fast muscle fibers. Slow muscle fibers develop from adaxial cells, which are adjacent to the notochord in the segmental plate. These cells are the first to express myogenic transcription factors such as MyoD (Weinberg et al., 1996) and the first to differentiate into muscle fibers (Devoto et al., 1996; van Raamsdonk et al., 1978). Adaxial cells begin to elongate while adjacent to the notochord and then migrate radially to the surface of the somite, forming a superficial monolayer of embryonic slow twitch muscle fibers (Devoto et al., 1996). A small subset of these cells express Engrailed proteins and are known as muscle pioneer slow muscle cells (Felsenfeld et al., 1991; Hatta et al., 1991). Muscle pioneers span the somite from its medial to its lateral surface at the future position of the horizontal myoseptum, which establishes a separation between the dorsal and ventral myotome (Waterman, 1969). The position of slow muscle precursors adjacent to the notochord suggests that notochord signaling may play a role in their development. In support of this, mutants with disrupted notochord development have a loss of muscle pioneers, and muscle pioneer development can be rescued by transplanting wild-type notochord cells into mutant embryos (Halpern et al., 1993). Non-pioneer slow muscle cells are also dependent on notochord signaling (Blagden et al., 1997). After adaxial cell migration to the surface of the myotome, fast muscle fibers develop from cells that were initially lateral to adaxial cells in the segmental plate. Their development does not depend on notochord signaling (Blagden et al., 1997).

Sonic hedgehog (Shh) is a secreted protein that underlies
many of the notochord signaling properties. Hedgehog (Hh) gene family members have been proposed to play critical roles in many diverse biological processes, ranging from segmentation in insects to cancer in humans (reviewed by Hammerschmidt et al., 1997). In every system that has been analyzed, components of the Hh signaling pathway are conserved. The Hh receptor Patched (Ptc) is a 12-pass transmembrane protein that forms a heterodimer with Smoothened, a 7-pass transmembrane protein with homology to G-protein coupled receptors (Chen and Struhl, 1998; Murone et al., 1999; Stone et al., 1996). When Hh binds to Ptc, it inhibits Ptc’s repression of Smoothened, enabling Smoothened to signal to downstream Hh signaling components such as Cubitus interruptus (Ci) in Drosophila and its homologs Glil, Glil2 and Glil3 in vertebrates (for recent reviews of the mechanism of Hh signal transduction, see Ingham, 1998; Johnston and Scott, 1998; McMahon, 2000). Hh signaling can be modulated by the activity of cAMP-dependent protein kinase (PKA), an antagonist to Hh signaling in vertebrates and in Drosophila (Hammerschmidt et al., 1997; Perrimon, 1995).

Ectopic overexpression of Hh in zebrafish is sufficient to transform the entire myotome into slow muscle (Blagden et al., 1997; Du et al., 1997). Inhibition of PKA signaling is also sufficient to induce slow muscle fibers, while hyperactivation of PKA blocks slow muscle development (Du et al., 1997; Hammerschmidt et al., 1996). From these data, we and others have proposed that Hh signaling specifies fiber type identity in the early zebrafish embryo (Blagden et al., 1997; Currie and Ingham, 1996; Du et al., 1997).

In this paper, we have used genetic and pharmacological approaches to further characterize the role of Hh in slow muscle development. We introduce a gene, slow-muscle-omitted (smu), that is necessary for slow muscle fiber type development. smu function is required for the response of muscle precursors to Hh, but not for their response to the inhibition of PKA or the overexpression of Smoothened. We discuss the implications of these data with respect to the Hh signaling pathway and the development of vertebrate muscle fiber type identity.

MATERIALS AND METHODS

Animals and mutagenesis

Wild-type embryos were obtained from the Oregon AB line, which was maintained in the Wesleyan University zebrafish colony (details of animal husbandry are available on request). Embryos were staged by hours (h) or days (d) post-fertilization at 28.5°C (Kimmel et al., 1995; available on the World Wide Web: http://zfish.uoregon.edu/).

Mutations were induced with N-ethyl-N-nitrosourea (ENU), following published procedures (Riley and Grunwald, 1995). Embryos were screened for morphological defects through the ongoing screen at the University of Oregon.

Two independent alleles of slow-muscle-omitted (smu) were identified, b577 and b641. Both alleles have been maintained on the AB background, with at least three outcrosses from the original stock of mutants. Both alleles appear to have identical phenotypes and all experiments were done at least once on each allele. Both mutations are recessive lethals inherited in simple Mendelian ratios, and all phenotypes are fully penetrant. Each phenotype that we have labeled as smu<sup>−/−</sup> was present in 1/4 of the embryos from a cross of two heterozygous carriers.

