Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 299 (2006) 12–21
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Regulation of *Gremlin* expression in the posterior limb bud

Sahar Nissim [a], Sean M. Hasso [b], John F. Fallon [b], Clifford J. Tabin [a,*]

[a] *Department of Genetics, Harvard Medical School, Boston, MA 02115, USA*
[b] *Department of Anatomy, University of Wisconsin-Madison, Madison, WI 53706, USA*

Received for publication 15 December 2005; revised 20 May 2006; accepted 23 May 2006
Available online 26 May 2006

Abstract

Proper outgrowth of the limb bud requires a positive feedback loop between *Sonic hedgehog* (*Shh*) in the zone of polarizing activity (ZPA) and *Fgfs* in the overlying apical ectodermal ridge. The Bmp antagonist *Gremlin* is expressed in a domain anterior to the ZPA and is thought to act as a signaling intermediate between *Shh* and *Fgf*. It is currently unclear whether *Shh* acts directly or indirectly to initiate and maintain *Gremlin*. In this study, we confirm that Bmp activity is necessary and sufficient for induction of *Gremlin*. Beads soaked in the Bmp antagonist Noggin downregulate *Gremlin*, while beads soaked in Bmp2 cause its upregulation. Furthermore, Bmp2 is also capable of upregulating Gremlin in *oligozeugodactyly* mutant limbs that lack Shh activity, demonstrating that Gremlin expression does not depend on the combined exposure to both these factors. In spite of the ability of Bmp2 to induce Gremlin, beads soaked in high concentrations of Bmp2 downregulate *Gremlin* around the bead without apparent induction of cell death, whereas another target gene *Msx2* is upregulated around the bead. Consistent with this concentration-dependent effect, we find that low concentrations of Bmp2 upregulate *Gremlin* while high concentrations of Bmp2 downregulate *Gremlin* in limb mesenchyme cultures. These data implicate Bmp activity as a required intermediate in the Shh–Fgf4 signaling loop. Though we show that Bmp activity is sufficient to upregulate *Gremlin*, *Gremlin* expression is excluded from a posterior domain of the limb, and expansion of this domain as limb outgrowth proceeds is important in terminating the Shh–Fgf4 signaling loop. We find that the posterior limb is refractory to *Gremlin* induction in response to Bmp2, suggesting that termination of the Shh–Fgf4 signaling loop results from inability of Bmp activity to induce *Gremlin* in the posterior. In contrast, in the *oligozeugodactyly* limb, we find that beads soaked in Bmp2 can induce *Gremlin* in the posterior, demonstrating that Shh activity is required for exclusion of *Gremlin* in the posterior. Finally, by blocking Shh activity with cyclopamine, we find evidence that continued Shh activity is also required to maintain refractoriness to *Gremlin* expression in response to Bmp activity.

Keywords: *Gremlin*; Bmp; Shh; Fgf; Bone morphogenetic protein; Sonic hedgehog; Fibroblast growth factor; Limb bud; *oligozeugodactyly*

Introduction

Proper outgrowth and patterning of the vertebrate limb require the coordination of several classical signaling centers. The Zone of Polarizing Activity (ZPA) in the posterior limb mesenchyme is a source of the secreted factor Sonic hedgehog (Shh) which is responsible for anterior–posterior patterning of the limb (Laufer et al., 1994; Riddle et al., 1993). Another signaling center, the apical ectoderm ridge (AER), is a specialized epithelial structure at the distal tip of the limb that is a source of several members of the fibroblast growth factor (FGF) family necessary to maintain proliferation of underlying mesenchyme and distal outgrowth of the limb (Fallon et al., 1994; Martin, 1998; Niswander et al., 1994). A positive feedback loop between Shh from the ZPA and Fgfs from the AER coordinates the activity of these two signaling centers. Misexpression of *Shh* in the anterior limb is sufficient to ectopically induce *Fgf4* in the anterior AER (Laufer et al., 1994; Niswander et al., 1994), and mice lacking *Shh* lose *Fgf4*, *Fgf9*, and *Fgf17* (Sun et al., 2000; Zuniga et al., 1999), demonstrating a direct requirement of *Shh* to maintain these Fgfs in the overlying AER. Likewise, Fgfs from the AER are required to maintain the expression of *Shh* (Laufer et al., 1994; Niswander et al., 1994). This interdependence of the ZPA and AER is critical in regulating limb outgrowth.

The bone morphogenetic protein (Bmp) antagonist *Gremlin* is an important intermediate in the signaling loop between Shh and Fgf. *Gremlin* is expressed in a domain anterior to the ZPA (Ganan et al., 1996) and is thought to block Bmps from

* Corresponding author.
E-mail address: tabin@genetics.med.harvard.edu (C.J. Tabin).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.05.026

downregulating Fgfs in the overlying AER (Capdevila et al., 1999; Pizette and Niswander, 1999; Zuniga et al., 1999). *Gremlin* activity is also required for the integrity of the AER itself and is thereby necessary for Fgf8 expression, although Fgf8 is not regulated by Gremlin at the transcriptional level (Khokha et al., 2003). *Shh* is both necessary and sufficient for *Gremlin* expression (Zuniga et al., 1999), and *Gremlin* in turn is both necessary and sufficient for expression of *Fgf4* in the AER (Khokha et al., 2003; Zuniga et al., 1999). While BMP antagonism by several disparate factors such as Noggin can mimic the ability of Gremlin to upregulate *Fgf4* in the AER, only Gremlin has been shown to have a physiologic role in early limb outgrowth; *Gremlin* mutant mice have distal skeletal defects resulting from a disrupted Shh–Fgf4 loop (Brunet et al., 1998; Khokha et al., 2003). Thus, the current model has Shh inducing the expression of *Gremlin* which, in turn, activates *Fgf* expression in the adjacent AER by antagonizing BMP activity.

