Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail

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The Hedgehog (Hh) family of secreted proteins controls many aspects of growth and patterning in animal development. The seven-transmembrane protein Smoothened (Smo) transduces the Hh signal in both vertebrates and invertebrates; however, the mechanism of its action remains unknown. We found that Smo lacking its C-terminal tail (C-tail) is inactive, whereas membrane-tethered Smo C-tail has constitutive albeit low levels of Hh signaling activity. Smo physically interacts with Costal2 (Cos2) and Fused (Fu) through its C-tail. Deletion of the Cos2/Fu-binding domain from Smo abolishes its signaling activity. Moreover, overexpressing Cos2 mutants that fail to bind Fu and Ci but retain Smo-binding activity blocks Hh signaling. Taken together, our results suggest that Smo transduces the Hh signal by physically interacting with the Cos2/Fu protein complex.

Keywords: Smo; Hh; Cos2; Fu; signaling; development

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kinases: the cAMP-dependent protein kinase (PKA), GSK3, and CKI (Jiang and Struhl 1998; Y. Chen et al. 1999; Price and Kalderon 1999, 2002; Wang et al. 1999; Jia et al. 2002, Jiang 2002). These kinases phosphorylate Ci at multiple sites in three clusters in its C-terminal region (Jiang 2002). Hyperphosphorylation of Ci targets it for Slimb/Proteasome-mediated proteolytic processing (Jiang and Struhl 1998; Jiang and Struhl 2002). Hh appears to induce dephosphorylation of Ci, leading to the blockage of its processing (C.H. Chen et al. 1999).

Hh regulates Ci at multiple levels. In addition to blocking its proteolysis, Hh also induces nuclear translocation of Ci155 and further stimulates its transcriptional activity (Ohlmeyer and Kalderon 1998, C.H. Chen et al. 1999; Wang and Holmgren 1999, 2000; Wang et al. 1999, 2000). In the absence of Hh, Ci155 is retained in the cytoplasm by forming a large protein complex that also includes the kinesin-related protein Costal2 (Cos2), the Ser/Thr kinase Fused (Fu), and the tumor suppressor protein Su(fu) (Methot and Basler 2000; Wang and Holmgren 2000; Wang et al. 2000). The Ci/Cos2/Fu complex binds microtubules, likely through Cos2, in an Hh-regulated manner (Robbins et al. 1997; Sisson et al. 1997; Stegman et al. 2000). The transcriptional activity of Ci155 appears to be further induced by PKA phosphorylation and by stoichiometric interaction with Su(fu) (Ohlmeyer and Kalderon 1997; Wang et al. 1999). Su(fu) prevents the maturation of Ci155 into a labile hyperactive form, and Hh alleviates such inhibition through Fu kinase activity (Ohlmeyer and Kalderon 1997). Su(fu) appears to inhibit Ci155 by both impeding its nuclear translocation and inhibiting its transcriptional activity after it enters the nucleus (Methot and Basler 2000; Wang et al. 2000). One possible mechanism for Su(fu) to inhibit the transcriptional activator activity of Ci155 is to recruit transcription corepressors (Cheng and Bishop 2002).

Different layers of negative regulation of Ci appear to be offset by distinct thresholds of Hh signaling activity. Low levels of Hh signaling activity suffice to block Ci processing but do not stimulate the transcriptional activity of Ci155 (Methot and Basler 1999; Wang and Holmgren 1999; Wang et al. 1999). As a consequence, dpp is derepressed, whereas other genes that are activated by Ci155 remain silent. High levels of Hh activate Ci155, at least in part, by alleviating Su(fu)-mediated repression (Ohlmeyer and Kalderon 1998). Cos2 was initially identified as a negative component in the Hh pathway; however, a recent study suggested that it also has a positive role in the pathway, as removal of cos2 function in Hh-receiving cells blocks the transduction of high levels of Hh signaling activity (Wang et al. 2000).

Here we address how Smo relays Hh signal to intracellular signaling components. Although Smo is related to the serpentine family of receptors that transduce signals through trimeric G-proteins (Alcedo et al. 1996; van-den-Heuval and Ingham 1996), no evidence for the involvement of a G-protein in physiological Hh signaling has been obtained (Ingham and McMahon 2001). Here we provide evidence that Smo transduces the Hh signal by physically interacting with the Cos2/Fu complex through its C-terminal tail.