By complementation analysis, smu is not allelic to helix, you-too, iguana or you; no mutant embryos were seen in a cross of an identified smu mutant carrier and carriers of mutant alleles of these genes. The only other mutants that mildly resemble smu mutants, ugly and sonic you, are rescued by shh mRNA injections (Schauerte et al., 1998), indicating that these genes are distinct from smu (see Figs 3, 4, below).

Plasmids, in vitro mRNA synthesis and microinjection

Zebrafish shh and mouse dnPKA RNAs were transcribed from DNA plasmids TTTSshh and CS2+dnPKA-bGFP, respectively (Ekker et al., 1995a; Ungar and Moon, 1996). Capped mRNAs were transcribed from linearized DNA template with a T7 (shh) and SP6 (dnPKA) RNA polymerase in vitro transcription kit (mMESSAGE mMACHINE T7 or SP6, Ambion, Inc., Austin, TX, USA) according to the manufacturer’s instructions. Rat smoothened cDNA under the control of the CMV promoter (pRK5.xmoflag) was obtained from Donna Stone at Genentech (Stone et al., 1996).

For microinjections, mRNA and DNA were dissolved in double distilled H2O to final concentrations of 150-200 μg/ml and 17 μg/ml, respectively. Phenol Red was added to the solution at a final concentration of 0.1% to facilitate visualization during microinjections. Approximately 2 nl of RNA or DNA was microinjected into the yolk of zebrafish embryos at the one- or two-cell stage using published procedures (Westerfield, 1995).

Forskolin treatment of wild-type embryos

Wild-type embryos at specific stages were treated with 0.3 mM forskolin dissolved in 4% DMSO in embryo medium. To examine the effect of forskolin on eye separation, slow muscle fibers, and myoD, ptc1 and shh expression in the notochord, wild-type embryos were dechorionated and soaked in forskolin solution from 5.5h to the desired stage. For shh expression in the limb buds, forskolin treatment began at 8h, whereas for cartilage staining and pectoral fin outgrowth, treatment began at 24h. 100% of the treated animals exhibited the phenotype shown. Wild-type controls consisted of corresponding treatments with embryo medium alone and 4% DMSO in embryo medium. There were no differences between controls in the phenotypes assessed.

Transplantations

We created genetic mosaics between wild-type and mutant embryos essentially as described (Ho and Kane, 1990). Donor embryos were injected at the 1- to 4-cell stage with lysinated rhodamine dextran (10,000 kDa, Molecular Probes). Between 3h and 5h, 10-50 cells were transplanted from these embryos into similarly staged embryos. Transplant pipettes were made on a sanding disk constructed from a discarded hard drive coated with diamond lapping film. Transplantations were done using an Olympus SZX12 dissecting microscope. At 24h, the smu<sup>−/−</sup> embryos were identified on the basis of partial cyclopia and the U-shape of their somites. Embryos were fixed and sectioned on a cryostat; sections were then labeled with F59 and S58, respectively. Slow muscle faintly in zebrafish (Devoto et al., 1996). Tissue culture supernatants of F59 and S58 are specific for slow isotypes of myosin heavy chain in chicken (Crow and Stockdale, 1986) and slow muscle fibers in zebrafish (Devoto et al., 1996). S58 is an IgA monoclonal antibody specific for slow muscle fibers in zebrafish; supernatants were generously provided by Monte Westerfield at the University of Oregon and used at a dilution of 1:5. 4D9 is an IgG
monoclonal antibody, generated against the Drosophila invected homeodomain (Patel et al., 1989), which recognizes Engrailed proteins in zebrafish (Ekker et al., 1992; Hatta et al., 1991). 4D9 supernatant was obtained from the Developmental Studies Hybridoma Bank and diluted 1:3. Secondary antibodies from Sigma were used as follows: horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG at a dilution of 1:200, HRP-conjugated goat anti-mouse IgA at 1:100, TRITC-conjugated goat anti-mouse IgG at 1:200, and FITC-conjugated goat anti-mouse IgA at 1:100.

Immunocytochemistry, in situ hybridization and histology

Antibody labeling with F59 and 4D9 was carried out as previously described (Du et al., 1997; Hatta et al., 1991), with a few minor modifications. Briefly, embryos were fixed in 4% paraformaldehyde in PB (0.05 M phosphate buffer, pH 7.0) for 3 hours at room temperature (RT), or overnight at 4°C. Embryos were washed in 0.1 M PB (2x 5 minutes), rinsed in 50% methanol and soaked in 100% methanol at -20°C for at least 20 minutes. Embryos were rehydrated with 50% methanol (1x 5 minutes) followed by PBS-Tw (0.1% Tween20 in PBS, 1x 5 minutes) and incubated with 0.5% Triton X-100 (45 minutes, RT). Subsequent steps of antibody labeling were as described (Du et al., 1997).

