Identification of a Tetrameric Hedgehog Signaling Complex*

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Hedgehog (Hh) signal transduction requires a large cytoplasmic multi-protein complex that binds microtubules in an Hh-dependent manner. Here, we show that three members of this complex, Costal2 (Cos2), Fused (Fu), and Cubitus interruptus (Ci), bind each other directly to form a trimeric complex. We demonstrate that this trimeric signaling complex exists in Drosophila lacking Suppressor of Fused (Su(fu)), an extragenic suppressor of fu, indicating that Su(fu) is not required for the formation, or apparently function, of the Hh signaling complex. However, we subsequently show that Su(fu), although not a requisite component of this complex, does form a tetrameric complex with Fu, Cos2, and Ci. This additional Su(fu)-containing Hh signaling complex does not appear to be enriched on microtubules. Additionally, we demonstrate that in response to Hh Ci accumulates in the nucleus without its various cytoplasmic binding partners, including Su(fu). We discuss a model in which Su(fu) and Cos2 each bind to Fu and Ci to exert some redundant effect on Ci such as cytoplasmic retention. This model is consistent with genetic data demonstrating that Su(fu) is not required for Hh signal transduction proper and with the elaborate genetic interactions observed among Su(fu), fu, cos2, and ci.

Hedgehog (hh) was first identified as a Drosophila melanogaster gene required during early embryogenesis (1). Hh family members were subsequently demonstrated to be involved in the patterning of a diverse array of vertebrate structures (for review see Ref. 2). Although the importance of Hh family members in development and various human pathologies continues to be uncovered, their mechanism of action remains poorly understood.

A combination of genetics and biochemistry has begun to delineate a pathway through which Hh acts. Hh binds to the multiple membrane-spanning receptor, Patched (Ptc) (3, 4), where it is thought to relieve the inhibitory influence of Ptc on the seven-transmembrane protein Smoothered (Smo) (4–8). Other intracellular signaling components genetically implicated in the Hh pathway include two Ser/Thr protein kinases, Fused (Fu) and protein kinase A (PKA) (9–14); a suppressor of fu called Su(fu) (15); a kinesin-related protein Costal2 (Cos2) (16, 17); and the transcription factor Cubitus interruptus (Ci) (10, 18–21). Cos2 associates with Fu and Ci in a large multi-protein complex (~1000 kDa) (22). This complex binds microtubules in an Hh-dependent manner, presumably through Cos2. It has been suggested that this Hh signaling complex (HSC) (1) is involved in the proteolytic processing of Ci, generating a transcriptional repressor form of Ci in the absence of Hh (23). This processing of Ci is inhibited when Hh is present, leading to an accumulation and activation of full-length Ci (24).

Su(fu) was originally described as an extragenic suppressor of fu that was able to completely rescue both embryonic and adult fu phenotypes (25). It has been previously reported, using a yeast two-hybrid approach, that Su(fu) may bind directly to Fu and Ci (26). Monnier et al. (26) suggested a model in which Su(fu) forms a bridge between Fu and Ci, which do not directly interact. Their model would require Su(fu) to be an integral part of the HSC, a role inconsistent with the observation that Su(fu) null flies have no apparent phenotype (25).

We provide here biochemical evidence for a tetrameric signaling complex consisting of Fu, Ci, Cos2, and Su(fu) that does not appear to be enriched on microtubules. Furthermore, we demonstrate that Cos2, Ci, and Fu can all associate directly with each other to form a competent trimeric Hh signaling complex, even in the absence of Su(fu). Although both of these complexes contain Ci, upon Hh stimulation Ci enriches in the nucleus whereas the other components do not.

### EXPERIMENTAL PROCEDURES

**Production of Su(fu) Antiserum—**A full-length Su(fu) cDNA was fused in frame to the cDNA for glutathione S-transferase (GST). The fusion protein was produced and purified by binding to glutathioneagarose as described previously (27). Antiserum was then produced to the purified protein by standard methods (Research Genetics, Huntsville, AL), followed by affinity purification (28).

**Cell Lysates—**cl8 and S2 cells were cultured as described previously (29, 30). Conditioned media from S2 cells expressing or not expressing Hh were incubated with cl8 cells for 16 h. The cells were washed twice with phosphate-buffered saline (PBS) at 4 °C and lysed in Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 50 mM NaF, 1 mM dithiothreitol, 1% Nonidet P-40, 1:100 protease inhibitor mixture (PI)). PI contains 1 mM benzamidine, 1 mM aprotinin, 1 mM leupeptin, and 1 mg/ml pepstatin A in 100% ethanol. The various cellular lysates were centrifuged at 5,000 × g for 10 min at 4 °C. The supernatants were analyzed by immunoblotting, following fractionation by SDS-PAGE, as described previously (22). Microtubule binding and subcellular fractionation were performed as described previously (22, 31).

