Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction

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Accepted 8 September 2003

Development 130, 6233-6243
Published by The Company of Biologists 2003
doi:10.1242/dev.00851

Summary

Sonic hedgehog (Shh) signaling is essential for sclerotome development in the mouse. Gli2 and Gli3 are thought to be the primary transcriptional mediators of Shh signaling; however, their roles in Shh induction of sclerotomal genes have not been investigated. Using a combination of mutant analysis and in vitro explant assays, we demonstrate that Gli2 and Gli3 are required for Shh-dependent sclerotome induction. Gli2–/–Gli3–/– embryos exhibit a severe loss of sclerotomal gene expression, and somitic mesoderm from these embryos cannot activate sclerotomal genes in response to exogenous Shh. We find that one copy of either Gli2 or Gli3 is required to mediate Shh induction of sclerotomal markers Pax1 and Pax9 in vivo and in vitro. Although Gli2 is generally considered an activator and Gli3 a repressor, our results also reveal a repressor function for Gli2 and an activator function for Gli3 in the developing somite. To further dissect the function of each Gli, we used adenovirus to overexpress Gli1, Gli2 and Gli3 in presomitic mesoderm explants. We find that each Gli preferentially activates a distinct set of Shh target genes, suggesting that the functions of Shh in patterning, growth and negative feedback are divided preferentially between different Gli proteins in the somite.

Key words: Sonic hedgehog, Somite, Mouse, Patterning, Gli

Supplemental data available online

Introduction

The axial musculoskeletal system of vertebrates derives from somites, segmented blocks of mesoderm flanking each side of the neural tube. Somites form from an unsegmented precursor tissue called the presomitic mesoderm (PSM). The anterior PSM forms the newest somite by undergoing a mesenchymal-to-epithelial transition, resulting in an epithelial sphere with a mesenchymal core. As the somite matures, the dorsal region remains epithelial, while the ventral region undergoes a second transition into mesenchyme. The dorsal epithelium forms the dermomyotome, which gives rise to dermis of the back and skeletal muscles, while the ventral somite forms sclerotome, which gives rise to the ribs and vertebrae (reviewed by Brent and Tabin, 2002).

The secreted signaling molecule Sonic hedgehog (Shh), expressed in the notochord and floorplate, is crucial for sclerotome development as Shh mutant mice lack vertebra columns, and form only a few rudimentary rib cartilages (Chiang et al., 1996). Consistent with this, Shh induces expression of sclerotomal markers including the paired-box containing transcription factors Pax1 and Pax9, and the HMG-box containing transcription factor Sox9 in PSM in vitro (Fan and Tessier-Lavigne, 1994; Murtaugh et al., 1999; Zeng et al., 2002). These target genes are essential for sclerotome development as Pax1/Pax9 double mutants have severe defects in formation of ribs and vertebrae (Peters et al., 1999), and Sox9 is required for the transcription of collagen α2, an extracellular component necessary for cartilage formation (Bell et al., 1997; Bi et al., 1999). In addition to the sclerotomal markers, Shh induces proliferation of the somitic mesoderm (Fan et al., 1995), possibly by upregulation of G1 cyclins, which are Shh targets in other tissues (Kenney and Rowitch, 2000; Mill et al., 2003). Shh also negatively regulates its own signaling by upregulation of its own binding receptor patched 1 (Ptc1) and a decoy receptor hedgehog interacting protein (Hhip) (Briscoe et al., 2001; Chuang and McMahon, 1999; Goodrich et al., 1996). Both the proliferation and negative feedback induced by Shh may help define the shape and size of sclerotome-derived skeletal components. Thus, the roles of Shh in the somite can be divided into three categories, patterning, proliferation and negative feedback.

Induction of Shh targets in the somite is thought to be carried out through the conserved Hedgehog (Hh) signaling pathway first described in Drosophila (reviewed by McMahon, 2000). In this pathway, Hh binds to its receptor Patched (Ptc) and relieves Ptc inhibition of the signaling component Smoothened (Smo). Smo then signals to the transcription factor Cubitus interruptus (Ci) to activate gene expression. Ci acts as a bipotential transcription factor, repressing some of the same target genes in the absence of Hh (Methot and Basler, 2001; Muller and Basler, 2000). In the mouse, there are three Ci homologs, Gli1, Gli2 and Gli3 (Hui et al., 1994). Gli1 and Gli2 are thought to act primarily as activators, while Gli3 acts...
primarily as a repressor (Bai et al., 2002; Lee et al., 1997; Ruiz i Altaba, 1998; Sasaki et al., 1997; Sasaki et al., 1999; Shin et al., 1999). Bipotential functions of Gli2 and Gli3 on reporter genes in cultured cells has been demonstrated (Sasaki et al., 1999), but in vivo evidence of bipotential Gli2 activity is lacking.

Genetic data supports a crucial role for Gli2 and Gli3 in formation of the axial skeleton as Gli2 and Gli3 mutants exhibit distinct vertebral and rib defects late in development, whereas Gli1 mutant mice exhibit no developmental defects. Gli2+/Gli3+/+ mice exhibit more severe defects than either single mutant, indicating some overlapping functions in skeletogenesis (Mo et al., 1997). These mice exhibit defects similar to Pax1/Pax9 double mutants, suggestive of defects early in sclerotome induction. However, the molecular basis for Gli2 and Gli3 mutant phenotypes and the role of each Gli in sclerome development have not been investigated.