**Results**

**Smo C-tail is essential for its activity**

To determine the mechanism by which Smo transduces the Hh signal, we generated epitope-tagged full-length and truncated forms of Smo [summarized in Fig. 1], and assessed their signaling activities in wing imaginal discs using the Gal4/UAS system [Brand and Perrimon 1993]. Overexpressing a full-length Smo tagged by GFP at its N terminus [GFP-Smo] by an MS1096 Gal4 driver resulted in ectopic albeit low levels of Hh pathway activation, as evidenced by the accumulation of high levels of Ci155 and ectopic expression of modest levels of dpp-lacZ in A-compartment cells away from the A/P compartment boundary [Fig. 2A–A’]. Coexpressing Ptc with GFP-Smo inhibited the ectopic Hh signaling activity caused by overexpressing GFP-Smo alone [Supplementary Fig. 1]. These observations suggest that high levels of Smo could at least partially titrate out the inhibitory activity of Ptc, resulting in constitutive Hh pathway activation. In contrast, overexpressing a truncated form of Smo that lacks the C-tail (GFP-SmoACT) failed to induce ectopic Hh pathway activation [Fig. 2B–B’], even though its level of expression is comparable to that of full-length Smo [Fig. 2, cf. B and A]. Moreover, wing discs expressing multiple copies of UAS-GFP-SmoACT by MS1096 did not ectopically activate the Hh pathway [data not shown].

We also examined if GFP-SmoACT possess any Hh-inducible activity by expressing UAS-GFP-SmoACT in wing discs carrying a smo mutant clone induced by FLP/FRT-mediated mitotic recombination [Xu and Rubin 1993]. smo mutant cells expressing GFP-SmoACT failed to activate ptc-lacZ expression near the A/P compartment boundary [Fig. 2D–D’], whereas GFP-Smo could functionally substitute the endogenous Smo to activate ptc-lacZ in A-compartment cells near the A/P border [Fig. 2C–C’]. In addition, expressing GFP-Smo but not GFP-SmoACT in smo zygotic null embryos rescued smo mutant phenotypes [Fig. 2F–H]. Similar results were obtained with Myc-tagged full-length [Myc-Smo] and C-terminally truncated Smo [Myc-SmoACT; Fig. 2I, data not shown]. These results demonstrate that the Smo C-tail is essential for its signaling activity.

**Membrane-tethered forms of Smo C-tail possess constitutive Hh signaling activity**

To further assess the role of the Smo C-tail in Hh signal transduction, we generated several Flag-tagged Smo deletion mutants that only contain the C-tail portion of Smo [Fig. 1]. Sve-SmoCT is a chimeric protein that contains the transmembrane and extracellular domain of Sevenless (Sev) protein fused to SmoCT, whereas Myr-SmoCT contains a myristoylation signal at the N terminus of SmoCT. Surprisingly, A-compartment cells ex-
pressing either Sev–SmoCT or Myr–SmoCT accumulate high levels of Ci155 and activate dpp–lacZ in a cell-autonomous manner even when they are distant from the A/P compartment boundary [Fig. 3A–B’], suggesting that both Sev–SmoCT and Myr–SmoCT can block Ci processing and the formation of Ci75. In contrast, cells expressing untethered SmoCT do not ectopically activate the Hh signaling pathway [data not shown], suggesting that membrane targeting of SmoCT is essential for its activity. As Sev–SmoCT and Myr–SmoCT activate the Hh signaling pathway to similar extents, we focused our analyses on Myr–SmoCT.

In addition to preventing Ci processing, Hh also promotes nuclear translocation of accumulated Ci155 (C.H. Chen et al. 1999; Wang and Holmgren 2000; Wang et al. 2000). To determine whether Myr–SmoCT stimulates Ci155 nuclear translocation, we treated wing discs expressing Myr–SmoCT with Leptomycin B (LMB), an inhibitor that blocks Ci155 nuclear export (C.H. Chen et al. 1999; Wang and Holmgren 2000; Wang et al. 2000). In wild-type wing discs, A-compartment cells near the A/P boundary accumulate Ci155 in the nucleus, whereas cells situated distantly from the A/P boundary keep Ci155 in the cytoplasm [Fig. 3C; Wang and Holmgren 2000; Wang et al. 2000]. In contrast, A-compartment cells expressing Myr-SmoCT accumulate Ci155 in the nucleus regardless of their locations [Fig. 3D,D’], indicating that Myr–SmoCT stimulates Ci155 nuclear translocation in addition to blocking its processing.