**Preparation of Baculovirus—**Sf21 cells were cultured in Grace’s medium (Life Technologies, Inc.) supplemented to 10% fetal bovine serum and 1% penicillin/streptomycin. The baculoviruses were produced, and infections were carried out according to the manufacturer’s directions (Bac-to-Bac, Life Technologies, Inc.). Sf21 cells were infected at a total multiplicity of infection of 5 using wild type baculovirus to normalize growth.
the various co-infections used in this study.

**Immunoprecipitation from S21 Lysates—**S21 cells were lysed 48 h post-infection by dounce homogenization in 325 mM NaCl, 20 mM Tris, pH 7.5, 0.5 mM EDTA, 5% glycerol, 0.006% Nonidet P-40. Cellular lysates were centrifuged at 5,000 × g for 15 min, and the supernatants were subsequently centrifuged at 100,000 × g for 30 min. The resulting cellular lysates were then immunoprecipitated as described previously (22).

A baculovirus expressing V5 epitope-tagged Ci was used in the experiment shown (see Fig. 1). Antibodies to V5 were used to immunoprecipitate Ci and are referred to as sCi in Figs. 1 and 2. Similar results were observed with a baculovirus expressing Ci without a V5 epitope (data not shown). The Ci immunoblots shown here represent full-length Ci. The Cos2 antibodies used in this report will be characterized elsewhere. The anti sera to Fu, Ci, and tubulin were used as described previously (22).

**GST-Su(fu) Co-sedimentation—**S21 cells were lysed as described above. The supernatants were pre-cleared with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 30 min at 4 °C. The various pre-cleared supernatants were incubated for 1 h with 5 μg of either GST alone or GST-Su(fu). Glutathione-Sepharose 4B was then added to each tube and incubated for an additional 30 min. Bound proteins were collected by centrifugation, washed 3 times with PBS, 1% Nonidet P-40, and eluted by boiling in 2x PAGE loading buffer (0.12 M Tris, 20% glycerol, 4% SDS, 0.2% bromphenol blue).

**Preparation of Drosophila Lysates—**Imaginal discs were isolated from wild type or Su(fu)Δ13/Su(fu)Δ13 third instar larvae (22). Su(fu)Δ13 is a null allele (25). The imaginal discs from approximately 40 larvae were homogenized in 325 mM NaCl, 20 mM Tris, pH 7.5, 0.5 mM EDTA, 5% glycerol, 0.0005% Nonidet P-40. Cellular lysates were then immunoprecipitated as described previously (22).

**RESULTS**

**Formation of a Trimeric Hedgehog Signaling Complex—**To determine the direct interactions among the members of the HSC we made baculoviruses that express Fu, Cos2, or Ci. These various recombinant viruses were used to infect S21 cells on their own, in pairs, or in triplcates. 48 h post-infection the cells were lysed, and aliquots of the lysates were separated by SDS-PAGE followed by immunoblotting. The various components of the HSC were highly and specifically expressed in the appropriately infected cells (Fig. 1A). These overexpressed proteins were then immunoprecipitated with antibodies to Fu, Cos2, and Ci or with normal rabbit serum. We demonstrate here that a trimeric Fu, Ci, and Cos2 complex can form (Fig. 1B). From the appropriately double-infected lysates, Fu co-immunoprecipitated with either Ci or Cos2 (lanes 14–16 and 8–10) and Cos2 co-immunoprecipitated with Ci (lanes 11–13), compared with the appropriate controls. Additionally, all three overexpressed proteins are immunoprecipitated from the triple-infected S21 lysates with antibodies to Fu, Ci, or Cos2 but not with control IgG (lanes 17–20). Thus, Fu, Cos2, and Ci bind to each other in a trimeric complex, in what we speculate is a direct fashion (see “Discussion”).

These results, especially the association between Fu and Ci, were contradictory to a previous report suggesting that Su(fu) tethered Ci to Fu (26). To verify the existence of a trimeric Fu, Ci, and Cos2 complex that does not require Su(fu), we immunoprecipitated with Fu antibodies from cellular lysates made from wild type Drosophila or Drosophila lacking Su(fu). We were also able to specifically co-immunoprecipitate Ci and Cos2 with Fu from both lysates (Fig. 1C). These data are consistent with our baculovirus data, which provide evidence for a complex of Fu, Ci, and Cos2 that forms and is stable in the absence of Su(fu). The existence of this trimeric signaling complex, in the absence of Su(fu), is consistent with the lack of an apparent phenotype in Su(fu) null flies (25). Because Hh signaling does not appear to be interrupted by the lack of Su(fu), Su(fu) can not be a requisite member of the HSC.

