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Slow muscle induction by Hedgehog signalling in vitro

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SUMMARY

Muscles are composed of several fibre types, the precise combination of which determines muscle function. Whereas neonatal and adult fibre type is influenced by a number of extrinsic factors, such as neural input and muscle load, there is little knowledge of how muscle cells are initially determined in the early embryo. In the zebrafish, fibres of the slow twitch class arise from precociously specified myoblasts that lie close to the midline whereas the remainder of the myotome differentiates as fast myosin expressing muscle. In vivo evidence has suggested the Sonic Hedgehog glycoprotein, secreted from the notochord, controls the formation of slow twitch and fast twitch muscle fates. Here we describe an in vitro culture system that we have developed to test directly the ability of zebrafish myoblasts to respond to exogenous Sonic Hedgehog peptide. We find that Sonic Hedgehog peptide can control the binary cell fate choice of embryonic zebrafish myoblasts in vitro. We have also used this culture system to assay the relative activities of different Hedgehog-family proteins and to investigate the possible involvement of heterotrimeric G-proteins in Hedgehog signal transduction.

Key words: Sonic Hedgehog, Zebrafish, Myoblast, Cell fate

INTRODUCTION

Vertebrate muscles are generated by the differentiation of mono-nucleate proliferating myoblast precursors into non-dividing myocytes, which in turn fuse to form mature multi-nucleate muscle fibres. Within each individual fibre constituent proteins of the contractile apparatus are induced and specific isoforms of these proteins determine the kinetic properties of individual muscles. In particular, expression of the myosin heavy chain (MyHC) class of proteins determines the contractile properties of fibres through its associated ATPase activity. Muscle fibres can be broadly classed into two types. Fibres that express the 'slow' class of MyHC isoforms and are specialised for slow contraction under conditions of oxidative metabolism. Fast fibres in turn express the 'fast' class of MyHC isoforms required for high force – short duration contractions directed by glycolytic metabolism. Each muscle contains a unique mix of each type of fibre, specific for the nature of the muscle load.

Attempts to understand the mechanisms that determine fibre type specification within the amniote embryo have been hampered by a lack of knowledge of the lineages of individual muscle cells. In the zebrafish embryo, by contrast, the elucidation of distinct embryonic origins for fast and slow myocytes has allowed an examination of the mechanisms that underlie their determination (Devoto et al., 1996; Blagden et al., 1997; reviewed by Currie and Ingham, 1998). Slow MyHC expressing myocytes arise from presomitic cells immediately adjacent to the notochord, termed the 'adaxial' cells. Midway through segmentation, adaxial cells differentiate as an axial layer of slow muscle flanking the notochord (Devoto et al., 1996; Blagden et al., 1997). By 24 hours of development, however, the majority of differentiated slow MyHC expressing cells have migrated from their origins next to the notochord traversing the entire extent of the myotome to form a superficial, subcutaneous layer of slow twitch muscle (Bone, 1978; Devoto et al., 1996; Blagden et al., 1997). The remainder of the myotome differentiates as fast twitch muscle behind the migration of the adaxially derived slow muscle cells (Devoto et al., 1996; Blagden et al., 1997).

The intimate association of the adaxial derived slow muscle precursors with cells of the notochord and the aberrant muscle development in mutant zebrafish embryos with defective notochords, suggested that notochord derived signals controlled specification of the adaxial lineage (Halpern et al., 1993; Currie and Ingham, 1996). Recent studies have implicated the secreted glycoprotein Sonic Hedgehog (SHH) as one notochord-derived signal that controls the specification of these cells (Currie and Ingham, 1996; Hammerschmidt et al., 1996; Weinberg et al., 1996; Concordet et al., 1996; Blagden et al., 1997; Du et al., 1997). Mutants that compromise *shh* signalling possess concomitant defects in adaxially derived slow twitch muscle, but leave lateral fast muscle cell fates unaffected (Blagden et al., 1997; Lewis et al., 1999).

Conversely, injection of *shh* sense mRNA induces slow MyHC expressing myocyte differentiation throughout the myotome and this occurs at the expense of the fast muscle cell fate (Blagden et al., 1997; Du et al., 1997). The observation that all lateral, non-adaxial, myoblasts can be induced to form slow muscle when exposed to ectopic SHH suggests that their fate is determined by the limited range of a localised SHH signal (Blagden et al., 1997). These observations suggest a model whereby high concentrations of SHH peptide are received by the single layer of adaxial cells flanking the notochord and these cells are consequently induced to express slow MyHC. Any SHH protein reaching the nearby prospective fast MyHC expressing myocytes would fall below the threshold required for induction of the slow muscle fate. This scenario differs from that postulated to occur within the neural tube: here, specific neuronal fates appear to be determined in a dose dependent manner, with ventrally derived neurons specified at higher concentrations of SHH peptide than more dorsal fates (reviewed by Briscoe and Ericson, 1999).

Unravelling the role of SHH in the zebrafish myotome is complicated by the presence of multiple HH-family proteins in midline structures of the zebrafish embryo. Previous studies have suggested that qualitative differences in HH activity could underlie the differentiation of different muscle cell types (Currie and Ingham, 1996). Muscle pioneer cells are the first elongating and striating cells of the zebrafish myotome and can be distinguished from other slow muscle cells by their expression of a number of *engrailed* (*en*) genes and a failure to migrate from their adaxial position flanking the notochord. Muscle pioneers are absent from embryos homozygous for the mutations *no tail* (*ntl*) and *floating head* (*flh)* which lack differentiated notochords (Currie and Ingham, 1996). Transplantation of wild-type notochord cells into these mutations can rescue the formation of muscle pioneer cells but only in those myotomes directly adjacent to the transplanted notochord cells (Halpern et al., 1993) thus implicating a notochord-derived signal in the induction of muscle pioneers from the presomitc mesoderm. These mutations still maintain significant expression of *shh* within the axial midline and consequently form near normal levels of slow twitch muscle suggesting that SHH activity alone cannot induce muscle pioneers (Halpern et al., 1993, 1997; Krauss et al., 1993; Talbot et al., 1995; Currie and Ingham, 1996; Blagden et al., 1997). However, a second member of the HH family of secreted glycoproteins, *echidna hedgehog* (*ehh*) is expressed exclusively in the notochord of wild-type embryos but is not expressed in *ntl* and *flh* mutants.