Antibody labeling with S58 and zm4 was performed on embryos fixed with Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid). Embryos were rehydrated through 95, 85, 70, 50, and 30% ethanol (each for 10 minutes, RT) into distilled H2O or PB. Whole-mount S58 labeling was performed as described for F59 above, except that a goat anti-mouse IgA secondary antibody was used (Du et al., 1997). Labeling on sections was carried out as previously described (Devoto et al., 1996).

To count slow muscle fibers for the general description of smu-/- and forskolin-treated embryos, 24h-26h embryos were labeled with F59 or S58 in whole mount. Slow muscle fibers were identified on the basis of position (superficial in the myotome) and intensity of antibody labeling. For the counts shown in Figs 4 and 7, only S58 was used, and any labeling was counted as a complete muscle fiber. In these cases, individual embryos were separated and coded; the number of fibers in each embryo was counted 'blind', i.e. without knowing whether the embryo had been injected or not.

In situ hybridization of zebrafish embryos was performed using published procedures (Jowett, 1997). Vertebral and jaw cartilages of 3.5d embryos were visualized with Alcian Blue as described (Schilling et al., 1996).

Imaging

Whole-mount embryos were viewed and photographed using either Nomarski (DIC) optics on a Zeiss Axioskop compound microscope or with an Olympus SZX12 stereo microscope. Sections were photographed at 40x magnification using a fluorescence microscope. Rhodamine, fluorescein and DAPI or Hoechst images were overlaid using Adobe Photoshop. For comparisons between wild-type, mutant and treated embryos, photography and image manipulations were done identically and on the entire image.

RESULTS

In all species and systems that have been tested, activation of PKA inhibits Hh signaling, while inhibition of PKA mimics Hh signaling (Ingham, 1998; Perrimon, 1995). PKA can be activated pharmacologically using agents such as forskolin that increase cellular cAMP levels by direct stimulation of adenyl cyclase (Seamon and Daly, 1981; Tesmer and Sprang, 1998). We reasoned that if mutations in slow-muscle-omitted (smu) disrupted all Hh signaling, the phenotype should resemble that of forskolin-treated embryos. We have therefore compared these two phenotypes in detail, focusing on muscle fiber type development.

Phenotypes in slow-muscle-omitted mutants resemble those resulting from a deficiency in Hedgehog signaling

24h wild-type embryos had straight tails and chevron-shaped somites (Fig. 1A). In contrast, smu-/- and forskolin-treated embryos had ventrally curved tails and U-shaped somites (Fig. 1B,C). Morphologically distinct muscle pioneers were absent in both smu-/- and forskolin-treated embryos (Fig. 1A-C; data not shown). Both smu-/- and forskolin-treated embryos had varying degrees of ventral cyclopia (Fig. 1D,F,H) and a loss of head cartilage as compared to wild-type embryos (Fig. 1E,G,J). They also both had circulation defects, leading to cardiac edema and death by 5d (data not shown).

Shh is expressed in the posterior limb bud region known as the zone of polarizing activity (Roelink et al., 1994) and is necessary for limb bud outgrowth and anterior-posterior polarity in the limb (Neumann et al., 1999; for a review, see Tickle, 1995). Shh was expressed appropriately in the fin buds of wild-type, smu-/- and forskolin-treated embryos (Fig. 1J,L,N). At about 3d, however, smu-/- and forskolin-treated pectoral fins were severely reduced in size as compared to wild-type fins (Fig. 1K,M,O).

In zebrafish, as in other vertebrates, Shh is expressed in the axial mesoderm just after the beginning of gastrulation (Krauss et al., 1993). As the axial mesoderm differentiates into the notochord during the segmentation period, it continues to express Shh. smu-/- and forskolin-treated embryos expressed Shh in the notochord at levels comparable to wild-type (Fig. 1P,R,T). Tiggy winkle hedgehog and echidna hedgehog were also expressed normally in mutant and treated embryos (data not shown). The development of the hypochord and floor plate was apparently normal in mutant embryos (data not shown, see also Schauerte et al., 1998).

The types of defects in smu mutant embryos and their similarity to defects in forskolin-treated embryos suggested that smu mutations are disrupting Hh signaling. An early response to Hh signaling in zebrafish is the transcriptional activation of patched1 (ptc1) (Concordet et al., 1996; Lewis et al., 1999a). In wild-type embryos, ptc1 was expressed at high levels in mesodermal cells immediately adjacent to the notochord (Fig. 1P,Q; Concordet et al., 1996). smu mutant and forskolin-treated embryos did not express detectable ptc1 mRNA in the paraxial mesoderm (Fig. 1R-U).

slow-muscle-omitted/- and forskolin-treated embryos have defects in fiber type development