**Myr–SmoCT activates Hh signaling independent of endogenous Smo**

To determine if Myr–SmoCT can elicit Hh signaling activity in the absence of endogenous Smo, we misexpressed Myr–SmoCT in wing discs carrying smo null mutant clones induced by FLP/FRT-mediated mitotic recombination. As shown in Figure 3, smo− cells expressing Myr–SmoCT accumulate high levels of Ci [Fig. 3E,E’], and activate dpp–lacZ even though they are situ-
Figure 2. The Smo C-tail is essential for Hh signaling. Wing discs expressing UAS–GFP–Smo [A–A'] or UAS–GFP–SmoΔCT by MS1096 [B–B'] were immunostained to show the expression of GFP (green in A,B), Ci155 (red in A',B'), and dpp–lacZ [blue in A',B']. Wing discs containing smo mutant clones and expressing UAS–GFP–Smo [C–C'] or UAS–GFP–SmoΔCT by MS1096 [D–D'] were immunostained to show the expression of ptc–lacZ [green] and CD2 (red). Smo mutant clones are recognized by the lack of CD2 expression (arrows). [E–I] Cuticles were prepared from embryos of the following genotypes growing at 18°C: wild type [E], smo1/smo1 [F], smo3/smo3; prd–Gal4/UAS–GFP–Smo [G], smo3/smo3; prd–Gal4/UAS–GFP–SmoΔCT [H], and smo3/smo3; prd–Gal4/UAS–Myr–Smo [I]. The wild-type embryo exhibits alternate naked cuticles and denticles [E], whereas the smo3 homozygote exhibits the typical zygotic null phenotype with a lawn of denticles covering most of the ventral surface [F]. Naked cuticles [indicated by arrows] are restored in alternate segments of smo1 homozygotes expressing either UAS–GFP–Smo [G] or UAS–Myr–Smo [I], but not in smo3 homozygotes expressing UAS–GFP–SmoΔCT [H].

Deletion analysis of Smo C-tail

To further define the region within the Smo C-tail that mediates Hh pathway activation, we generated a series of deletion mutants that remove various parts of the Smo C-tail and examined their abilities to activate the Hh pathway [Fig. 1]. We found that Myr–SmoCTΔ625–730 and Myr–Smo730–1035 can elicit Hh pathway activation, as indicated by the accumulation of Ci155 and ectopic expression of dpp–lacZ [Supplementary Fig. 2; Fig. 3H–I]. In contrast, Myr–Smo556–730, Myr–Smo730–818, Myr–Smo556–818, and Myr–SmoCTΔ625–818 do not possess Hh signaling activity [Supplementary Fig. 2; Fig. 3H–I].

Myr–SmoCT does not fully activate the Hh pathway

Although Myr–SmoCT can stabilize Ci155 and promote its nuclear import, it does not fully stimulate the transcriptional activity of Ci155, as wing discs expressing UAS–Myr–SmoCT by act > CD2 > Gal4 does not activate ptc–lacZ and en in most of the A-P compartment cells (Fig. 4B, data not shown). Under this condition, ptc–lacZ is only activated at low levels in anterior-most regions of wing discs [Fig. 4B], where cells are more responsive to low levels of Hh signaling activity [Jia et al. 2002].

The inability of Myr–SmoCT to fully activate the Hh pathway is not caused by the lack of enough expression. As a matter of fact, the levels of Myr–SmoCT expressed by act > CD2 > Gal4 is much higher than those of endogenous Smo at the A/P compartment boundary, as determined by immunostaining with an antibody against the Smo C-tail [data not shown]. In addition, increasing the dose of Myr–SmoCT by expressing multiple copies of UAS–Myr–SmoCT does not further enhance the Hh signaling activity elicited by Myr–SmoCT [data not shown].

It has been shown that Su[fu] can inhibit Ci155 activity via a mechanism that is independent of Ci nuclear localization [Wang et al. 2000]. Hence, one possible reason that Myr–SmoCT fails to activate Ci155 is that it does not alleviate the inhibition of Ci155 by Su[fu]. If so, removal of Su[fu] should allow activation of Ci155 accumulated in Myr–SmoCT-expressing cells. We therefore misexpressed Myr–SmoCT in Su[fu] homozygous wing discs. Although Su[fu] homozygous wing discs do not exhibit any discernible phenotypes [Ohlmeyer and Keller 1998; overexpressing Myr–SmoCT in Su[fu] wing discs ectopically activated high levels of ptc–lacZ in most A-compartment cells [Fig. 4C].