Previous studies have indicated that *ehh* and *shh* can act synergistically when over-expressed in the early embryo, inducing supernumerary muscle pioneers within the ventral portion of the myotome (Currie and Ingham, 1996). Other authors, however, have reported that expression of high levels of *shh* or other HH family members alone are sufficient to induce muscle pioneers in vivo (Du et al., 1997; Hammerschmidt et al., 1996; Schauerte et al., 1998). In addition we found that EHH is a weak inducer of adaxial fates in vivo (Currie and Ingham, 1996; Blagden et al., 1997) though Du et al. (1997) subsequently reported EHH to be equally effective as SHH in inducing slow muscle.

To investigate the control of myoblast specification further, we have established conditions for the culture of zebrafish myoblasts in vitro and analysed the response of these cells to exogenous recombinant HH peptides. We find that addition of SHH peptide to cultured zebrafish myoblasts induces slow MyHC expression within these cells in a concentration dependent manner. Different HH peptides show varying levels of inductive activity in this assay, suggesting that they may act in an additive manner in vivo to specify cell fate within the embryonic myotome. We have also exploited this culture system to investigate the role of putative downstream effectors of HH signalling. We find in common with previous studies that PKA antagonises the effects of SHH on muscle specification but find no evidence for an involvement of heterotrimeric G-proteins in this process.

MATERIALS AND METHODS

Culture, treatment and immunohistochemistry of zebrafish primary myoblasts

Zebrafish embryos (150, 3 hours old) were washed twice in 20 ml of sterile 10% Hanks' buffered saline. Embryos were sterilised by incubation in ice-cold 0.1% sodium hypochlorite for 2 minutes. This was followed by two washes in sterile 10% Hanks' buffered saline after which embryos were transferred in a minimal volume to a 1 mg/ml solution of pronase (Sigma) in embryo medium (Westerfield, 1995) and dechorionated enzymatically. Dechorionated embryos were then placed in Ca^{2+}-free Ringers saline and squashed with a 10 mm square glass coverslip. The coverslip was rinsed with sterile Ringers saline and this cell suspension was centrifuged at 1000 rpm for 7 minutes. The resulting cell pellet was resuspended in 0.3 ml of Ca^{2+}-free Ringers and triturated gently with a Pasteur pipette. 3 ml of L15 medium containing 3% FCS was added and the mixture centrifuged as before. The cell pellet was then resuspended in 5 ml of growth medium containing zebrafish embryo extract. Embryo extract was produced from 200, 3-day embryos, which were dechorionated in 1 mg/ml pronase diluted in embryo medium, sterilised in 0.5% hypochlorite for 2 minutes in Ca^{2+}-free Ringers, washed twice with 10% Hanks' buffered saline and transferred to a Dounce homogeniser with a minimum of liquid and homogenised well. Following resuspension in L15, 1 ml of extract was added to 9 ml of L15 containing 3% FCS, 100 μg/ml penicillin/streptomycin, 2 mM glutamine and 0.8 mM $CaCl_2$ to produce growth medium. 150 μl of the cell suspension in growth medium was added per well in a series of laminin coated 8-well chamber slides (Labtek, NUNC). HH peptides, were diluted in growth medium immediately prior to use and were added at the time of plating (recombinant mouse SHH peptide, produced from over-expression in bacteria, was a gift from D. Bumcrot and A. McMahon, Harvard; see Marti et al., 1995, for method of production). The chamber slides were then incubated at 28°C in a humidified box for the time required. Comparative western blots were produced from combined cell scrapings of 4, 8-well chamber slides and protein prepared essentially as described (Blagden et al., 1997). SDS-PAGE, western blot and immuno-detection were carried out as described (Blagden et al., 1997). Antibody blocking experiments were carried out with concentrated anti-SHH antibody 5E1 (a gift from T. Jessel, Columbia). Concentration was achieved via centrifugation through Centricon filtration units as described by the manufacturer. Estimation of cell numbers were performed on whole field counts of cultures incubated with the antibodies A4-1025 (a gift from S. Hughes London), which recognises all MyHC expressing myocytes, BA-D5 (a gift from S. Hughes, London), which specifically recognises slow MyHC expressing myocytes and EB-165 (a gift from E. Bandman, Univ. of California Davis) which specifically recognises fast MyHC expressing myocytes (for a description of the specificity of these antibodies see Blagden et al., 1997). Individual wells in the

same chamber slide were incubated separately with specific antibodies, except in experiments illustrating the mutually exclusive nature of fast and slow MyHC expressing myocyte differentiation (Fig. 1D) where antibodies were incubated in combination. Antibody binding was detected by standard methods (Blagden et al, 1997) and stained cells were visualised under 50× magnification, revealing 50-300 myocytes per field depending on the experiment. Cell counts were compared within individual chamber slides and the percentage of fast or slow cells determined as a percentage of total myocytes in untreated culture as revealed by the detection of A4-1025 binding. The standard error of the mean was calculated for all cell counts in different experiments with a minimum n=5.

Generation of zebrafish SHH and EHH peptides

SHH peptide prepared by different methodologies can result in recombinant proteins with vastly different activities (Roelink et al., 1995; Marti et al., 1995). In order to directly compare the activities of the zebrafish SHH and EHH these peptides were prepared by identical methods. The N-terminal domain of SHH and EHH comprising cDNA encoding amino acids 1-198 representing the mature N-terminal cleavage product with an intact signal sequence was cloned into the late stage promoter baculovirus vector pAcMP2 (Pharmingen). SF9 cells were infected and high-expressing transfected cell lines were selected by immunoblot assay using polyclonal sera raised against SHH protein (Bumcrot et al., 1995). Supernatants of unlysed SF9 cells were collected and concentrated through microcentaur concentrators. Peptide concentration was determined by quantitative western blots, utilising zebrafish SHH peptide that was previously isolated from bacterial expression systems as standards.