Three muscle fiber types develop during the segmentation period of zebrafish embryogenesis: fast muscle, slow muscle and muscle pioneer slow muscle cells. These three fiber types can be unambiguously identified using monoclonal antibodies. The S58 antibody exclusively labels muscle pioneer and non-pioneer slow muscle cells. The F59 antibody preferentially labels both types of slow muscle fibers, but also weakly labels fast muscle cells (Devoto et al., 1996). Using these two antibodies to label slow muscle fibers at 24-26h for counting, we found that all wild-type embryos had over 1000 slow muscle fibers per embryo (approximately 20 fibers per somite), whereas smu mutants had on average 11.3±0.5 (n=498) and
forskolin-treated wild-type embryos had on average 10.8±4.6 (n=23) fibers per embryo (Fig. 2A-C) (values are means ± s.e.m.). Engrailed proteins were expressed in muscle pioneer slow muscle fibers, but not in other slow muscle fibers, in 24h wild-type embryos (Fig. 2D; Hatta et al., 1991). No Engrailed expression was detectable in the trunk of smu+/- or forskolin-treated embryos (Fig. 2E,F), though Engrailed was expressed appropriately at the mid-hindbrain junction in these animals (data not shown).

Slow muscle fibers develop from adaxial cells adjacent to the notochord (Devoto et al., 1996). To determine if adaxial cells are initially present in smu+/- embryos, we examined myoD mRNA levels in wild-type and smu+/- embryos. At the 6-somite stage, myoD was expressed in the adaxial cells still adjacent to the notochord in the segmental plate (Fig. 2G; Weinberg et al., 1996). Mutant embryos lacked all adaxial cell staining except for very faint labeling in the posteriormost region of the segmental plate (Fig. 2H), whereas somitic labeling was apparently normal. Forskolin-treated embryos had a similar loss of adaxial myoD expression (Fig. 2I), although somitic staining was somewhat more disrupted in forskolin-treated embryos than in smu+/- embryos.

We used slow and fast muscle cell-specific antibodies to examine the differentiation of fast muscle fibers and to further characterize slow fibers. S58 is specific for slow muscle in zebrafish (Devoto et al., 1996), while zm4 is specific for fast muscle in zebrafish (Fig. 2J; M. Westerfield, personal communication). In sections of wild-type embryos approximately 20 slow muscle fibers in each somite formed a superficial monolayer bordering the zm4 staining of fast muscle fibers (Fig. 2J). Sections of smu+/- and forskolin-treated embryos were almost entirely devoid of S58-labeled slow muscle fibers (Fig. 2K,L). In contrast, zm4-labeled fast muscles were still present, suggesting that fast muscle development is not as dependent on smu function as is slow muscle. However, there was a variable reduction in the amount of fast muscle in both smu+/- and forskolin-treated embryos (compare Figs 2K,L, 3E,F, 6E,F); further experiments will be necessary to determine if this is due to an effect on cell proliferation or cell growth, leading to a smaller number or a smaller size of fast muscle fibers.

**Shh overexpression does not rescue slow muscle in slow-muscle-omitted+/- or forskolin-treated embryos**

Ectopic overexpression of Shh in wild-type embryos is sufficient to induce ectopic slow muscle cells in the paraxial
2193 Slow muscle requires slow-muscle-omitted mesoderm (Blagden et al., 1997; Du et al., 1997). To determine if smu−/− muscle precursors are able to respond to Hh, we overexpressed Shh by microinjection of mRNA at the 1- to 4-cell stage. We also microinjected shh into embryos that were subsequently treated with forskolin at 5.5h. Embryos were labeled by whole-mount RNA in situ hybridization for myoD at 12h, and at 30h sections were labeled with S58 and zm4 monoclonal antibodies to identify slow and fast muscle fibers, respectively. Overexpression of Shh did not have any effect on smu−/− mutant embryos. In a cross of two heterozygous carriers, 3/4 of the embryos had both normal adaxial myoD labeling and ectopic labeling in the lateral presomitic cells induced by the ectopic shh (Fig. 3A), whereas the remaining 1/4 of the embryos had no myoD labeling in adaxial cells and no expansion of myoD labeling into the lateral presomitic cells (Fig. 3B). Labeling of slow and fast muscle fibers at 30h showed the same patterns: smu mutant embryos did not respond to ectopic overexpression of Shh (Fig. 3D,E). We also