To determine whether Gli2 and Gli3 are required for Shh-dependent sclerome induction, we examined Shh target gene expression in Gli2/Gli3 compound mutants. We find that scleromeal gene expression is severely reduced in Gli2−/−Gli3−/− mice and that at least one copy of either Gli2 or Gli3 is required in the somitic mesoderm to confer Shh-responsiveness. We also investigated the specific role of each Gli in activating Shh target genes by overexpression in the PSM in vitro. We find that each Gli displays preferential activation of different scleromeal targets involved in Shh-directed patterning, proliferation and negative feedback.

Materials and methods

Mice
Gli2+/ mice in a CD1 background (Mo et al., 1997) and Gli3XtJ+/ mice in a C3H background (Jackson Laboratory) were crossed to obtain Gli2−/−Gli3−/− mice, which were then mated to obtain various mutant combinations. Genotyping using yolk sac DNA was performed by the polymerase chain reaction (PCR) using primers described elsewhere (Mo et al., 1997; Maynard et al., 2002).

Radioactive action in situ hybridization
Mice at embryonic day 9.5 (E9.5) were fixed overnight in 4% paraformaldehyde and cryosectioned at 12 μm. Riboprobes were labeled with [α-35S]-UTP (Amersham Pharmacia) using T3 or T7 RNA polymerase (Promega). Radioactive action in situ hybridization was performed as described (Frohman et al., 1990). Slides were exposed to NBT emulsion (Kodak), developed, stained by Hematoxylin, and mounted in Permount (VWR). Images were photographed under dark field illumination to visualize silver granules and presented with the corresponding bright-field images.

Whole-mount in situ hybridization
Whole-mount in situ hybridization using digoxigenin (DIG)-UTP (Roche) labeled riboprobes was performed as described (Butitta et al., 2003). Probes for Pax1, Sim1 and Pax3 have been described previously (Fan and Tessier-Lavigne, 1994). Myf5 probe was a gift from Dr. M. Buckingham. Pax9 and Sox9 probes were generated by reverse transcription coupled with PCR (RT-PCR) of 0.45kb and 1kb regions of the respective transcripts. Primers used for RT-PCR are available upon request. Embryos hybridized to Pax9, Shh and Sox9 probes exhibited higher background and were destained in methanol resulting in a bluish signal. Embryos were photographed using an AxioCam camera. Selected embryos were cryosectioned at 20 μm and mounted in Crystal Mount (Biomeda).

Explant induction assays
E9.5 mouse PSM explants from CD1 mice, or Gli2/Gli3 progeny, were cultured in collagen gels as described (Fan and Tessier-Lavigne, 1994). For inductions in the presence of cycloheximide, PSM was cultured for 10 hours prior to exposure to Shh-N conditioned media, 1 μg/ml cycloheximide (Sigma), or the combination for 8 hours. Shh-N and control conditioned media were collected from COS cells as described (Fan et al., 1995) and used at 500 μg/ml Shh-N. RNA was isolated from explants by RNAsol and used for RT-PCR with 30 cycles of amplification. Primer sequences are available at http://www.ciwemb.edu/labs/fan/index.html. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining. Images of gels were captured using a UVP 7500 Gel Documentation System and quantified using Image-Quant v.1.2 (Amersham Pharmacia). All RT-PCR assays were performed within the linear range of amplification for each product as determined by quantitative real-time PCR.

Adenovirus production and explant infection
Adenoviruses carrying full-length mouse Gli1, mouse Gli2, human Gli3 or N-terminally truncated Gli2 as C-terminal EGFP (Clontech) fusion proteins; wild-type Smoothen (Smo); an activated form of Smo (Smo-M2 designated here as Smo*) (Xie et al., 1998); or EGFP driven by a CMV promoter were constructed using the AdenoX system (Clontech). Production of fusion proteins was confirmed by western blot using anti-EGFP (Molecular probes) or anti-Gli1, Gli3 and Smo antibodies (Santa Cruz). For infection, explants were cultured in the presence of 0.5-1.0×10⁶ plaque-forming units (pfu) of adenoovirus for ~100% infection. Protein expression levels appeared similar as assessed visually by GFP fluorescence. Infected explants were used for RT-PCR or fixed and processed for immunofluorescence as described (Lee et al., 2001).

Results

Gli gene expression in the early somites
Despite the importance of Gli genes in axial skeletal development, a thorough examination of their expression in the early mouse somites has not been reported. To determine which Gli genes might play a role in sclerome induction, we performed radioactive in situ hybridization on posterior regions of mice at E9.5 to investigate Gli expression within the early somite.