expressing smo3; prd–Gal4/UAS–Myc–Smo (G2712 GENES & DEVELOPMENT/11033 boundary (Fig. 3G–Gigated in A-compartment cells distant from the A/P boundary [Fig. 3G–G2], suggesting that Myr–SmoCT possesses constitutive Hh signaling activity independent of endogenous Smo. To determine if Myr–SmoCT is refractory to Ptc inhibition, UAS–Ptc and UAS–Myr–SmoCT were co-overexpressed in wing discs by the act > CD2 > Gal4 driver [Pignon et al. 1997]. As shown in Figure 3F, coexpression of Ptc does not block the ectopic Hh signaling activity caused by overexpressing Myr–SmoCT. Hence, Myr–SmoCT appears to escape the inhibitory regulation by Ptc, which explains why it is constitutively active.
Deleting the C-terminal sequence from full-length Smo (GFP-SmoΔ818 and GFP-SmoΔC730) also abolishes Smo activity as overexpressing these deletion mutants failed to ectopically activate dpp–lacZ (Supplementary Fig. 2; data not shown). These results suggest that the C-terminal half of the Smo C-tail (amino acids 730–1035) is critical for Smo activity.

Smo physically interacts with Cos2/Fu through its C-tail

Interestingly, expressing Myr-SmoCT at high levels inhibits the expression of endogenous ptc and en near the A/P compartment boundary [Fig. 4D–D’], suggesting that Myr-SmoCT can interfere with the ability of endogenous Smo to transduce high levels of Hh signaling activity. The inhibition of ptc and en expression by Myr-SmoCT can be suppressed by coexpressing GFP-Smo [Fig. 4E–E’]. One hypothesis that accounts for these observations is that Myr-SmoCT may compete with endogenous Smo for a downstream signaling effector(s).

Several known intracellular Hh signaling components, including Cos2, Fu, and Ci, form large protein complexes [Robbins et al. 1997; Sisson et al. 1997]. As no signaling intermediates between Smo and the Cos2 complex have been identified, we sought to determine if Smo transduces Hh signal by physically interacting with the Cos2 complex. To facilitate the detection of complex formation, we transfected S2 cells with DNA constructs expressing Myc-tagged full-length Smo [Myc-Smo] or its C-tail deletion mutant [Myc-SmoΔC730], HA-tagged Cos2, Fu, and Ci. Cell extracts were immunoprecipitated with a mouse anti-Myc antibody, followed by Western blot analysis with aCos2, aFu, or aCi [2A] antibody. Myc-Smo but not Myc-SmoΔC730 pulled down Cos2 and Fu [Fig. 5B], suggesting that Myc-Smo binds the Cos2/Fu complex and the Smo C-tail is essential for this interaction. Ci was not consistently pulled down by Myc-Smo (data not shown). It is possible that Ci/Smo interaction is very dynamic. For example, Ci may dissociate from the Cos2/Fu complex after the complex binds Smo. We also found that Myc-Smo expressed in S2 cells can pull down en-
high levels of most A-compartment cells expressing Myr–SmoCT activate SmoCTS of the lack of CD2 staining. Myc–SmoCT only activates low levels wing discs expressing a strong line of Myr–SmoCT (red). Myr–SmoCT-expressing cells are recognized by ptc–lacZ were immunostained to show the expression of CD2 (green) and interferences with endogenous Smo. (A) A wild-type wing disc. Wild-type (B) or Su(fu)ji. Homozygous (C) wing discs expressing UAS–Myc–SmoCT by act > CD2 > Gal4 were immunostained to show the expression of CD2 (green) and ptc–lacZ (red). Myr–SmoCT-expressing cells are recognized by the lack of CD2 staining. Myc–SmoCT only activates low levels of ptc–lacZ in anteriormost cells (arrow in B). (C) In contrast, most A-compartment cells expressing Myr–SmoCT activate high levels of ptc–lacZ in Su(fu) homozygous discs. (D–E’) Wing disc expressing a strong line of Myr–SmoCT (UAS–Myc–SmoCT) alone (D–D’) or in conjunction with GFP-Smo (E–E’) were immunostained to show the expression of ptc–lacZ (red), Flag (green), and En (blue). High levels of Myr–SmoCT inhibit the expression of ptc–lacZ and en in A-compartment cells near the A/P boundary (arrows in D,D’), which is reversed by coexpressing GFP-Smo (arrows in E,E’).

dogenous Cos2 and Fu [Fig. 5C]. As S2 cells do not express Ci, this result indicates that Smo can interact with Cos2 and Fu independently of Ci.