Fig. 2. smu−/− and forskolin-treated embryos have defects in slow muscle fiber type development. (A-C) Side views of S58 antibody labeled 24h embryos. S58 specifically labels slow muscle fibers. In wild-type embryos (A) approximately 20 slow muscle fibers span each chevron-shaped somite very clearly. In smu−/− (B) and forskolin-treated (C) embryos, slow muscle is markedly reduced; individual slow muscle fibers are marked with arrowheads. (D-F) Side views of 4D9 Engrailed antibody labeling of muscle pioneer slow muscle fibers at 24h. 4D9 labels 2-5 muscle pioneer nuclei per somite in wild-type (D), while smu−/− (E) and forskolin-treated (F) embryos have a complete loss of muscle pioneer slow muscle cells. (G-I) Dorsal view of 12h-14h embryos hybridized to show myoD (red) and shh (dark blue) expression. MyoD is expressed in wild-type embryos (G) in the somites and in the adaxial cells of the presomitic mesoderm. smu−/− (H) and forskolin-treated (I) embryos express myoD in the somites, but do not have any expression in cells adjacent to the notochord. (J-L) Transverse sections through the trunk of 30h embryos labeled with S58 (green) and zm4 (red) monoclonal antibodies to identify slow and fast muscle fibers, respectively. In sections of wild-type embryos (J), slow muscle fibers form a monolayer around the deeper fast muscle fibers. smu−/− (K) and forskolin-treated (L) embryos have a nearly complete loss of S58 labeling throughout the entire trunk, whereas zm4 staining is still present. Bars, 50 μm (A-F); 100 μm (G-I).

Fig. 3. Shh overexpression in smu−/− and forskolin-treated embryos does not rescue slow muscle development. (A-C) shh mRNA injected embryos were fixed at 12h and labeled by in situ hybridization for myoD; dorsal views are shown. In 3/4 of the embryos from a cross of 2 smu heterozygotes (60/86), myoD expression is expanded into the segmental plate as a result of Shh overexpression (A). In 1/4 of the embryos (26/86), myoD expression is not affected by Shh overexpression (B). 100% of the forskolin-treated embryos failed to respond to Shh overexpression (C). (D-F) shh mRNA injected embryos were fixed at 30h and transverse sections were labeled for S58 (green) and zm4 (red). In wild-type embryos (D), most of the zm4-labeled fast muscle (red) is replaced by S58-labeled slow muscle (green), compare to Fig. 2J. Slow muscle is not rescued by Shh overexpression in smu−/− (E) or in forskolin-treated (F) embryos; compare to Fig. 2K,L. See also Fig. 4. Bars, 100 μm (A-C); 50 μm (D-F).
Fig. 4. Quantitative analysis of slow muscle fiber numbers in uninjected, shh injected and dnPKA injected embryos from a cross of two smu<sup>+/−</sup> animals. SS8-labeled slow muscle fibers in uninjected and injected embryos were counted blind, and fiber tallies binned in sets of 100 for clarity of presentation. Uninjected siblings of shh injected, dnPKA injected and rsmo injected embryos (Fig. 7) were pooled. shh injection (n=453) has no effect on the expected Mendelian proportion of mutant embryos; as in uninjected embryos (n=1219), approximately 25% had <100 fibers, and approximately 75% had >1000 fibers. Conversely, only 5% of the embryos injected with dnPKA exhibited <100 fibers (black bars, n=421). Numbers of dead and malformed embryos (in which we did not count fibers) for injected embryos and their siblings were very similar (shh injected: 58 of 511, siblings 34 of 607; dnPKA injected 10 of 431, siblings 29 of 651).

Although we saw variation between clutches of uninjected embryos in the number of slow muscle fibers per embryo, among embryos with <1000 slow muscle fibers shh injection did not affect the number of fibers within clutches, whereas dnPKA injection consistently increased the mean number of slow muscle fibers. shh-injected embryos had an average of 3.9±0.14 slow muscle fibers per fish (n=114), and their uninjected siblings had an average of 3.2±0.3 (n=154). This difference is not significant (t-test, P≥0.10). dnPKA injected embryos had an average of 259±25.6 slow muscle fibers per fish (n=71), while their uninjected siblings had an average of 8.3±0.6 (n=162). This difference is significant (t-test, P=0.0005). Values are means ± s.e.m. Insets depict representative embryos for uninjected controls with 0-99 (a) and >1000 (b) fibers, shh injected embryos with 0-99 (c) and >1000 (d) fibers, and dnPKA injected embryos with 300-399 (e) and >1000 (f) fibers.

counted whole-mount, SS8-labeled slow muscle fibers in shh-injected embryos to test for any quantitative rescue by Shh overexpression. 25% of both uninjected and shh-injected embryos had <100 fibers, while 75% had >1000 fibers (Fig. 4). We conclude that slow muscle in mutant embryos is unaffected by shh overexpression.

Shh overexpression in forskolin-treated embryos also had no effect on myoD expression at 12h (Fig. 3C), or on slow muscle differentiation at 30h (Fig. 3F). The observation that treatment with forskolin at 5.5h blocked the effect of very early overexpression of Hh resulting from mRNA injections at the 1-2 cell stage suggests that cells are unable to respond to Hh overexpression until after 5.5h, when endogenous Hh is normally expressed. Neither of the likely Hh receptors, Ptc1 or Ptc2, is expressed before 8h, supporting this conclusion (Concordet et al., 1996; Lewis et al., 1999a).