Expression of Gli1 is absent throughout the PSM. Upon formation of the somites, Gli1 is expressed in the ventral domain (Fig. 1A, A'). Transverse sections through the early somites reveal expression in the most ventromedial domain of the sclerome (Fig. 1D, D'). By comparison, Gli2 is more widely expressed. Gli2 is weak in the posterior two-thirds of the PSM, but becomes stronger in the anterior domain (Fig. 1B, B'). Gli2 expression is absent from the dermomyotome but found in the sclerome, expanding more dorsally and laterally than Gli1 (Fig. 1E, E'). Gli3 exhibits the broadest expression of all three genes. Gli3 is expressed throughout the anterior two-thirds of the PSM as well as both dorsal and ventral domains of the early somites (Fig. 1C, C'). Transverse sections reveal that Gli3 is expressed throughout the sclerome, extending laterally like Gli2, but has stronger expression in the dermomyotome (Fig. 1F, F'). Thus, all three Gli genes are expressed in the ventral domain of the early somites. As Gli2 and Gli3 are expressed in the anterior PSM, they may act earliest in sclerome patterning.
Skeletal defects in Gli2+/−, Gli3−/− and Gli2+/−Gli3+−/− mice are not due to an initial defect in sclerotome induction

The skeletal defects observed in Gli2 and Gli3 mutants are suggestive of defects in sclerotome gene induction (Mo et al., 1997). We therefore examined the expression of the sclerotomal markers Pax1, Pax9 and Sox9 in Gli2, Gli3 and Gli2/Gli3 mutant mice by whole-mount in situ hybridization. Surprisingly, Pax1 and Pax9 expression appeared normal in Gli2+/−, Gli3−/−, Gli2+−/Gli3+−/− and Gli2+−/Gli3−/− embryos at E9.5 (Fig. 2A–D,A′–D′; see Fig. S1A at http://dev.biologists.org/supplemental/) and E10.5 (data not shown). Sox9 expression also appeared normal in the early somites of Gli2+/−, Gli3−/− and Gli2+−/Gli3+−/− embryos, but we did observe a loss of Sox9 specifically in the mature anterior somites at E9.5 (Fig. 2, inset A′′−D′′) and E10.5 (data not shown). Sox9 expression is normally found in both ventral and dorsal domains of the sclerotome in anterior somites (arrowheads in inset Fig. 2A′′). Although we observed the most severe loss of Sox9 in Gli3−/− embryos to be specific to the dorsal sclerotome domain, Gli2+/− and Gli2+−/Gli3+−/− embryos exhibited a reduction of Sox9 expression in both ventral and dorsal sclerotome domains (arrowheads in inset Fig. 2B′′−D′′; see Fig. S1B at http://dev.biologists.org/supplemental/). This is consistent with the finding that Gli2+/− and Gli2+−/Gli3+−/− embryos exhibit more severe skeletal defects than Gli3−/− embryos (Mo et al., 1997), and suggests that the skeletal defects observed in these mice are due to an inability to maintain high levels of Sox9 expression in maturing somites.

Gli2−/−Gli3−/− mice have severely reduced sclerotomal gene expression

In contrast to the other allelic combinations, Gli2−/−Gli3−/− embryos display dramatically reduced Pax1 and Pax9 expression and undetectable Sox9 expression in the somites at E9.5 (Fig. 2E−E′). Both the initiation and maintenance of Pax1 and Pax9 were affected, as only a low level of expression is restricted to a small number of interlimb somites (arrowheads in Fig. 2E,E′). This demonstrates that loss of both Gli2 and Gli3 leads to a severe defect in sclerotomal gene expression and that one copy of either Gli2 or Gli3 is sufficient to substantially restore Pax1 and Pax9 expression in Gli2+−/Gli3+−/− and Gli2+−/Gli3−/− embryos.

Somite morphology is abnormal in Gli2+/−Gli3−/− embryos

The severe loss of sclerotomal gene expression in Gli2+−Gli3−/− embryos prompted us to investigate the somite morphology in these embryos. In wild-type, Gli2+−Gli3+−/− and Gli2+−/Gli3+−/− embryos, the dermomyotome (dm), myotome (my) and sclerotome (scl) are visible and organized (Fig. 3A, see Fig. S1C at http://dev.biologists.org/supplemental/). By contrast, in Gli2+/−Gli3−/− somites, the dermomyotome had an abnormal upside down U-shape, owing to ectopic epithelium that extends ventromedially adjacent to the neural tube (Fig. 3B, open arrowheads). We also frequently observed a closed sphere of epithelium in the trunk somites of these embryos (indicated by broken lines in Fig. 3H,J,L,P). In Gli2+/−Gli3−/− embryos, mesenchyme resembling the sclerotome was present but reduced in size, and the myotome was not clearly distinguishable.

Dermomyotomal and myotomal gene expression is abnormal in Gli2+/−Gli3−/− embryos

The abnormal epithelium in Gli2+/−Gli3−/− embryos suggested defects in dermomyotome patterning. We therefore investigated the expression of the dermomyotomal marker Pax3 in Gli2+−Gli3−/− embryos by whole-mount in situ hybridization. Expression of Pax3 in Gli2+/−Gli3−/− embryos appeared largely normal by wholomount at E9.5 (Fig. 3C,D). However transverse sections through the trunk revealed weak

Fig. 1. All three Gli genes are expressed in the ventral somite. Gene expression within the PSM and early somites was examined by radioactive section in situ hybridization on sagittal (A–C) and transverse (D–F) sections through E9.5 embryos. Bright-field images (A–F) are shown with corresponding dark field images (A′–F′). Radioactive section in situ hybridization signal corresponds to silver granules highlighted by dark-field illumination. Dotted lines indicate the boundary between the PSM and first fully formed somite (A–C′). Gli1 is expressed upon somite formation (A,A′) in the sclerotome (D,D′). Gli2 is expressed in the anterior PSM and the somites (B,B′,E′,E′). Gli3 is expressed in the PSM (C,C′) and the somites (F,F′). nt, neural tube; dm, dermomyotome; scl, sclerotome.
Pax3 expression in the ectopic ventromedial epithelium (Fig. 3H).