To determine if Smo binds Cos2 and Fu in vivo, we expressed Myc-Smo or Myc-SmoACT in wing discs by the MS1096 Gal4 driver. Wing disc extracts were immunoprecipitated with the aMyc antibody, followed by Western blot analysis with aCos2, aFu, and aCi antibodies. As shown in Figure 5D, Myc-Smo but not Myc-SmoACT pulled down Cos2 and Fu. Myc-Smo did not pull down detectable levels of Ci [data not shown]. We also coexpressed UAS–Hh with UAS–Myc–Smo to determine if the interaction between Myc-Smo and Cos2/Fu is affected. As expected, ectopic expression of Hh stabilizes coexpressed Myc-Smo, as more Myc-Smo was pulled down by the aMyc antibody [Fig. 5D]. However, the amounts of Cos2 and Fu pulled down by Myc-Smo only increase modestly [Fig. 5D], suggesting that overexpressing Myc-Smo alone may already saturate Cos2/Fu binding. Interestingly, a significant fraction of Fu bound to Myc-Smo became phosphorylated when Hh was co-expressed, as indicated by its mobility shift [Fig. 5D]. Hence, overexpressed Myc-Smo binds Cos2/Fu in vivo, but appears to stimulate Fu phosphorylation only when Hh is coexpressed.

When expressed in S2 cells, both Myr–SmoCT and Myr–Smo730–1035 pulled down endogenous Cos2/Fu [Fig. 5E, lane 2, data not shown], suggesting that SmoCT is sufficient for binding to the Cos2/Fu complex. Consistent with their inability to activate the Hh pathway, both Myr–Smo556–730 and Myr–Smo556–818 pull down diminishing levels of Cos2/Fu [Fig. 5E, lanes 3,4]. Intriguingly, Myr–SmoCTΔ625–818, which does not possess Hh signaling activity, appears to bind Cos2/Fu with affinity similar to that of Myr–SmoCT [Fig. 5E, lane 5]. In addition, the untethered SmoCT also binds Cos2/Fu [Fig. 5E, lane 1]. These observations suggest that binding of Smo to Cos2/Fu per se does not trigger Hh pathway activation. It seems that membrane association and the Smo sequence between amino acids 730 and 818 are also critical for SmoCT to activate the Hh pathway.

To define the Cos2 domain[s] that mediates Smo binding, we generated a series of HA-tagged Cos2 deletion mutants [summarized in Fig. 5A] and examined their ability to bind Myc-Smo in S2 cells by coimmunoprecipitation. It appears that Myc-Smo binds Cos2 through at least two regions of Cos2: the microtubule-binding domain and the C-tail, as HA-Cos2MB, HA-Cos2CT1, and HA-Cos2CT2 were pulled down robustly by Myc-Smo [Fig. 5F, lanes 5,6,8]. HA-Cos2ΔN2 also binds strongly to Myc-Smo [Fig. 5F, lane 3]. In contrast, HA-Cos2CC was not pulled down by Myc-Smo [Fig. 5F, lane 4]. Intriguingly, relatively less HA-Cos2, HA-Cos2ΔN1, and HA-Cos2ΔC were pulled down by Myc-Smo [Fig. 5F, lanes 1,2]. One possibility is that Smo-binding domains in these large proteins could be partially “masked.” Alternatively, they might be unstable when bound to Smo in the absence of Fu. Consistent with the latter hypothesis, we found that coexpression of Fu with HA-Cos2 dramatically increases the amount of HA-Cos2 pulled down by Myc-Smo [data not shown].

Blockage of Hh signal transduction by Cos2 deletion mutants

To access the physiological significance of Smo/Cos2 interaction in Hh signal transduction, we expressed Cos2 mutants that lack Ci- and Fu-binding domains but retain Smo-binding activity in wing discs and examined their effects on Hh signaling. We reasoned if Smo transduces Hh signal by recruiting the Cos2/Fu complex, overexpressing such Cos2 deletion mutants should titrate out endogenous Smo and prevent it from interacting with the endogenous Cos2/Fu complex. As a consequence, Hh signal transduction should be blocked. The Ci- and Fu-binding domains have been mapped to the N-terminal half of Cos2 [Fig. 5A; Monnier et al. 2002], and Cos2ΔN2 failed to bind Ci and Fu in yeast [G. Wang and J. Jiang, unpubl.]. As shown in Figure 6A, overexpressing Cos2ΔN2 in wing discs blocks Hh signaling, as indicated by the loss of ptc–lacZ. The inhibition of Hh signaling
activity by Cos2ΔN2 correlates with Smo binding as Cos2CC, which does not bind Smo, fails to block Hh signaling (Fig. 6B). Moreover, overexpressing the Cos2 C-tail [Cos2CT1] also results in pathway inhibition, as manifested by the reduction of ptc–lacZ and anterior en expression [Fig. 6D–E] as well as fu-like wing phenotypes [Fig. 6F]. If the inhibition of Hh signaling by Cos2 deletion mutants is caused by titrating out endogenous Smo and preventing it from binding to endogenous Cos2/ Fu complex, then increasing the amounts of Smo should reverse such a blockage. Indeed, coexpressing GFP-Smo with Cos2ΔN2 restores ptc–lacZ expression [Fig. 6C–E].