### Table 1. smu is required cell-autonomously

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<tr>
<th>Donor→Host</th>
<th>Slow</th>
<th>Fast†</th>
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<tr>
<td>wt*→wt* (n=31)</td>
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<td>1176</td>
</tr>
<tr>
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<td>201</td>
</tr>
<tr>
<td>smu&lt;sup&gt;+/−&lt;/sup&gt;→wt (n=17)</td>
<td>0</td>
<td>663</td>
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The fiber type identity of every donor-derived muscle cell was determined by labeling with F59. The number of individual slow and fast fibers are shown. n, the number of host embryos examined.

*wt, wild type: includes both smu<sup>+/−</sup> and smu<sup>+/+</sup> embryos.

†This is a conservative estimate of the number of fast muscle fibers; individual fast fibers were sometimes difficult to distinguish from each other.

**slow-muscle-omitted function is required in slow muscle precursors**

The above results suggest that smu function is required for muscle precursor response to Shh. To determine if the smu gene product is necessary in muscle cells, we transplanted 10-50 cells from wild-type into mutant embryos, from mutant into wild-type embryos and from wild-type into wild-type embryos. We then assayed the fiber type identity of donor-derived muscle cells by F59 antibody labeling. We expected that donor cells that developed adjacent to the notochord would develop into slow muscle fibers, whereas donor cells positioned more laterally in the segmental plate would develop into fast muscle cells. As displayed in Table 1, we found that about 1/3 of transplanted wild-type muscle precursor cells developed into slow muscle fibers when placed into wild-type hosts. Roughly the same proportion of wild-type donor muscle precursors developed into slow muscle fibers when placed into smu<sup>+/−</sup> hosts. In striking contrast, none of the muscle precursors derived from a smu<sup>−/−</sup> donor developed into slow muscle fibers in wild-type hosts. The rescue of slow muscle by wild-type cells in mutant hosts occurred irrespective of whether other, non-muscle, donor cells were present. In some cases, wild-type donor cells differentiated only into slow muscle fibers in a smu mutant host embryo (Fig. 5A-C), whereas in other cases both slow and fast muscle fibers developed from wild-type cells in the mutant host (Fig. 5D-F). No rescue of slow muscle was observed when wild-type cells differentiated into notochord or floor plate cells (data not shown), consistent with the observation that mutant cells are unable to differentiate into...
slow muscle in wild-type hosts (Table 1). We conclude that smu gene function is required only in muscle precursors in order for slow muscle development to occur.

**Fig. 5.** Transplanted wild-type muscle cells rescue slow muscle development in smu−/−. Transverse sections from two different smu−/− embryos show donor-derived, wild-type muscle cells. Sections were labeled with the F59 antibody, detected with a fluorescein-conjugated secondary antibody, and counterstained with Hoechst 33258 (blue). (A,D) Rhodamine-labeled wild-type cells (red, arrowheads). (B,E) F59-labeled slow muscle fibers (green, arrowheads). (C,F) merged micrographs. In A-C, four transplanted wild-type cells have developed into slow muscle fibers in an approx. 22h smu−/− host (C, yellow, arrowheads). Cells are still migrating through the somite. In D-F, transplanted wild-type cells have differentiated into both slow (4 cells; F, yellow, arrowheads) and fast (approx. 10 cells) muscle fibers in a 24h smu−/− host. Slow fibers can be distinguished from fast on the basis of the intensity of F59 labeling. Bar, 50 μm.

dnPKA overexpression rescues slow muscle in slow-muscle-omitted−/− and forskolin-treated embryos

Overexpression of a dominant negative form of PKA (dnPKA) leads to the induction of ectopic muscle pioneer and non-pioneer slow muscle cells (Du et al., 1997; Hammerschmidt et al., 1996). If the Smu protein acts upstream of the PKA target, then microinjecting dnPKA should rescue slow muscle in mutant embryos. Forskolin-treated embryos should also respond to dnPKA, as dnPKA does not respond to elevated cAMP levels (Clegg et al., 1987). smu mutant embryos did respond to dnPKA. In a cross of two heterozygous carriers, greater than 90% of the embryos injected with dnPKA had normal myoD labeling in adaxial cells and/or the expansion of myoD labeling into the lateral presomitic cells (Fig. 6A,B).

Similarly, dnPKA overexpression reversed the effects of forskolin on adaxial myoD expression at 12h (Fig. 6C). Labeling of slow and fast muscle fibers at 30h showed the same patterns: wild-type, forskolin-treated and smu mutant embryos all responded to ectopic overexpression of dnPKA (Fig. 6D-F). Some of these embryos have rescue of myoD and slow muscle on only one side of the trunk; we suspect that these are partially rescued smu−/− embryos (Fig. 6B,E), a phenotype never observed following shh mRNA injections.

Unlike shh overexpression, dnPKA overexpression led to a large reduction in the proportion of embryos with less than 100 fibers, and an increase in the proportion with greater than 100 fibers (Fig. 4). We conclude that dnPKA rescued the mutant embryos (see also Fig. 4 insets).