We next examined the expression of the myotomal marker and Gli target gene Myf5 (Gustafsson et al., 2002) by whole-mount in situ hybridization. Myf5 expression is normally restricted to the dorsomedial lip of the dermomyotome and the developing myotome. In Gli2−/−Gli3−/− embryos, Myf5 expression appeared diffuse and laterally expanded throughout the somites (arrowheads in Fig. 3E,F). In contrast to Pax3 and Myf5, expression of the lateral somite marker Sim1 appeared normal in Gli2−/−Gli3−/− embryos (arrowheads in Fig. 3I,J). Somites of Gli2−/−Gli3−/− embryos exhibit more severe defects than somites of Shh−/−Gli3−/− embryos

The somite defects we observed in Gli2−/−Gli3−/− mutants appear less severe than those described for Shh mutants (Chiang et al., 1996). Loss of Gli3 in a Shh mutant background rescues specific aspects of neural tube patterning due to the removal of Gli3 repressor function (Litingtung and Chiang, 2000), but whether Gli3 also acts as a repressor in the somites has not been examined. To test whether Gli2−/−Gli3−/− embryos more closely resemble those in which both the Shh signal and Gli3 repressor activity is lost, we compared the expression of Pax1 and Myf5 in Gli2−/−Gli3−/−, Shh−/− and Shh−/−Gli3−/− embryos.

Transverse sections through the trunk reveal that the weak Pax1 expression observed in Gli2−/−Gli3−/− embryos is restricted to a group of medially located mesenchymal cells within an epithelial sphere (Fig. 3L). In Shh−/− embryos Pax1 expression was undetectable in the ventral mesenchyme (Fig. 3M). Strikingly, strong Pax1 expression was restored in Shh−/−Gli3−/− embryos (Fig. 3N). In wild-type embryos, Myf5 expression is restricted to the developing myotome (Fig. 3O). In Gli2−/−Gli3−/− embryos, Myf5 expression was observed throughout mesenchymal cells within the epithelial spheres without forming a distinct layer (Fig. 3P). Sox9 appears normal in the posterior somites of these embryos (indicated by the black line A′′-D′′). Sox9 is undetectable in Gli2−/−Gli3−/− embryos. Scale bar: 0.5 mm.

Fig. 2. Gli2−/−Gli3−/− mutant mice exhibit a severe loss of sclerotomal gene expression. Expression of Pax1, Pax9 and Sox9 was assessed by whole-mount in situ hybridization in wild type (A,A′,A″), Gli2−/− (B,B′,B″), Gli3−/− (C,C′,C″), Gli2−/−Gli3+/− (D,D′,D″) and Gli2−/−Gli3−/− (E,E′,E″) E9.5 embryos. Pax1 and Pax9 are expressed normally in all allelic combinations (A-D,A′-D′) except for Gli2−/−Gli3−/− embryos, which exhibit a severe reduction and delay in Pax1 and Pax9 (between arrowheads in E and E′) expression. In Gli3−/− embryos Sox9 expression is reduced in the anterior somites, with the dorsal region most affected (compare arrowheads in inset of A″ and C″). Gli2−/− and Gli2−/−Gli3+/− embryos exhibit reduced Sox9 in both ventral and dorsal domains of anterior somites (arrowheads in B″ and D″). Sox9 appears normal in the posterior somites of these embryos (indicated by the black line A′′-D′′). Sox9 is undetectable in Gli2−/−Gli3−/− embryos. Scale bar: 0.5 mm.

At least one copy of either Gli2 or Gli3 is required for Shh-dependent sclerotome induction tissue autonomously

Motoyama et al. (Motoyama et al., 2003) have demonstrated
that Gli2+/Gli3−/− embryos have severely reduced expression of Shh in the brain and brachial regions. Thus, the reduced sclerotomal gene expression in these embryos might be due to a loss of Shh expression. As shown in Fig. 4A, Shh expression in the notochord and floorplate of double mutants is significantly weaker than in wild type. This finding questions whether the reduction in sclerotome gene expression in Gli2+/Gli3−/− embryos is due to a reduction of Shh, or an inability to transcriptionally activate Shh targets in the somites. To distinguish between these possibilities, we tested whether PSM isolated from Gli2+/Gli3−/− embryos can activate sclerotomal genes in response to exogenously provided Shh. If loss of Shh is the cause of the sclerotomal defects, PSM from Gli2+/Gli3−/− embryos will be able respond to exogenous Shh. If an inability to transcriptionally activate Shh target genes is the cause of the sclerotomal defects, exogenous Shh will not be able to induce Shh target genes in the PSM from Gli2+/Gli3−/− embryos.