Pharmacological manipulation of SHH signalling in vitro

Forskolin (Sigma) was prepared by the addition of DMSO to produce a stock concentration of 25 mM. After initial dilution in PBS, forskolin was added to cultures at the time of plating to a final concentration of 100 μM. Cells were allowed to differentiate for 24 hours and the percentage of differentiated slow myocytes determined. After incubation, cultures were fixed directly in −20°C methanol for 2 minutes and then rinsed in PBS. Cultures were incubated for 2 hours at room temperature with primary, secondary and tertiary antibodies using methods previously described (Blagden et al., 1997). Mastoparan (Sigma) and cholera toxin (Sigma) were re-hydrated both to stock concentrations of 1 mg/ml. Pertussis toxin (membrane permeable A and B subunits, Sigma) was rehydrated to a concentration of 50 mg/ml. Initial dilutions were done in PBS as required and toxins were added at the time of plating to the final concentrations described. The range of concentrations chosen spanned those at which G-protein dependent signalling has been shown to be regulated by addition of these peptides and increased incrementally to the point where cell survival was compromised (Kelvin et al., 1989; Horgan et al., 1995). The addition of AlF_4 to differentiating myocyte cultures utilised 100 μM $AlCl_3$ to 10 mM NaF to create an active solution. Cultures were allowed to differentiate for 24 hours and the percentage of slow myocytes determined. 5′bromo 3′deoxy uridine (BRDU) (2 mM) was added at the time of plating and incorporation into cultured myoblasts detected using an antibody raised against BRDU as described by the manufacturer (Sigma). Cell counts were performed as described above.

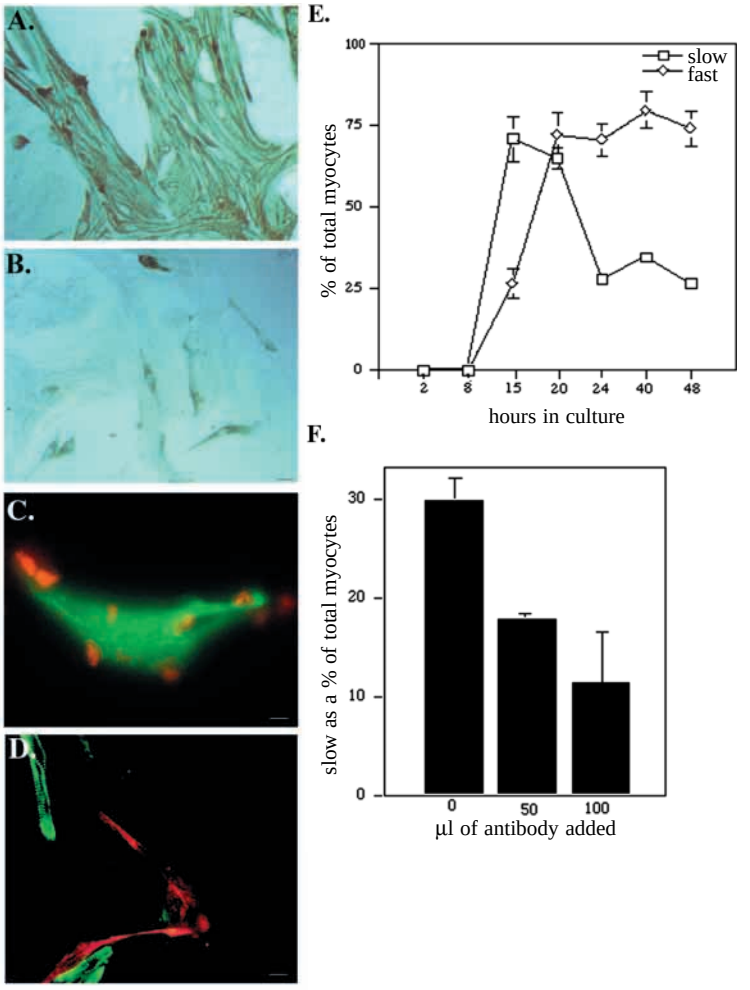

Fig. 1. Zebrafish blastomeres differentiate into slow and fast myosin expressing muscle cells. (A) Cells differentiate as muscle within zebrafish blastomere cultures. Dissociated zebrafish blastomeres were cultured in conditions of low serum and allowed to differentiate. Cells were then incubated with the antibody A4.1025 which recognizes all muscle cell types in all species tested. Up to 80% of cells are able to express myosin heavy chain when differentiated for 48 hours in culture.
(B) Approximately 30% of differentiated muscle cells cultured in this manner express slow MyHC within blastomere cultures. Cells were incubated with the antibody BA-D5 which specifically recognises slow muscle cells in all vertebrate species examined (Blagden et al., 1997).
(C) Slow MyHC expressing cells differentiate next to SHH expressing cells within zebrafish blastomere cultures. Around 10% of slow myosin cells seem to be adjacent to SHH expressing cells in these cultures. Cells were incubated with BA-D5 (Red) and anti-SHH antibody (Green) (Bumcrot et al., 1995). (D) Blastomere cultures incubated with slow (Red) and fast (Green) myosin heavy chain specific antibodies recognise two non-overlapping populations of muscle cells. (E) Timing of fast and slow MyHC expressing myocyte differentiation in vitro. Slow expressing myocytes differentiate early and form the majority of cells within 15 hour cultures. Fast MyHC expressing myocytes differentiate later and form the majority cells within 24 hour cultures. After 24 hours the ratio of fast and slow expressing muscle cells becomes stable, with approximately one quarter of cells expressing slow MyHC and the remainder expressing fast MyHC. (F) Addition of the anti-SHH monoclonal antibody 5E1 (Ericson et al., 1996) lowers the number of slow MyHC expressing cells within zebrafish blastomere cultures. All error bars in this and remaining figures refer to the standard error of the mean. Bars: 10 μm (A,B); 4 μm (C,D).