**Rat smoothered overexpression rescues slow muscle in slow-muscle-omitted−/− embryos**

Since smu mutant cells can respond to dnPKA overexpression, we next focused on Smoothened, a component in the Hh signaling pathway upstream of the action of PKA. Because the zebrafish homologue(s) of the smoothered gene has not yet been identified and members of the Hh signaling pathway are widely conserved, we tested rat smoothered cDNA for its ability to rescue slow muscle development in smu mutant embryos. We injected 1-2 cell embryos with rat smoothered cDNA under the control of the CMV promoter (Stone et al., 1996), allowed the embryos to develop to 24h, and then labeled for slow muscle fibers (S58). Like dnPKA overexpression, overexpression of smoothered led to a large reduction in the
proportion of embryos with less than 50 fibers and an increase in the proportion with greater than 50 fibers (Fig. 7). We conclude that smoothened rescued the mutant embryos (see also Fig. 7 insets).

**DISCUSSION**

Mutations in the slow-muscle-omitted gene lead to the loss of hedgehog signaling and the consequent loss of slow muscle fibers. smu functions cell-autonomously in the responding cells, and smu mutant cells do not respond to overexpression of Shh. Slow muscle development is rescued in smu mutant embryos by inhibition of PKA, as well as by expression of rat Smoothened. Below, we discuss these results in the context of what is known about Hh signaling.

**The role of slow-muscle-omitted in Hedgehog signaling**

The first step in Hedgehog signal transduction is fairly well-established: Hh binds to its receptor Patched and inhibits Patched’s repression of Smoothened (for reviews, see Ingham, 1998; McMahon, 2000). Less is known about transduction of the Hh signal from the Patched-Smoothened complex to Hh target genes. The homology of Smoothened to G-protein linked receptors suggests that Smoothened could have an as-yet-unidentified extracellular ligand. Recent work in *Drosophila* has demonstrated that cells homozygous for smoothened null mutations are unable to respond to Hh signaling and that inactivation of PKA yields a Hh response in smoothened null cells (Chen and Struhl, 1998). These results suggest that all Hh signaling in *Drosophila* depends on Smoothened and that at least some functions of Smoothened can be replaced by the repression of PKA activity. Recently, evidence has emerged that Ci is directly phosphorylated by PKA (Chen et al., 1999b, 1998; Wang et al., 1999) and that Hh signaling may depend on the activity of a protein phosphatase (Chen et al., 1999a; Krishnan et al., 1997). Vertebrate Gli proteins appear to function similarly to Ci (Aza-Blanc and Kornberg, 1999; Ruiz i Altaba, 1999; von Mering and Basler, 1999).

Our data eliminate some potential candidates for the smu gene. The lack of rescue by Hh and the cell-autonomy results indicate that smu is not required for Hh synthesis, processing or presentation to muscle precursor cells. Rescue by dnPKA makes it also very unlikely that smu function is required downstream of the target of PKA action. Thus, smu is probably not one of the gli homologues, as the products of these genes act downstream of PKA (Aza-Blanc and Kornberg, 1999). Moreover, mutations in yot, the zebrafish homologue of gli2 (Karlstrom et al., 1999), are not rescued by inhibition of PKA with dnPKA (Schauerte et al., 1998).

We favor a model in which smu encodes a component directly within the Hedgehog pathway, acting upstream or at the same point as Smoothened. The Hedgehog receptor Patched (Concordet et al., 1996; Hooper and Scott, 1989; Nakano et al., 1989; Stone et al., 1996), and the Hh binding protein Hip (Chuang and McMahon, 1999) are both inhibitory components of the Hh signaling cascade. Thus, unless both alleles of smu are gain of function mutations, smu is unlikely to encode these components. Our data do not exclude the possibility that smu encodes a ligand or other cofactor required for Smoothened function. However, Smu protein must be required cell-autonomously (Fig. 5), and must not be required in the context of dnPKA or rat Smoothened overexpression (Figs 6, 7). As in the *Drosophila* smoothened mutant (Chen and Struhl, 1998), the simplest model that accounts for the rescue of smu mutant embryos by smoothened and the lack of rescue by shh is that smu encodes a zebrafish homologue of the smoothened gene.
Other possibilities for smu function can be envisaged. If both alleles of smu are partial loss of function mutations, it is possible that Smu functions downstream of Smoothened. In this case, overexpression of Smoothened compensates for the partial loss of Smu function. If the outcome of Hh signaling depends on a quantitative balance between the activity of Smoothened and the activity of PKA, it is also possible that smu encodes a inhibitor of PKA activity. In this case, smu mutations would lead to an increase in PKA activity, which could be overcome by a greater increase of Smoothened signaling following smoothened overexpression. Although we cannot formally exclude these possibilities, we believe that they are less likely than the model we propose above. Conclusive evidence of the nature of the smu gene product will require the cloning of the smu gene.