E9.5 PSM isolated from various Gli2/Gli3 allelic combinations was cultured either in the absence or presence of 500 ng/ml Shh-N for 24 hours, and Shh target gene induction was assessed by RT-PCR. ß-actin expression was assessed as a control for normalization and the Shh target genes tested include Ptch, cyclin D2 (Cend2), Hhip, Pax1 and Pax9. As shown in Fig. 4B, wild-type PSM responded normally to Shh-N by inducing all target genes tested. PSM from Gli3−/− embryos also responded to Shh-N by inducing target genes. However, a 2.3-fold increase in the level of Pax9 induction in the absence of Shh was observed. Gli2+/− PSM exhibited normal Shh-responsive to exogenous Shh-N, as the expression of all target genes was unchanged in the presence of Shh-N,
indicating that Gli2 and Gli3 are required for Shh-induction of these genes. Interestingly, the expression of Ptc, Gli1 and Pax9 in the absence of Shh-N was increased (3.5-, 2.0- and 10-fold respectively) above wild-type control levels suggesting one copy of Gli3 is required for repression of these genes in the absence of Shh. These findings demonstrate a tissue autonomous requirement in the PSM for one copy of either Gli2 or Gli3 to mediate Shh induction of target genes.

Fig. 4. One copy of either Gli2 or Gli3 is required for tissue autonomous Shh responsiveness. (A) Shh expression was assessed by whole-mount in situ hybridization at E9.5. Transverse sections through the trunk reveal dramatically reduced Shh in the notochord and floorplate of Gli2–/–Gli3–/– embryos (compare arrowheads in A). (B) PSM tissue was isolated from E9.5 wild-type, Gli3–/–, Gli2–/–, Gli2–/–Gli3+/– and Gli2+/–Gli3+/– embryos and cultured in the presence (+) or absence (−) of Shh-N for 24 hours. Induction of target genes was assessed by RT-PCR. β-Actin serves as a control for normalization. Compared with wild-type PSM, Gli3–/– PSM shows Shh-responsiveness with an increase in Pax9 expression in the absence of Shh-N (n=4). Gli2–/– PSM shows reduced responsiveness in activating Ptc and Pax9, but normal induction of other targets (n=4). In Gli2+/–Gli3+/– PSM, all targets are induced with some reduction in Pax9 induction (n=2). In Gli2–/–Gli3–/– PSM, all targets tested are expressed identically in the presence and absence of Shh-N. There is increased basal expression of Ptc and Pax9 in Gli2+/–Gli3–/– PSM independent of Shh-N (n=3).

Fig. 5. ΔNG2 can mimic Shh and Smo* induction of targets in presomitic mesoderm. (A) PSM explants were cultured alone (−Shh), with Shh-N (+Shh) or infected with adenoviruses carrying EGFP, Smo or Smo* for 24 hours and analyzed for induction of Shh target genes by RT-PCR. Shh-N induces target genes, while infection with EGFP virus does not. Expression of Smo* induces all Shh targets tested (n=8). (B) PSM explants were infected with adenoviruses carrying either EGFP, full-length Gli1, Gli2, Gli3 or ΔNG2 for 24 hours and analyzed for induction of Shh targets by RT-PCR. Gli1 induced Ptc, Hhip and moderate levels of Ccnd2. Gli2 induced Ccnd2, and moderate levels of Ptc. Gli3 did not induce any Shh targets, while ΔNG2 activated all targets tested (n=8). (C) PSM was cultured for 24 hours in the absence (−) or presence (+) of Shh-N and adenovirus as indicated. Expression of Ptc and Pax1, was assessed by RT-PCR. Infection with EGFP and Gli1 does not affect induction of target genes by Shh, while infection with Gli2 does seem to have a moderate inhibitory affect specifically on Pax1. Infection with Gli3 acts to repress Shh induction of both Ptc and Pax1 (n=3). (D) PSM infected with Gli2 and ΔNG2 induce Gli1 at 24 hours (n=8). (E) PSM explants cultured alone, with Shh-N, with 1 μg/ml cycloheximide (CHX) or the combination for 9 hours were analyzed for Gli1, Ccnd2 and Hhip by RT-PCR. In the presence of CHX, Shh activates only Ccnd2 and Gli1, but not Hhip (n=2). CHX (1 μg/ml) effectively inhibits over 90% of new protein synthesis under similar culture conditions (Fu et al., 2002).
NG2 mimics Shh and Smo* signaling in the somitic mesoderm

To further dissect the function of each Gli in mediating specific target gene expression, we generated adenoviral vectors for overexpression of the Gli genes in the PSM. Adenoviral vectors contained either full-length Gli1, Gli2, Gli3 or an activated N-terminally truncated form of Gli2 (ΔNG2) (Sasaki et al., 1999), as C-terminal fusions to EGFP driven by a cytomegalovirus (CMV) promoter (see Fig. S2A at http://dev.biologists.org/supplemental/). Additional vectors serving as negative and positive controls contained EGFP alone, wild-type Smo or a constitutively active form of Smo (Smo*) (Xie et al., 1998). Production of the desired protein products upon adenoviral infection was confirmed by western analysis (see Fig. S2B-D at http://dev.biologists.org/supplemental/) and functionality of Gli-EGFPs was tested using a Shh-responsive cell line and a luciferase reporter downstream of eight Gli binding sites (see Fig. S2E at http://dev.biologists.org/supplemental/). Virally expressed Gli genes, Smo and Smo* functioned as predicted from previous studies (Sasaki et al., 1997; Sasaki et al., 1999; Shin et al., 1999; Taipale et al., 2000).