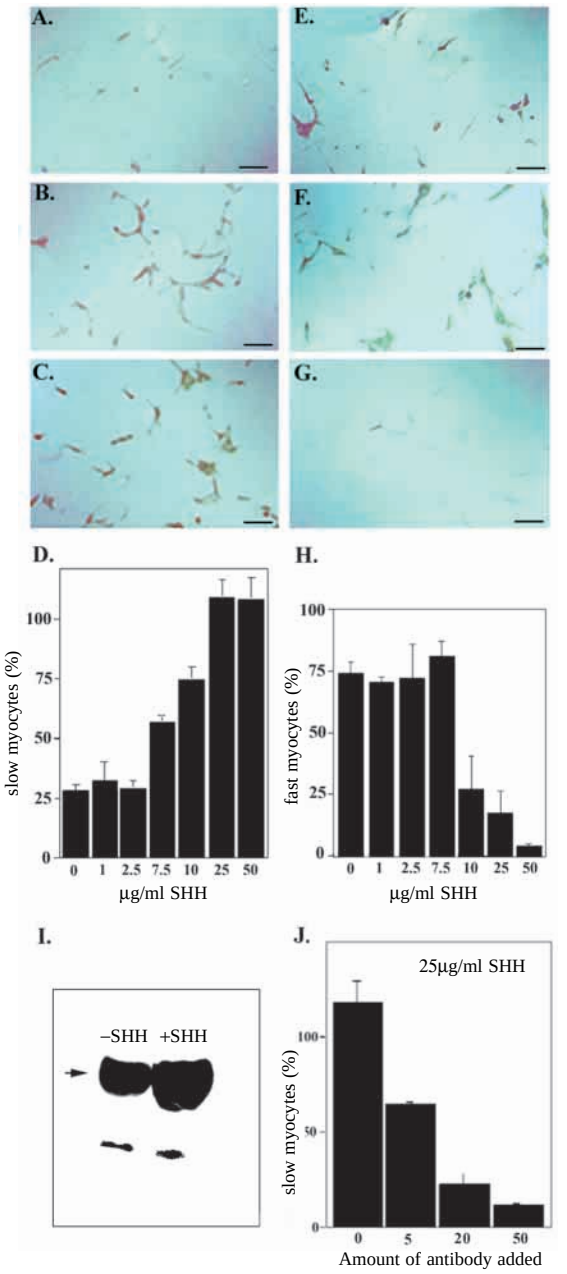

Fig. 2. Bacterially produced N-terminal SHH induces slow MyHC expression at the expense of fast MyHC in zebrafish myocytes. (A) Typical culture without addition of SHH peptide reacted with the slow MyHC specific antibody BA-D5. (B) Typical culture without SHH peptide incubated with the all myosin reacting antibody A4-1025. (C) Typical culture with the addition of 25 mg/ml SHH peptide reacted with the slow MyHC specific antibody BA-D5. (D) The percentage (%) of slow MyHC expressing cells is plotted against increasing concentration of SHH peptide. SHH can induce slow MyHC expressing myocytes at 7.5-10 µg/ml. (E) Typical culture without addition of SHH peptide reacted with the fast MyHC specific antibody EB-165. (F) Typical culture without SHH peptide incubated with the all myosin reacting antibody A4-1025. (G) Typical culture with the addition of 25 µg/ml SHH peptide reacted with the fast MyHC specific antibody EB-165. H. The percentage of fast MyHC expressing cells is plotted against increasing concentrations of SHH peptide. I. Western blot of protein derived from untreated and 25 µg/ml SHH treated cultures incubated with an antibody against slow MyHC (arrow) and actin (lower band). A four- to fivefold increase in the levels of slow MyHC expression is evident in treated cultures. (J) The anti-SHH antibody 5E1 can block the induction of slow MyHC expression by SHH N-terminal peptide. Increasing amounts of concentrated 5E1 was added to cultures to which 25 µg/ml SHH peptide has been added. Amounts refer to the volume of unpurified 5E1 that would have been required prior to concentration. Bars: 25 µm (A-C and E-G).

RESULTS

SHH induces slow muscle in vitro

To analyse effects of SHH on fibre type in vitro, we developed techniques for the culture of zebrafish primary myoblasts. Zebrafish embryos exhibit no restriction in cell lineage until the late blastula stage (Kimmel et al., 1990). When pre-late blastulae are dissociated and cultured the majority of blastomeres differentiate as muscle (Fig. 1A). After one day in culture, two mutually exclusive stable populations of myocytes are evident, the majority expressing fast MyHC and a small percentage expressing slow MyHC (Fig. 1B-D). Differentiation in vitro mirrors the temporal sequence of myogenesis in vivo, with the majority of fast expressing myocytes appearing only after slow expressing myocytes have differentiated (Fig. 1E; Devoto et al., 1996; Blagden et al., 1997). We note that at the 20 hours time point the high percentage of both fast and slow expressing myocytes within these cultures suggests that some differentiating myocytes may be expressing both MyHC isoforms. However, we cannot detect co-expression of fast and slow MyHC in differentiating myocytes at this time point (data not shown). As the temporal sequence of fast and slow MyHC expressing myocyte differentiation was determined in separate experiments we suggest that this discrepancy most likely stems from variations in the timing of differentiation within cultures. We further note that the total number of slow myocytes did not vary between 15 hours and 24 hours in culture indicating that there was no cell death or loss of slow MyHC expressing cells over time (data not shown), revealing the drop in percentage of slow

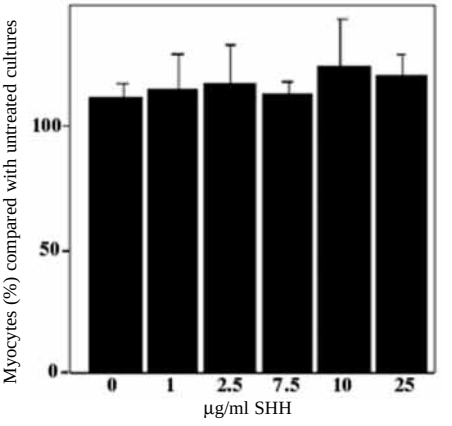

Fig. 3. Increasing amounts of SHH peptide does not influence myocyte number in differentiating blastomere cultures. Increasing amounts of SHH peptide were added to differentiating blastomere cultures. Myocyte number was then plotted as a percentage of myocytes present in untreated cultures.

myocytes over time is a consequence of the increasing number of fast MyHC cells differentiating within these cultures.