**Discussion**

The seven-transmembrane protein Smo plays a central role in transducing Hh signal. In this study, we demonstrated that the Smo C-terminal tail [C-tail] is essential for its function. Surprisingly, we found that the Smo C-tail possesses constitutive albeit low levels of signaling activity when it is tethered to plasma membrane. We provided biochemical evidence that Smo interacts with the Cos2/Fu complex through its C-tail, and Smo/Cos2 interaction is mediated at least in part through the C-terminal region of Cos2. In addition, we provided evidence that Smo/Cos2 interaction is essential for Hh sig-
A previous structure–function study on mammalian Smo transduces Hh signal through its C-tail protein family. An involvement of a G-protein in a physiological Hh signaling process (Ingham and McMahon 2001). Second, Myr–SmoCT-expressing cells must rely on a mechanism that is independent of inhibiting Ci nuclear translocation.

**Physical interaction between Smo and Cos2/Fu**

Several observations prompted us to determine whether Smo could transduce Hh signal by physically interacting with the Cos2/Fu complex. First, although Smo is related to G-protein-coupled receptors, no genetic or pharmacological evidence has been obtained to support the involvement of a G-protein in a physiological Hh signaling process (Ingham and McMahon 2001). Second, Myr–SmoCT can interfere with the ability of endogenous Smo to transduce signal in Hh-receiving cells. Hence, the Smo C-tail is absolutely required for Smo activity. It is not clear what causes the discrepancy. One possibility is that Smo may act differently from mammalian Smo. Alternatively, Smo may behave differently in vivo versus in vitro. Consistent with this, we found that mouse Smo without a C-tail fails to induce ventral markers in chick spinal cords, whereas a control full-length Smo does (W. Zhang, R. Lu, and J. Jiang, unpubl.).

Perhaps a more surprising finding of our study is that the Smo C-tail suffices to induce Hh pathway activation. We found that overexpressing the membrane-tethered Smo C-tail [Myr–SmoCT, Sev–SmoCT] blocks Ci processing, induces dpp–lacZ expression, and stimulates nuclear translocation of Ci155. Myr–SmoCT is refractory to Ptc inhibition and activates Hh-pathway independent of endogenous Smo. Membrane tethering appears to be crucial for the Smo C-tail to activate the Hh pathway, as untethered SmoCT has no signaling activity. This is consistent with previous observations that cell surface accumulation of Smo correlates with its activity [Denef et al. 2000; Zhu et al. 2003].

Although the Smo C-tail has constitutive Hh signaling activity, it does not possess all the activities associated with full-length Smo. For example, overexpressing Myr–SmoCT in A-compartment cells away from the A/P compartment boundary does not significantly activate ptc and en, which are normally induced by high levels of Hh. In addition, Myr–SmoCT cannot substitute endogenous Smo at the A/P compartment boundary to transduce high levels of Hh signaling activity, as boundary smo mutant cells expressing Myr–SmoCT failed to express ptc in response to Hh [data not shown].

The failure of the Smo C-tail to transduce high Hh signaling activity is due to its inability to antagonize Su(fu). Although Myr–SmoCT blocks Ci processing to generate Ci75, the activity of Ci155 accumulated in Myr–SmoCT-expressing cells is still blocked by Su(fu), as removal of Su(fu) function from Myr–SmoCT-expressing cells allows Ci155 to activate ptc to high levels. As Myr–SmoCT stimulates nuclear translocation of Ci155, the inhibition of Ci155 by Su(fu) in Myr–SmoCT-expressing cells must rely on a mechanism that is independent of impeding Ci nuclear translocation.

**Smombo**

A previous structure–function study on mammalian Smo using cultured cells suggested that the Smo C-tail is not essential for Smo activity [Murone et al. 1999]. Here we showed that Smo deletion mutants lacking the Smo C-tail (SmoΔC730) or part of it (SmoΔC818 and SmoΔC818 and SmoΔC730) are inactive when overexpressed in wing discs. Moreover, SmoΔCT does not substitute endogenous Smo to transduce signal in Hh-receiving cells. Hence, the Smo C-tail is absolutely required for Smo activity. It is not clear what causes the discrepancy. One possibility is that *Drosophila* Smo may act differently from mammalian Smo. Alternatively, Smo may behave differently in vivo versus in vitro. Consistent with this, we found that mouse Smo without a C-tail fails to induce ventral markers in chick spinal cords, whereas a control full-length Smo does (W. Zhang, R. Lu, and J. Jiang, unpubl.).