As Shh signaling in the somite is thought to occur via the conserved HH-PTC-Smo signaling pathway (Zhang et al., 2001), we first wanted to establish whether overexpression of Smo* by adenovirus can activate Shh target genes in cultured PSM. To test this, PSM from E9.5 mice was cultured alone, with Shh-N (500 ng/ml), or infected with adenovirus carrying EGFP, Smo or Smo* for 24 hours and analyzed for induction of the Shh target genes Ptc, Ccnd2, Hhip, Pax1, Pax9 and Sox9. As shown by RT-PCR in Fig. 5A, treatment with Shh-N induces all Shh target genes tested, while infection with EGFP adenovirus does not affect any Shh targets. Expression of Smo induces Ptc, Ccnd2, Hhip and weak Pax1, while expression of Smo* induces all Shh target genes tested. In conclusion, Shh target gene induction in the somite can be recapitulated by overexpression of Smo* in the PSM.

As all three Gli genes are expressed in the sclerotome, we next investigated whether overexpression of Gli genes can mimic Shh transcriptional target gene induction in the PSM. PSM explants were infected with adenovirus carrying EGFP alone or each of the Gli genes, for 24 hours and analyzed for Shh target gene expression. As shown in Fig. 5B, Gli1 and Gli2, each induced a subset of Shh target genes, whereas Gli3 did not induce any targets. Gli1 acted as a strong activator of Ptc and Hhip expression, but had a moderate effect on Ccnd2 expression. By contrast, Gli2 was a stronger activator of Ccnd2 (6.5-fold induction by Gli1 compared to 8.0-fold by Gli2), but had a weaker effect on Ptc (34-fold induction by Gli1 compared with 15-fold by Gli2) and no effect on Hhip. As none of the full-length Gli genes activated expression of Pax1, Pax9 and Sox9, we tested an N-terminally truncated form of Gli2, ΔNG2, previously shown to strongly activate Shh target genes in the neural tube (Sasaki et al., 1999) and skin (Mill et al., 2003). Indeed, infection with ΔNG2 adenovirus for 24 hours activated all Shh target genes tested in the PSM, suggesting that an activated form of Gli2 may mediate Shh signaling in the somite.

We next tested the activity of full-length Gli genes in the presence of Shh-N to determine whether the Gli genes can also exhibit repressive effects when overexpressed. PSM was cultured for 24 hours with each Gli adenovirus in the absence or presence of 500 ng/ml Shh-N, and expression of Ptc and Pax1 was assessed by RT-PCR. As shown in Fig. 5C, infection with EGFP and Gli1 adenoviruses does not affect induction of Pax1 and Ptc genes by Shh, while infection with Gli2 seems to have a moderate inhibitory affect (threefold) specifically on Pax1. By contrast, infection with Gli3 strongly represses Shh induction of both Ptc and Pax1. These results confirm the repressive ability of Gli3, and suggest that although full-length Gli2 can activate some Shh targets, full-length Gli2 expressed at high levels can also repress specific targets.

In other tissues Gli2 has been shown to be critical for induction of Gli1 expression (Bai et al., 2002; Ding et al., 1998). Similarly, we found that Gli2 and ΔNG2 can induce the expression of Gli1 in the PSM, although neither Gli1 nor Gli3 induce Gli1 expression (Fig. 5D and data not shown), demonstrating that Gli1 and Gli2 can preferentially activate different target genes.

Ptc, Pax1 and Pax9 are direct transcriptional targets of Shh signaling in the PSM that can be induced in the absence of protein synthesis (Dockter, 2000) (C.M.F., unpublished). We next tested whether Gli1, Ccnd2 and Hhip are also direct targets of Shh signaling in the PSM. PSM explants were treated with Shh-N in the absence or presence of 1 μg/ml cycloheximide to inhibit protein synthesis. In the absence of protein synthesis Shh-N induces only the expression of Gli1 and Ccnd2, but not of Hhip (Fig. 5E). Together with the Gli2 and ΔNG2 overexpression, these data support a linear pathway where Gli2 may be activated upon Shh signaling to induce Gli1, which in turn can induce the secondary target Hhip.

Gli1 cannot restore Shh-responsiveness in Gli2−/− Gli3−/− PSM

Although PSM from Gli2−/−Gli3−/− embryos does not activate Shh target genes in response to exogenous Shh-N, there is a low level of Pax1 and Pax9 expressed in Gli2−/−Gli3−/− somites (Fig. 2E,E'). It is possible that Gli1 expressed in the somites of Gli2−/−Gli3−/− embryos can mediate some Shh target gene expression. We measured the level of Gli1 expression in Gli2−/−Gli3−/− embryos by quantitative real-time PCR and found it was reduced to 9% of wild-type levels (data not shown). To address whether this low level of Gli1 is responsible for the Pax1 and Pax9 expression observed we tested whether expression of Gli1 in the PSM could restore Shh-responsiveness. PSM explants from wild-type or Gli2−/−Gli3−/− embryos were cultured in the presence of 500 ng/ml Shh-N and infected with either EGFP or Gli1 adenovirus for 24 hours. As shown in Fig. 6, infection with Gli1 adenovirus did not restore Shh-responsiveness in Gli2−/−Gli3−/− PSM. We then extended these results by testing the Shh-responsiveness of somites from Gli2−/−Gli3−/− embryos expressing endogenous Gli1 (data not shown). Gli2−/−Gli3−/− somites cultured with Shh-N also did not exhibit any Shh-responsiveness (data not shown). These findings demonstrate that in the absence of Gli2 and Gli3, Gli1 cannot restore Shh-responsiveness in the somitic mesoderm. Surprisingly, we found that overexpression of Gli1 failed to increase Ptc and Hhip expression in Gli2−/−Gli3−/− PSM, as it did in wild-type tissues (3.2- and 2.0-fold respectively), suggesting that Gli1 somehow requires Gli2 or Gli3 for transcriptional activity.