That zebrafish blastomeres, dissociated prior to any lineage restriction within the embryo, can differentiate into fast and slow MyHC expressing myocytes in vitro implies that their fate cannot be determined by a lineage-based mechanism. However, if an inductive signal controls their specification, such a signal must be secreted by cells present within the culture. A small proportion of cells in these cultures differentiate into non-muscle cell types and show characteristics of notochord, as revealed by antibodies directed against the zebrafish Bracyhury-related protein, No Tail (NTL; data not shown). Notochord cells are the source of a number of secreted signalling peptides, including the secreted glycoprotein, SHH. Within differentiating blastomere cultures, cells expressing SHH are often located next to cells which have initiated slow MyHC expression (Fig. 1C). Furthermore, addition of the anti-SHH monoclonal antibody 5E1, which blocks SHH-mediated responses in other vertebrate cells (Ericson et al., 1996), lowers the number of cells differentiating as slow myocytes within these cultures without lowering the total number of myocytes in culture (Fig. 1H). Thus, the slow myocytes present in vitro are most likely induced by SHH secreted by the NTL expressing cells, as observed in vivo (Blagden et al., 1997; Du et al., 1997).

To test whether all myoblasts can respond to SHH in this way, increasing levels of recombinant mouse protein were added to differentiating zebrafish myoblasts in primary culture. The number of slow MyHC expressing cells increased as a function of concentration of recombinant N-terminal SHH peptide, with concentrations above 10 μg/ml inducing all myocytes to express slow MyHC (Fig. 2A-D); and this response is inhibited by the addition of the anti-SHH antibody 5E1 (Fig. 2J). A comparative western blot prepared from treated and untreated cultures incubated with an anti-slow muscle antibody demonstrates a 4- to 5-fold induction of slow muscle MyHC at a concentration of SHH peptide that induces all myocytes in culture to express slow MyHC (Fig. 2I). Addition of SHH peptide leads to a reciprocal loss of fast MyHC expressing myocytes at similar concentrations of peptide which induced slow muscle (Fig. 2E-H). Furthermore, we note that the increase in slow MyHC expressing cells occurs without an increase in overall myocyte number, indicating that SHH peptide does not induce a mitogenic expansion of slow-fated myoblasts or act as a survival factor, activities previously ascribed to SHH with respect to myoblast formation (Duprez et al., 1998; Teillet et al., 1998; Fig. 3).

Comparison of slow muscle inducing activities of different HH peptides

Previous analysis has suggested that different members of the zebrafish HH family of peptides have different inducing activities for muscle cell fates in vivo (Currie and Ingham, 1996; Blagden et al., 1997). To test the ability of these different peptides to induce slow muscle in vitro we added identically prepared EHH and SHH peptide to differentiating blastomere cultures. Addition of EHH can induce slow MyHC in zebrafish blastomere cultures (Fig. 4B) but only at a threefold higher concentration than SHH (Fig. 4A).

It has previously been postulated that muscle pioneer cells, a particular subclass of zebrafish slow muscle cells which express the homeodomain containing protein Engrailed (En), require the activity of both SHH and EHH to be induced in vivo (Currie and Ingham, 1996). En expressing myocytes could never be detected within zebrafish primary myoblast cultures, either prior to, or after, incubation with any concentration of SHH and EHH peptides (data not shown).

Analysis of SHH signal transduction in zebrafish myoblasts

If SHH signalling is required for zebrafish myoblasts to initiate slow myosin expression, modulating the activity of components of the SHH signal transduction pathway with pharmacological agents should affect myocyte fibre type. Protein kinase A has been shown to be a negative regulator of SHH signal transduction in a number of vertebrate cell types (Fan et al., 1995; Concordet et al., 1996; Hammerschmidt et al., 1996; Epstein et al., 1996). Addition of the PKA agonist forskolin at a concentration of 100 μM effectively blocks the slow muscle inducing activity of 25 μg/ml SHH (Fig. 5A-D) but alone has no effect on the ability of zebrafish primary myoblasts to differentiate and does not affect the proportion of

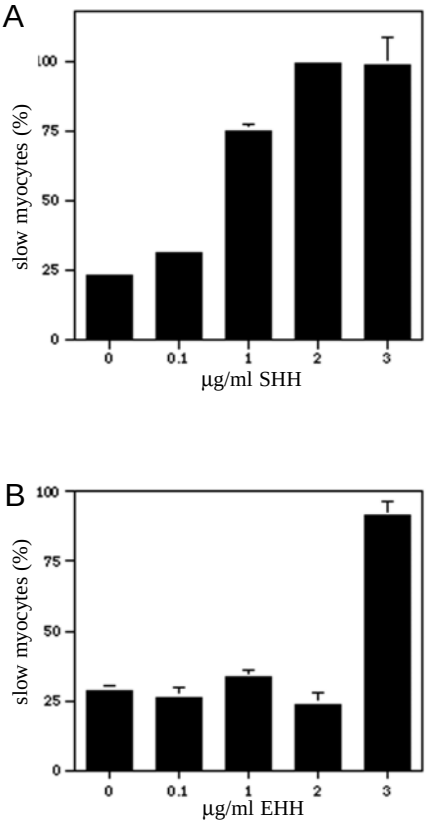

Fig. 4. Induction of slow muscle by different HH peptides. Increasing amounts of identically prepared HH peptide were added at the time of plating to zebrafish primary myoblast cultures. (A) Addition of zebrafish SHH peptide to primary zebrafish myocytes induces slow muscle at a concentration of 1 μg/ml. The percentage of slow myosin expressing myocytes is plotted against concentration of SHH peptide added. (B) The addition of identically prepared EHH peptide to primary zebrafish myoblasts induces slow muscle differentiation at 3 μg/ml. The percentage of slow myosin expressing myocytes is plotted against concentration of EHH peptide added.