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to transduce high levels of Hh signaling activity, which can be offset by increasing the amount of full-length Smo. This implies that Myr–SmoCT may compete with full-length Smo for binding to limiting amounts of downstream signaling components [Fig. 7E,F]. Third, extensive genetic screens failed to identify Hh signaling components that may link Smo to the Cos2/Fu complex [Amanai and Jiang 2001; Vegh and Basler 2003].

Using a coimmunoprecipitation assay, we demonstrated that Smo interacts with the Cos2/Fu complex both in S2 cells and in wing imaginal discs, and the Smo C-tail appears to be both necessary and sufficient to mediate this interaction. We narrowed down the Cos2/Fu-binding domain to the C-terminal half of the Smo C-tail [between amino acids 818 and 1035]. Furthermore, we found that both the microtubule-binding domain (amino acids 1–389) and the C-terminal tail (amino acids 990–1201) of Cos2 interact with Smo. As none of these Cos2 domains binds Fu, this implies that the Cos2/Smo interaction is not mediated through Fu. Ci is also dispensable for Smo/Cos2/Fu interaction, as Smo binds Cos2/Fu in S2 cells in which Ci is not expressed [Aza-Blanc et al. 2000]. However, our results did not rule out the possibility that Smo could interact with the Cos2/Fu complex through multiple contacts. For example, Smo could simultaneously contact Cos2 and Fu. Nor did we demonstrate that binding of Cos2 to Smo is direct. Indeed, we failed to detect direct protein–protein interaction between Smo and Cos2 in yeast (data not shown). It is possible that a bridging molecule(s) is required to link Smo to the Cos2/Fu complex. Alternatively, Smo needs to be modified in vivo in order to bind Cos2. It has been shown that Hh stimulates phosphorylation of Smo [Deneff et al. 2000], hence, it is possible that phosphorylation of Smo might be essential for recruiting the Cos2/Fu complex.

Several lines of evidence suggest that Smo/Cos2/Fu interaction is important for Hh signal transduction. (1) Deletion of the Cos2-binding domain from Smo, either in the context of full-length Smo or the Smo C-tail, abolishes Smo signaling activity. (2) Overexpressing Cos2 deletion mutants that no longer bind Fu and Ci but retain

![Figure 7. Signaling by Smo and its C-tail in response to different thresholds of Hh.](Image)
a Smo-binding domain intercept Hh signal transduction. We previously provided genetic evidence that Cos2 has a positive role in transducing Hh signal in addition to its negative influence on the Hh pathway, as Ci155 is no longer stimulated into labile and hyperactivity forms by high levels of Hh in cos2 mutant cells (Wang et al. 2000). In light of our finding that Smo interacts with Cos2/Fu, the simplest interpretation for a positive role of Cos2 is that it recruits Fu to Smo and allows Fu to be activated by Smo in response to Hh.

Of note, interaction between SmoCT and Cos2/Fu per se is not sufficient for triggering Hh pathway activation. For example, Myr–SmoCTΔ625–818, which binds Cos2/Fu to the same extent as Myr–SmoCT, does not possess Hh signaling activity. The fact that Myr–SmoCTΔ730–818 and Myr–Smo730–1035 can activate the Hh pathway suggests that Smo sequence between amino acids 730 and 818 is essential. This domain may recruit factors other than Cos2/Fu to achieve Hh pathway activation. Alternatively, it might target SmoCT to an appropriate signaling environment.

Implications for threshold responses to Hh

An important property of Hh family members in development is that they can elicit distinct biological responses via different concentrations. How different thresholds of Hh signal are transduced by Smo to generate distinct transcriptional outputs is not understood. Our results suggest that Smo can function as a molecular sensor that converts quantitatively different Hh signals into qualitatively distinct outputs (Fig. 7). In the absence of Hh, the cell surface levels of Smo are low (Denef et al. 2000; Zhu et al. 2003). In addition, the Smo C-tail may adopt a “closed” conformation that prevents it from binding to Cos2/Fu (Fig. 7A). Low levels of Hh partially inhibit Ptc, leading to an increase of Smo on the cell surface. In addition, the Smo C-tail may adopt an “open” conformation, which allows Smo to bind the Cos2/Fu complex and inhibit its Ci-processing activity (Fig. 7B). Low levels of Hh signaling activity can be mimicked by overexpression of either full-length Smo or membrane-tethered forms of the Smo C-tail (Fig. 7D). High levels of Hh completely inhibit Ptc, resulting in a further increase in Smo signaling activity. Hyperactive Smo stimulates the phosphorylation and activity of bound Fu, which in turn antagonizes Su(fu) to activate Ci155 (Fig. 7C). Consistent with this, we found that Fu bound to Myc-Smo became phosphorylated in response to ectopic Hh.