Discussion

Several reports have suggested Gli-independent mechanisms of Shh signaling in vertebrates (Krishnan et al., 1997a; Krishnan et al., 1997b; Lewis et al., 1999). However, our analysis of Gli function has unequivocally demonstrated a requirement for Gli2 and Gli3 in mediating Shh-induced sclerotomal gene expression. Through a combination of mutant studies and explant analysis, we reveal distinct functional activities for each Gli and identify cooperative roles for Gli genes in regulation of Shh target genes during mouse sclerotome development.

Skeletal defects in Gli2−/−, Gli3−/− and Gli2−/−Gli3−/− mice are likely due to loss of Sox9 expression

We demonstrate that Gli2−/−, Gli3−/− and Gli2−/−Gli3−/− embryos have reduced expression of Sox9 in the sclerotome of anterior somites at E9.5 and E10.5. A small reduction in Sox9 expression can result in severe chondrogenic defects, as Sox9+ cells cannot contribute to cartilage or bone in chimeric animals (Bi et al., 1999). We suggest that the loss of Sox9 is thus a likely cause of the skeletal phenotypes observed in these mutants (Mo et al., 1997), as the domains of Sox9 loss largely correspond to the specific skeletal abnormalities observed in each mutant. The normal Sox9 expression in the early somites of these mutants suggests that the defect is due to an inability to maintain proper levels of Sox9 expression in the mature somites. Recent work has shown that although Shh initiates Sox9 expression, BMP signals maintain a positive regulatory loop leading to sustained Sox9 expression (Zeng et al., 2002). Our data is consistent with either the establishment or maintenance of this positive regulatory loop being affected in these mutants.

Gli2 can act as a repressor and Gli3 can act as an activator for Shh target genes in the somites

Although Gli3 has been shown to activate Gli1 and Ptc promoters in cultured cells (Dai et al., 1999; Shin et al., 1999), the physiological role of this activator function is not clear. We provide two lines of evidence supporting a Shh-induced activator function for Gli3 in the somites. First, Gli2−/−Gli3−/−...
embryos express levels of *Pax1* and *Pax9* comparable with wild-type levels. Second, *Gli2*/*Gli3* PSM can activate expression of Shh target genes while *Gli2*/*Gli3* PSM cannot. This indicates that one copy of *Gli3* is sufficient to mediate Shh-dependent gene induction.

Although Gli2 is generally considered to be an activator of Shh signaling, *in vitro* studies suggest that it may also possess repressor function (Sasaki et al., 1999). Recent studies in zebrafish embryos have uncovered a possible repressor function for Gli2 specifically in telencephalon and muscle development (Karlstrom et al., 2003; Wolff et al., 2003). We provide evidence of a repressor function for Gli2 on direct targets of Shh in the sclerotome. Adenoviral overexpression of Gli2 in PSM can repress Shh induction of *Pax1* (Fig. 5C), and *Gli2*/*Gli3* PSM does not exhibit the same level of Shh target de-repression as *Gli2*/*Gli3* PSM (Fig. 4B; see Fig. S1D at http://dev.biologists.org/supplemental/), indicating that one copy of Gli2 is sufficient to mediate some repressive functions similar to those carried out by Gli3. The finding that the activator *Gli1* can replace Gli2 in vivo supports the notion that Gli2 functions solely as an activator (Bai and Joyner, 2001). However, our data illustrate that Gli2 repressor function is probably masked by the strong Gli3 repressor and is revealed only in the absence of Gli3, or when overexpressed in vitro. Thus, in addition to their overlapping activator functions, we suggest Gli2 and Gli3 also share repressor functions during somite development in mice.

**Gli genes and somite patterning**

The temporal and spatial expression of Gli genes in the developing paraxial mesoderm is consistent with Gli2 and Gli3 mediating initial Shh-induced sclerotome gene expression. Our overexpression and mutant analysis together support a model for Gli function in the somite as illustrated in Fig. 7A. In wild-type somites, Gli2 is modified to a strong activator (similar to ANG2) upon Shh signaling, and functions as the primary mediator of Shh signaling to promote both proliferation and patterning programs. Gli1 then acts downstream of Gli2, primarily activating Shh-dependent negative feedback mechanisms. However, as loss of *Gli1* does not affect somite development (Park et al., 2000), this function of Gli1 may be compensated by the activated form of Gli2. In the absence of Shh or when overexpressed, Gli3 represses Shh-induced programs. If Gli2 is lost, Gli3 activator function is revealed that compensates for the loss of Gli2. Conversely, in the absence of Gli3, Gli2 repressor function is revealed which compensates for the loss of Gli3. In the absence of both Gli2 and Gli3, the low level of Gli1 is non-functional and Shh responsiveness, as well as target repression, are lost, resulting in a low level of Shh-independent target gene expression.