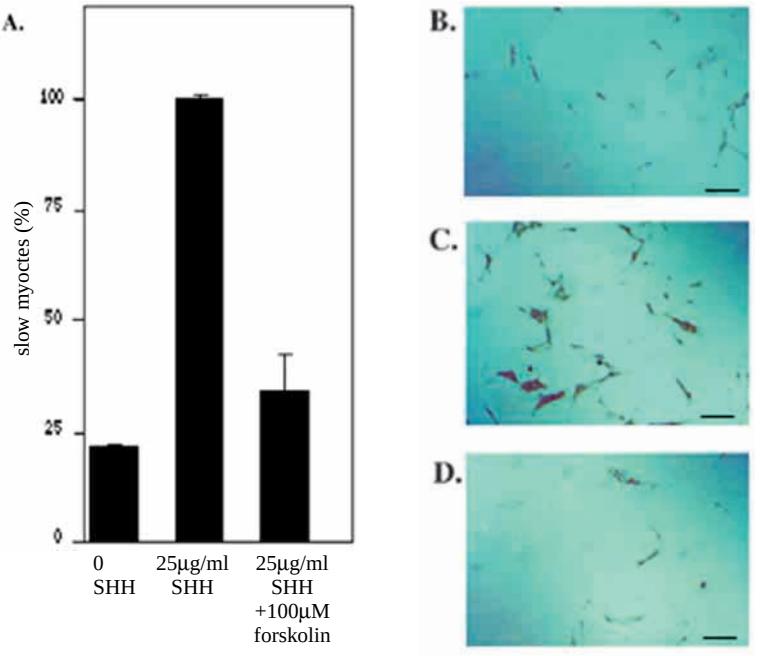

Fig. 5. Forskolin regulates slow muscle formation in vitro. (A) Percentage of slow myosin expressing myocytes within cultures to which no SHH peptide, 25 µg/ml SHH peptide and 25 µg/ml SHH together with 100 µM forskolin have been added. (B) Typical cultures to which no SHH has been added and reacted with the slow MyHC specific antibody BAD5. (C) Typical culture to which 25 µg/ml SHH has been added and reacted with BAD5. (D) Typical culture to which 25 µg/ml of SHH together with 100 µM forskolin have been added and reacted with BAD5. Bars, 25 µm (B-D).

slow myosin expressing myocytes present within these cultures (Fig. 5D).

The ability of cultured zebrafish myoblasts to respond to intracellular changes in PKA activity suggested that these cells may be amenable to further pharmacological manipulations on other components of the HH signal transduction pathway. The transmembrane protein Smoothened (SMO), the signalling element of the HH receptor complex, exhibits structural homology to seven membrane pass G-protein coupled receptors (van den Heuvel and Ingham, 1996; Alceldo et al., 1996; Chen and Struhl, 1998), suggesting that heterotrimeric G-proteins may play a role in HH signal transduction (reviewed by Quirk et al., 1997). Some support for this possibility comes from the report that injection of the G-protein inhibitor pertussis toxin into zebrafish embryos perturbs the formation of some but not all cell types known to be dependent upon HH signalling within the zebrafish embryo (Hammerschmidt et al., 1998). We investigated this further using our in vitro culture system to examine the effects of G-protein antagonists and agonists on HH dependent slow muscle specification. A number of different classes of G-proteins are coupled to a wide variety of receptors with G-proteins of the G_i, G_o and G_s classes representing a large proportion of G-proteins bound to receptors. All these sets of G-proteins have characterised roles in controlling the rate of cell division in a number of cell types (Andersen et al., 1993; Meininger and Granger, 1990; Sellers, 1999; Sarbassov et al., 1997) and both pertussis and cholera toxins have been shown to lower the rate of cell division within cultured myoblasts (Kelvin et al., 1989; Yoshida et al., 1996). Zebrafish primary myoblasts cultured in vitro normally divide before differentiating, an event that can be visualised by every differentiating myocyte incorporating BRDU (Fig. 6D-F). The addition of 1 µg/ml of either membrane permeable pertussis toxin, a general inhibitor of the G_i and G_o class of G-proteins or cholera toxin, an activator of the G_s class of G-proteins, leads to a significant decrease in the incorporation of BRDU into treated cells indicating that both of these toxins can modulate G-protein dependent cell division events in primary zebrafish myoblasts (Fig. 6G-L). Despite this effect on division, the differentiation of slow MyHC expressing myocytes cells was unaffected by treatment with cholera toxin (Fig. 6C). Similarly, the addition of pertussis toxin, or of mastoparan, a small, membrane-soluble insect venom peptide activator of G_i and G_o class G-proteins, failed to affect slow MyHC fibre type formation, even when these peptides were added at concentrations that affected cell survival (Fig. 6A,B; data not shown). Furthermore, neither of these two peptides affected the ability of 25 µg/ml SHH peptide to induce slow myosin expressing myocytes within these cultures (n=6 at 0.6, 3 and 9 nM mastoparan; n=2 at 1 µg/ml pertussis toxin). Finally, in experiments with the general G-protein activator, AlF_4, we also failed to detect a change in the fate of cultured zebrafish myocytes (data not shown).

DISCUSSION

SHH and myoblast cell fate determination

Previous studies have implicated SHH as one determinant of muscle cell fate in the developing zebrafish embryo (Weinberg et al., 1996; Currie and Ingham, 1996; Blagden et al., 1997; Du et al., 1997). Slow twitch muscle differentiates from the adaxial cells (Devoto et al., 1996) and initially forms a single layer of muscle cells flanking the notochord and floor plate, the sources of SHH peptide in the developing midline. Mutant embryos that lack these structures exhibit a failure of slow twitch muscle formation, which can be rescued by injection of *shh* mRNA (Blagden et al., 1997). These same mutants form fast twitch muscle normally however, indicating that SHH is not required for muscle differentiation per se (Blagden et al., 1997). Injection of *shh* mRNA into zebrafish blastomeres results in the formation of ectopic slow MyHC expressing myocytes throughout the myotome and this differentiation occurs at the expense of fast MyHC expressing myocytes. This