The Smo sequence N terminus to SmoCT [SmoN] appears to be essential for conferring high Smo activities. It is not clear how SmoN modulates the activity of SmoCT. SmoN might recruit additional effector[s] or target SmoCT to a microdomain with a more favorable signaling environment. Alternatively, SmoN might function as a dimerization domain that facilitates interaction between two SmoCTs, as in the case of receptor tyrosine kinases (Schlessinger 2000). It is also not clear how Smo/Cos2/Fu interaction inhibits Ci processing. One possibility is that Smo/Cos2 interaction may cause disassembly of the Cos2/Ci complex, which could prevent Ci from being hyperphosphorylated, as Cos2/Ci complex formation might be essential for targeting Ci to its kinas. Consistent with this view, we found that Ci is barely detectable in the Cos2/Fu complex bound to Smo.

Physical association of the receptor complex with a downstream signaling component has also been demonstrated for the canonical Wnt pathway whereby the Wnt coreceptor LRP-5 interacts with Axin, a molecular scaffold in the Wnt pathway (Mao et al. 2001). Hence, Hh and Wnt/Wg pathways appear to use a similar mechanism to transmit signal downstream of their receptor complexes.

Materials and methods

Mutants and transgenes

Su(fu)1P and smo1 are null alleles (Preat 1992; Chen and Struhl 1998). MS1096, act > CD2 > Gal4, prd-Gal4, UAS–ptc, UAS–hh, dpp–lacZ, and ptc–lacZ have been described (Pignoni et al. 1997; Chen and Struhl 1998; Wang et al. 1999). To construct UAS–GFP-Smo, the coding sequence for GFP was amplified by PCR and inserted into the Sphi site (amino acid 35) of smo cDNA. The resulting fusion gene was subcloned into a pUAST vector between the NotI and Xhol sites. GFP-SmoCT, GFP-SmoΔ730, and GFP-SmoΔ818 were derived from GFP-Smo by introducing a stop codon at amino acids 555, 730, and 818, respectively. Myc-Smo and Myc-SmoCT contain six copies of Myc-tag inserted at amino acid 35. To construct UAS–FlagSmoCT, smo cDNA encoding the C-terminal region of Smo from amino acids 556 to 1035 was amplified by PCR and subcloned in the BgIII and Xhol sites of a pUAST-Flag vector. To tether SmoCT to the membrane, a myristoylation signal from Src [MGNKCCSKRQ] or the Sevenless transmembrane and extracellular domains (Struhl and Adachi 1998) were inserted at the N terminus of Flag-SmoCT to generate UAS–Mry–SmoCT or Sev–SmoCT. Membrane-tethered deletion mutants of SmoCT were generated by a PCR-based approach using appropriate primers. HA-Cos2N1 was derived from HA-Cos2 by introducing a stop codon after amino acid 993. HA-Cos2ΔN1, HA-Cos2N2, HA-Cos2CT1, and HA-Cos2CT2 contain two copies of HA tag fused N-terminal to amino acids 389, 642, 906, and 991, respectively. HA-Cos2CC was derived from HA-Cos2 by introducing a stop codon after amino acid 993. Flag-Cos2CT1 contains one copy of Flag tag N-terminal to amino acid 906. Transformants were generated using standard P-element-mediated transformation. Multiple independent transformant lines were generated and examined for each construct.

Cell culture, transfection, immunoprecipitation, and Western blot analysis

S2 cells were cultured in the Schneider’s Drosophila Medium (Invitrogen) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Transfection was carried out using the Calcium Phosphate Transfection Kit (Specialty Media) according to the manufacturer’s instructions. Immunoprecipitation and Western blot analyses were performed using standard protocols as previously described (Robbins et al. 1997; C.H.
Chen et al. 1996). The antibodies used were mouse αMyc, 9E10 and αHA, F7 (Santa Cruz), mouse αFlag, M2 (Sigma); mouse αCos2 and rabbit αFu (Ascano et al. 2002); and rat αCi, 2A (Motzny and Holmgren 1995).

Immunostaining of imaginal discs

Standard protocols for immunofluorescence staining of imaginal discs were used (Jiang and Struhl 1995). LMB treatment of imaginal disc was done as described (Wang et al. 2000). The antibodies used in this study were rat αCi, 2A (Motzny and Holmgren 1995), rabbit anti-βGal (Cappel), mouse αCD2 (S erotec), rabbit αGFP (Clontech), and mouse αEn and Arm (DSHB, Iowa University).

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Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail

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