Previous studies of Gli genes in the somites have centered on specification of myogenic precursors. In zebrafish *Gli1* and Gli2 are required to specify most myogenic cell types in a semi-redundant fashion (Karlstrom et al., 2003; Wolff et al., 2003), whereas in the chick, Gli3 is specifically upregulated in the myotome, suggesting it contributes to myogenesis (Borycki et al., 1998). Although *Myf5* has been shown to be a target of Gli regulation (Gustafsson et al., 2002), *Myf5* expression is detectable in *Gli2*/*Gli3* embryos (Fig. 3F), suggesting a Gli-independent pathway for *Myf5* expression in the mouse. As roles for Gli genes in chick and zebrafish sclerotome are not known, our data provides the first description of Gli functions in sclerotome induction.

**Shh functions are divided preferentially amongst different Gli genes in the somite**

Although previous analyses of *Gli2* and *Gli3* have revealed cooperative functions in several contexts, including the neural tube, lung, trachea and teeth (Hardcastle et al., 1998; Motoyama et al., 1998; Motoyama et al., 2003; Persson et al., 2002), how multiple Gli proteins cooperate together in mediating Shh response is not well understood. Our analysis of Gli function extends the previous studies by addressing the tissue autonomous contributions of each Gli gene to regulation of several direct Shh targets in the somitic mesoderm. Using mutant explants, we find that Shh targets display different sensitivities to loss of each Gli gene. For example, *Pax9* is the most sensitive to loss of Gli repressor function, as it is abnormally expressed in the absence of Shh in *Gli3*–/– and *Gli2*/*Gli3* PSM (Fig. 4B). By comparison, *Ptc1* is the most sensitive to loss of Gli2 activator function, as it cannot be induced in *Gli2*–/– PSM by Shh. Thus, Gli genes may mediate Shh induction of target genes in at least two ways. One way is for Shh to inhibit formation of Gli repressors, which may be the primary method of Shh induction of *Pax9*. A second way is for Shh to stabilize formation of Gli activators, which compete with Gli repressors for target gene activation. This is consistent with the regulation we observe for *Ptc1*, which cannot be activated in the absence of Gli2 until one copy of Gli3 is lost, reducing the concentration of Gli repressor. For *Pax1* and *Gli1* a small amount of activator, such as that possibly formed by Gli3 in the presence of Shh in *Gli2*/*Gli3*–/– embryos, is sufficient to mediate gene expression. This demonstrates additional distinctions in the functional output of each Gli and suggests a previously unappreciated ability of Gli genes to distinguish between different promoter/enhancer contexts.

**Mis-patterning of the *Gli2*/*Gli3* somite results from a complete loss of responsiveness to Hedgehog signaling**

Our data suggest that the somite phenotypes of *Gli2*/*Gli3*–/– embryos result from a loss of Shh responsiveness and Gli3 repressor function. However, the somitic phenotypes observed in these embryos are more severe than those of *Shh*/*Gli3*–/– embryos. Comparisons of *Shh* and *Smo* mutants have revealed a compensatory role in the somite for another HH family member, Indian hedgehog (Ihh). As *Smo* mutants lose all Hh-responsiveness, they cannot be compensated by Ihh, and thus exhibit more severe phenotypes than *Shh* mutants (Zhang et al., 2001). We suggest that *Gli2*/*Gli3*–/– embryos also lose all Hh-responsiveness based upon the following evidence. First, *Pax1* expression in *Gli2*/*Gli3*–/– somites is weaker than that in *Shh*/*Gli3*–/– somites, suggesting a lack of rescue by Ihh. Second, we observe ectopic *Pax3* in the ventromedial somite of *Gli2*/*Gli3*–/– embryos, consistent with recent studies of neural tube patterning in *Smoo*–/– chimeras where dorsal markers are ectopically expressed in the most ventral regions (Wijgerde et al., 2002). Last, we find *Myf5* and *Pax1* both expressed in the mesenchymal cells of *Gli2*/*Gli3*–/– somites, suggesting mixing of *Pax1*-positive and *Myf5*-positive cells (illustrated in Fig. 7B). This is consistent with the mixing of ventral cell types
observed in the neural tube of Smo+/+Gli3+/+ embryos (Wijgerde et al., 2002). Thus, the patterning and gene expression observed in Gli2+/+Gli3+/+ somites probably represents a default state, independent of all Hh signaling and Gli repressive activity.

How does transcriptional activation by Gli1 occur?
We present the unexpected result that in the absence of Gli2 and Gli3, Gli1 cannot transcriptionally activate its downstream targets Ptc and Hhip in the PSM. This suggests that Gli1 transcriptional activation somehow requires either Gli2 or Gli3. This is surprising in light of previous studies that strongly suggest direct transcriptional activation by Gli1. For example, Gli1 has been shown to bind a conserved Gli enhancer sequence in vitro (Sasaki et al., 1997). Furthermore, Gli1 can largely replace Gli2 function in vivo, suggesting a level of functional equivalency between the two Gli genes (Bai and Joyner, 2001). However, our result does not necessarily contradict these findings. One possibility is that Gli1 activation requires physical interaction with Gli2 or Gli3. Alternatively, Gli2 or Gli3 is required to provide a downstream factor necessary for Gli1 activator function. Further molecular and biochemical analyses of the Gli1 transcriptional activation mechanism will help to explain our finding.

We thank J. Lovejoy and A. Wells for technical assistance, and members of the Fan Laboratory and Dr A. Fire for suggestions on this manuscript.

References


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