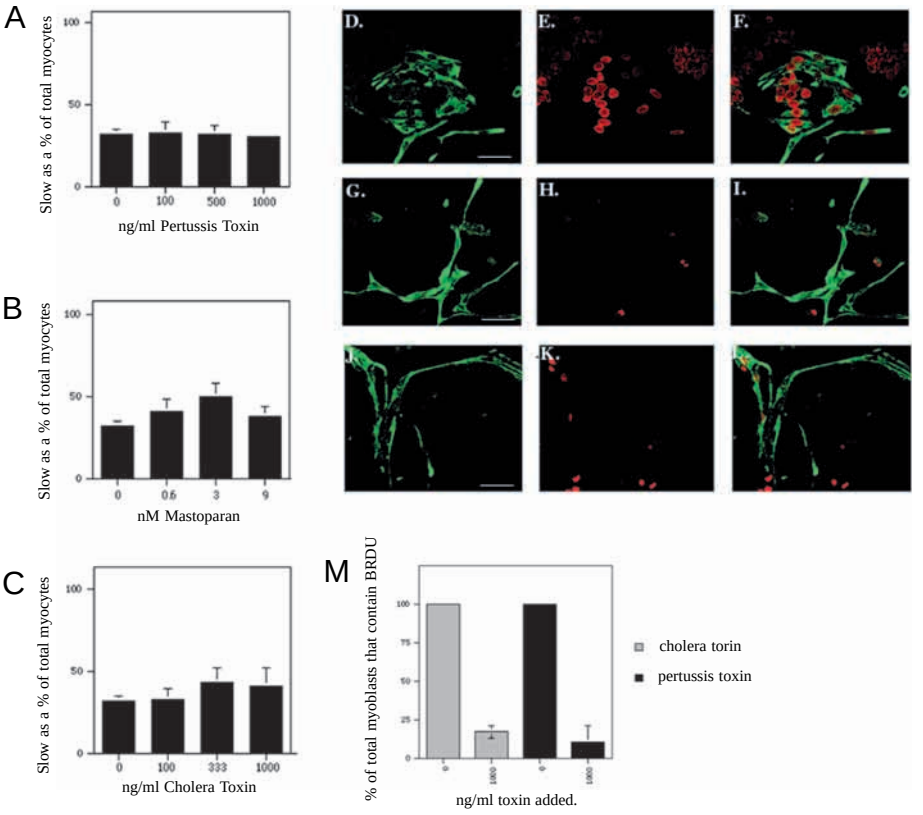

Fig. 6. Addition of G-protein inhibitors or activators has no effect on the induction of slow muscle within zebrafish myocytes but can inhibit cell division. (A) Addition of membrane permeable pertussis toxin, a general inhibitor of the G_i and G_o classes of G-proteins does not affect the amount of slow myocytes present within these cultures. (B) Addition of mastoparan, a small membrane soluble insect venom peptide activator of the G_i and G_o classes of G-proteins, fails to affect fibre type formation within treated cultures. (C) Addition of membrane-soluble cholera toxin, an activator of the G_s class of G-proteins, fails to stimulate slow muscle induction. (D) Typical culture to which BRDU (red) has been added at the time of plating and incubated with the all muscle recognising antibody 1025 (green). (E) The same culture as in D incubated with an antibody specific for BRDU. (F) The combination of panels D and E. (G) Typical culture to which 1000 ng/ml of pertussis toxin and BRDU have been added incubated with the antibody 1025. (H) Same culture as in G incubated with an antibody specific for BRDU. (I) The combination of H and G. (J) Typical culture to which 1000 ng/ml of cholera toxin and BRDU have been added incubated with the 1025 antibody. (K) Same culture as in J incubated with an antibody specific for BRDU. (L) The combination of J and K. Bars, 10 μm. (M) Plot of the percentage of total myocytes that contain BRDU with or without the addition of 1000 ng/ml of pertussis and cholera toxins.

in vivo analysis has suggested a model whereby zebrafish myoblasts are presented with a binary cell fate choice that is controlled by a threshold level of SHH activity secreted from the embryonic midline. Above this threshold all myoblasts are specified to be slow, while below it or in the complete absence of SHH, myoblasts follow the default pathway and differentiate into fast MyHC expressing cells. The in vitro assay system for zebrafish myoblasts that we have developed has allowed us to test this model directly.

Blastomeres of many vertebrate species, when dissociated and plated in vitro give rise to muscle cells. This can occur without replication, within blastomeres plated at clonal density in protein free medium (Holtzer et al., 1990; Godsave and Slack, 1991; George-Weinstein et al., 1996). We have exploited this property to develop methods to culture zebrafish myoblasts in vitro. Differentiation of cultured zebrafish primary myoblasts proceeds in a similar fashion to that which occurs in vivo, with slow muscle appearing first and the majority of cells differentiating later as fast MyHC expressing myocytes. These cells are responsive to SHH peptide and above a specific threshold level all myocytes within these cultures can be induced to express slow MyHC. This threshold concentration, 10 μg/ml, is similar to the concentration of the identical peptide required maximally to induce motor neurons in chick neural tube explant assays (Marti et al., 1995). These in vitro data, combined with the results of previously published in vivo studies (Blagden et al., 1997; Du et al., 1997), strongly support a model whereby high levels of SHH peptide, secreted from the notochord and floor plate, induce a single row of cells immediately flanking the notochord, the adaxial cells, to become slow MyHC expressing myocytes. This does not rely on contact with the notochord as myoblasts both in vivo and in vitro can respond to exogenous SHH peptide without association with the notochord. These findings suggest that the absolute level of HH peptide experienced by the target myoblast cell is the sole determinant of the slow muscle fate.

Numerous models have been invoked to explain the role of SHH during vertebrate myogenesis. One specific model suggests that SHH simply acts as a mitogenic survival factor for myoblasts during embryogenesis (Telliet et al., 1998; Duprez et al., 1998). However, our results indicate that zebrafish myoblasts are not stimulated to divide upon addition of SHH peptide nor do they require SHH for growth or differentiation. Rather the principal role SHH signalling plays in zebrafish myogenesis is to determine the type of muscle cell a myoblast will become. This conclusion is supported by observations in vivo, where zebrafish embryos in which HH signalling has been compromised or eliminated are still able to undergo myogenesis and form muscle. In this instance the only defect in terms of myogenesis is a lack of slow myosin expressing myocytes (Blagden et al., 1997; Lewis et al., 1999).