While *Gremlin* expression is lost in *Shh* null mice and anterior misexpression of Shh induces ectopic *Gremlin* expression (Zuniga et al., 1999), it is currently not clear whether Shh acts directly or indirectly to initiate and maintain *Gremlin*. It has been previously hypothesized that members of the Bmp family regulate their own activity by induction of *Gremlin* (Capdevila et al., 1999). Evidence for this hypothesis comes from the observation that implantation of a bead soaked in Bmp7 is sufficient to upregulate *Gremlin* (Merino et al., 1999); moreover, retroviral infection of *Noggin*, another Bmp antagonist, in presumptive limb mesenchyme in an HH stage 10 embryo entirely abolishes *Gremlin* expression in the limb at HH stage 22–23 (Capdevila et al., 1999). However, as the retroviral infection is very broad and is applied very early, it is unclear whether the loss of *Gremlin* is a direct or indirect consequence. Moreover, if Bmp activity is indeed necessary for *Gremlin* expression, it is not clear if Shh and Bmps are both required in concert for *Gremlin* expression or if Bmps act as a secondary signal downstream of Shh to regulate *Gremlin*.

Another aspect of *Gremlin* regulation that remains unclear is the basis for the exclusion of *Gremlin* expression from the posterior-most limb. While Shh and Bmps have been implicated in the induction and/or maintenance of *Gremlin*, *Gremlin* is not expressed in the posterior limb where expression levels of *Shh*, *Bmp2*, and *Bmp7* are highest. Because the region of cells excluding *Gremlin* expands over time to distance the domain of *Shh* expression from the domain of *Gremlin* expression, this refractoriness plays an important role in the eventual breakdown of the Shh–Fgf4 signaling loop and therefore regulates limb outgrowth (Scherz et al., 2004). By tracing the descendants of cells expressing *Shh*, it has been demonstrated that former *Shh*-expressing cells cannot express *Gremlin* (Scherz et al., 2004). This refractoriness appears to be cell-autonomous. However, it is not clear if establishment of the block in *Gremlin* expression in ZPA cells depends upon Shh activity.

In this study, we find that Bmp activity is necessary and sufficient for induction of *Gremlin*. Moreover, in the context of the *oligozeugodactyly* (*ozd*) mutant limb in which Shh activity is absent, Bmp2 is sufficient to induce *Gremlin*. Using beads containing varying concentrations of Bmp2 in vivo or culturing limb mesenchyme with varying concentrations of Bmp2 in vitro, we find evidence supporting the idea that Bmp activity regulates *Gremlin* in a concentration-dependent fashion. These data implicate Bmp activity as a required intermediate in the Shh–Fgf4 signaling loop. We further demonstrate that the Shh–Fgf4 signaling loop breaks down when Bmp activity can no longer upregulate *Gremlin* in the posterior. In the posterior of *ozd* mutant limbs, *Gremlin* can be induced by Bmp2, suggesting that refractoriness to *Gremlin* induction is dependent on Shh activity. By blocking Shh activity using cyclopamine, we provide evidence that Shh activity is also required to maintain refractoriness to express *Gremlin* in response to BMP signaling.

Results

Previous studies have demonstrated that Shh signaling is both necessary and sufficient for *Gremlin* induction (Capdevila et al., 1999; Zuniga et al., 1999). To test if Shh acts directly or indirectly to upregulate *Gremlin*, we applied a bead soaked in Shh protein to the anterior of an HH stage 21 limb bud in the presence of cycloheximide, an inhibitor of de novo protein synthesis. Whereas Shh normally results in robust upregulation of *Gremlin* (n = 5/5) (Fig. 1A), Shh fails to upregulate *Gremlin* in the presence of cycloheximide (n = 10/10) (Fig. 1B), suggesting that Shh acts indirectly, either by a secondary secreted signal or secondary intracellular signal.

If Shh regulates *Gremlin* via a secondary secreted signal, good candidates for that signal include Bmp2 and Bmp7, established downstream targets of Shh (Laufer et al., 1994; Chiang et al., 2001). Expression of *Gremlin* appears adjacent to mesenchymal expression of *Bmp2* and *Bmp7* in the HH stage 22 limb (Figs. 1C, D, F). Though it is not believed to be regulated by *Shh*, *Bmp4* is also expressed in limb mesenchyme at this stage (Fig. 1E). To test if Bmp activity is necessary for *Gremlin* expression in a more controlled way than the previously published retroviral misexpression studies (Capdevila et al., 1999), we applied a bead soaked in Noggin protein in HH stage 22 chick limbs. Consistent with the retroviral results, we observed downregulation of *Gremlin* immediately around the bead within 6 h (n = 10/10) (Fig. 1G). These results reinforce the conclusion that *Gremlin* expression is directly dependent on Bmp activity.

In the Shh null mouse, *Gremlin* expression is initially present but disappears by E10.25, indicating that Shh activity is necessary to maintain *Gremlin* (Zuniga et al., 1999). Therefore, Shh and Bmp activity may both be independently required to maintain *Gremlin* in the posterior. Alternatively, the requirement of Shh activity in *Gremlin* maintenance may be solely to induce Bmp activity. To distinguish these possibilities, we tested whether Bmp activity is sufficient to induce *Gremlin* in the anterior limb mesenchyme far from Shh activity. Indeed, a Bmp2-soaked bead applied to the anterior is sufficient to induce *Gremlin* (Figs. 2A, B). At a lower concentration (0.1 mg/ml), Bmp2