Is slow-muscle-omitted required for all Hedgehog signal transduction?

Vertebrates have several homologues of each of the genes involved in Hedgehog signaling and many of them have overlapping expression patterns. For example, in zebrafish, tiggy winkle hedgehog, echidina hedgehog and sonic hedgehog are all expressed in the midline (Currie and Ingham, 1996; Ekker et al., 1995b; Krauss et al., 1993), and ptc1 and ptc2 are both expressed in the paraxial mesoderm (Concordet et al., 1996; Lewis et al., 1999a). The extent of functional overlap, crosstalk and redundancy between the different homologues is not clear.

A variety of mutants exhibit a disruption in the development of tissues dependent on midline signaling. These include mutations in sonic you (suy), which encodes zhl, you-too (yot), which encodes gli2, you, chameleon (con) and uboot (ubo), which have all been proposed to be part of the Hh signaling pathway (Karlstrom et al., 1999; Schauerte et al., 1998; van Eeden et al., 1996). These mutants show a set of common phenotypes, many of which are also seen in mutants with notochord defects. Phenotypes include loss of muscle pioneers, adaxial cells and dorsal aorta, partial cyclopia, ectopic lens in the ventral head midline, and pectoral fin and jaw cartilage defects (Beattie et al., 1997; Blagden et al., 1997; Halpern et al., 1993; Karlstrom et al., 1999; Lewis, 1999b; Schauerte et al., 1998; Talbot et al., 1995; van Eeden et al., 1996). The severity of each phenotype varies considerably between the different mutants, with some phenotypes absent in some mutants. smu-/- embryos show all of the phenotypes seen in these mutants, and in every case the phenotype is as strong or stronger than that seen in other mutants. Moreover, the phenotypes seen in smu mutants are analogous to many of the phenotypes seen in chick and mouse embryos and in humans that are deficient in Hh signaling, including defects in the development of limbs, head cartilage, eye spacing and somite patterning (Ahlgren and Bronner-Fraser, 1999; Chiang et al., 1996; Ha and Helms, 1999; Marcelle et al., 1999; Ming and Muenke, 1998). Furthermore, the smu-/- phenotypes closely resemble the extreme Hh-deficient phenotypes seen in forskolin-treated embryos (Figs 1, 2). These observations together support the hypothesis that smu encodes a necessary component for all Hh signaling, which acts downstream of the three different Hh proteins and the two different Ptc proteins. If this is true, slow-muscle-omitted mutant embryos provide a very useful genetic tool for dissecting both the multifaceted role of Hh signaling in vertebrate embryogenesis and the complex nature of Hh signal transduction.

The role of Hedgehog signaling in vertebrate slow muscle development

Hh is a critical component in the development of vertebrate muscle (for a recent review, see Hughes et al., 1998). We and others have suggested that it also regulates the development of fiber type identity (Blagden et al., 1997; Cann et al., 1999; Currie and Ingham, 1996; Du et al., 1997; Lewis et al., 1999b). In zebrafish, overexpression of Hh is sufficient to induce slow muscle cells in the paraxial mesoderm, and mutations in yot, the zebrafish homolog of gli2, show a deficiency in slow muscle development (Du et al., 1997; Blagden et al., 1997; Lewis et al., 1999b). Moreover, slow muscle development can be blocked by either the activation of PKA, which is known to disrupt Hh signal transduction, or overexpression of ptc1, which represses Hh signaling (this paper; Du et al., 1997; Lewis et al., 1999b). If smu mutants have an almost complete loss of slow muscle, as we have shown, and if smu is required for all Hh signaling, as we propose, then this confirms that Hh signaling is necessary for slow muscle development in zebrafish.

Two important issues are raised by our results. First, we do not know the fate of smu-/- cells adjacent to the notochord that receive but cannot transduce the Hh signal. They may differentiate into fast muscle or sclerotome, remain undifferentiated, or die. Second, a small number of slow muscle fibers remain in smu-/- embryos. These fibers may develop from adaxial cells if the smu mutants that we have characterized do not lead to a complete loss of Hh signaling. Alternatively, they may develop from a population of slow muscle precursors that is distinct from the adaxial population. If a small number of slow fibers are also present in other mutants with a disruption in Hh signaling, then this would suggest that there is a Hh-independent population of slow muscle precursors. However, our results clearly show that the vast majority of embryonic slow muscle fibers are dependent on Smu mediated Hh signaling.

The development of muscle fiber type has not been examined in mice or chicken that have deficits in Hh signaling due to mutations or experimental perturbations. The molecular identification of the smu gene and the identification of smu homologues in mouse and chicken will help to indicate whether slow muscle development in other vertebrates also depends on Slow-muscle-omitted mediated Hh signaling.

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