Specificity of HH peptides in muscle cell fate determination

The results of Lewis et al. (1999) indicate that HH proteins, other than SHH, also play a role in specifying slow muscle.

Embryos homozygous for mutations at the SHH locus still retain significant levels of slow muscle differentiation, whereas *you-too* homozygous mutant embryos, which lack activity of the *gli2* gene, are devoid of all slow muscle (Lewis et al., 1999). The simplest interpretation of these phenotypes is that other HH signals transduced by GLI2 activity contribute to slow muscle specification. We have used our cell-based assay to compare directly the different activities of the two HH peptides present within the zebrafish notochord, SHH and EHH. Both peptides can induce slow MyHC expressing myocytes to form in culture, but SHH possesses a higher inductive activity than EHH. It is therefore likely that both these peptides contribute, in cumulative fashion, to the induction of slow MyHC expressing myocytes in the zebrafish myotome. This is consistent with the finding that slow muscle is depleted but not eliminated in the absence of SHH activity and suggests that EHH enhances the overall levels of HH activity emanating from the notochord (Lewis et al., 1999). We also note, however, the existence of a third HH peptide encoding gene, *tiggywinkles hedgehog (twhh)*, closely related to *shh* that is expressed exclusively within floorplate during the period of embryogenesis when slow muscle is specified (Ekker et al., 1995). Previous analysis has suggested that in the absence of the notochord, the floorplate can act as a potent inducer of slow muscle (Blagden et al., 1997). While we have not directly assayed the inductive potential of TWHH, the DNA sequence encoding its open reading frame suggests it arose as a recent duplication of the *shh* gene (Ekker et al., 1995). Thus, TWHH may also compensate for the lack of SHH activity in *shh* null mutants and contribute to the cumulative HH activity emanating from the midline.

Other observations also suggest that in the context of normal embryogenesis, EHH is likely to play a minor role in the cell fate determination of adaxial myoblasts to become slow MyHC expressing myocytes. The levels of *ehh* transcripts within the notochord are much lower that those of *shh* (Currie and Ingham, 1996), and this coupled with the lower inductive ability of EHH peptide suggests an over all lower potential for slow muscle induction. This notion is reinforced by the observation that the mutations *ntl* and *flh*, which do not express *ehh* (Currie and Ingham, 1996) possess near wild-type levels of slow muscle, except in the very caudal somites of *ntl* mutants which are adjacent to midline cells which do not express *shh* (Blagden et al., 1997).

We also assayed the ability of EHH and SHH peptides either alone or in combination to induce muscle pioneer differentiation in vitro. Muscle pioneer cell differentiation, assayed by the presence or absence of En positive myocytes, was never detected under any experimental condition adopted. We conclude that HH signalling is insufficient to induce muscle pioneer differentiation in this in vitro context; this may be because the activity of the recombinant HH proteins (which do not undergo the same lipophilic modifications as the endogenous proteins) are too low to induce MP differentiation: alternatively, it is possible that additional factors, not present within our blastomeric cultures are required for their formation.

SHH signal transduction within target myoblasts

Cloning of the genes required to transmit the HH signal in *Drosophila* cells has revealed a complex array of positive and negative effectors. The notion that the paradigm of transduction of the HH signal may be similar in vertebrate cells has been strongly reinforced by the identification of homologous vertebrate genes which act in a broadly equivalent fashion to transduce the vertebrate HH signal (reviewed by Ingham, 1998).

The generation of a SHH responsive cell-based assay provided an opportunity to analyse the intracellular signal transduction pathway that is utilised within zebrafish myoblasts to control induction of slow MyHC expression. Pharmacological activation of PKA, a negative regulator of SHH signal transduction (Fan et al., 1995; Kos et al., 1998; McMahon et al., 1998), inhibits slow muscle induction by SHH peptide within zebrafish myoblasts in vitro. We find no evidence for a positive role of activated PKA analogous to that demonstrated in *Drosophila* cells (Ohlmeyer and Kalderon, 1997) in specifying slow muscle cell fate. Our results reinforce in vivo observations that demonstrate that ectopic slow muscle markers are activated by the injection of mRNA encoding a dominant negative form of the catalytic subunit of PKA into the developing zebrafish embryo (Concordet et al., 1996; Hammerschmidt et al., 1996).

Reception of the HH signal requires a complex of two transmembrane proteins, PTC which is a large multi-membrane pass protein and SMO, which possess homology to serpentine G-protein coupled receptors (reviewed by Ingham, 1998). PTC has been shown to be a negative regulator of HH signalling in *Drosophila* and vertebrate cells, and the binding of HH to PTC is believed to relieve an allosteric inhibition of PTC on the constitutive signalling activity of SMO. It is unknown how SMO effects signal transduction but one very simple model suggests that PTC inhibits the release of G-proteins coupled to SMO that are required for transmission of the HH signal and this inhibition is released upon HH binding to PTC. Mapping of the domains of SMO required for SHH dependent signalling revealed that the third intracellular loop, a known G-protein interacting region in homologous serpentine receptors, plays a critical role in signal transduction (Murone et al., 1999). However, none of the second messenger systems typically deployed downstream of G-protein activation could be implicated in SHH dependent signalling (Murone et al., 1999). We have directly tested the involvement of G-proteins, of the G_i, G_o and G_s class in mediating the SHH signal transduction cascade in zebrafish myoblasts and we can find no evidence for their involvement. We cannot rule out the possibility that these G-proteins may be required to act at many different points in the growth and specification of zebrafish myoblasts and the inhibition or activation of a particular class of these proteins at one stage in myoblast determination may mask or prevent its role in SHH mediated signal transduction. Such a role may explain why injection of pertussis toxin into developing zebrafish embryos results in such a broad range of developmental defects, amongst which are deletion of muscle pioneer cells within a subset of somites (Hammerschmidt et al., 1998). It also remains possible that a unique class of G-proteins, unaffected by the pharmacological interventions we have used, control SHH signal transduction.

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