**Su(fu) Is Part of a Tetrameric Signaling Complex—**Although Su(fu) is not a requisite component of the HSC, it might still associate with it. To test this possibility we made antibodies to Su(fu) to use to co-immunoprecipitate the other components of the HSC. These antibodies were tested for their specificity by immunoblotting cellular lysates made from wild type Drosophila or Drosophila lacking Su(fu). The Su(fu) antibodies detected an immunoreactive protein of the predicted size, approximately 54 kDa, which was absent in lysates from Su(fu) flies (Fig. 2A). Tubulin was immunoblotted to verify protein normalization. Thus, our Su(fu) antibodies specifically recognize Su(fu).

Cellular lysates from cl8 cells, treated with or without Hh, were immunoprecipitated with antibodies to Su(fu), Fu, or control IgG (Fig. 2B). We show here that Ci and Fu co-immunoprecipitate with Su(fu) and that this association is Hh-independent. We further demonstrate that Cos2 associates with these Su(fu)-containing complexes and that these associations are not detected in immunoprecipitations performed with an equivalent amount of control IgG (compare lanes 1 and 4 to 2 and 5). Thus, Su(fu) can exist complexed with all the currently identified members of the HSC, and the bulk of these associations are independent of Hh.

To determine which components of the HSC associate directly with Su(fu) we added GST-Su(fu) or GST to lysates of baculovirus-infected S21 cell as indicated in Fig. 2C. These proteins were then collected by sedimentation with glutathione-agarose beads, fractionated by SDS-PAGE, and immunoblotted. Cos2 did not co-sediment with GST-Su(fu) significantly more than with GST alone, suggesting that Su(fu) does not
bind Cos2 directly. However, when Su(fu) was incubated with lysates from cells co-infected with Cos2 and either Ci or Fu, the amount of Cos2 that co-sedimented was increased. Thus, Fu and Ci appear to bind directly to Su(fu) (lanes 9 and 12), whereas Cos2 binds to Su(fu) through either Fu or Ci (lanes 15 and 18). Finally, we demonstrate that all three known components of the HSC co-sediment with GST-Su(fu) from lysates of Sf21 cells infected with Fu, Ci, and Cos2. Thus, we are able to recreate the tetrameric signaling complex seen in vivo, by mixing recombinant Su(fu) with lysate from Fu, Cos2, and Ci triple-infected cells.

Identification of a Microtubule-independent Tetrameric Signaling Complex—To determine whether Su(fu) is part of the previously described microtubule-bound HSC (17, 22) we used Drosophila embryo extracts to perform a microtubule co-sedimentation assay. As described previously, Fu, Cos2, and Ci enrich on microtubules, compared with a control assay performed in the absence of taxol-stabilized microtubules (Fig. 3). However, Su(fu) did not appear to co-sediment on microtubules with the other members of the HSC. Thus, although Su(fu) is part of a tetrameric signaling complex, this Su(fu)-containing complex does not appear to be enriched on microtubules. This suggests that there are at least two HSCs, one containing Su(fu) and one not, which may be in equilibrium with each other.

Ci Translocates to the Nucleus Independently of the HSC—To test the possibility that the non-microtubule bound HSC containing Su(fu) might translocate with Ci to the nucleus we treated cl8 cells with either Hh or the nuclear export inhibitor leptomysin B (LMB). As previously reported (31), both Hh and LMB were able to induce nuclear accumulation of Ci (Fig. 4). However, neither agent had any effect on the nuclear accumulation of the other components of the HSC, including Su(fu).

DISCUSSION

Although new components of the Hh signaling pathway continue to emerge, the biochemical mechanisms underlying Hh signal transduction remain poorly understood. We have previously demonstrated that several components of the Hh signaling pathway form a large cytoplasmic complex that binds microtubules in an Hh-dependent manner (17, 22). We show here that Fu, Cos2, and Ci bind directly to each other to form a stable trimeric signaling complex, which exists and functions in Drosophila that lack Su(fu). Additionally, we provide evidence for a second HSC that contains Su(fu) and does not appear to be enriched on microtubules. Su(fu) binds to this second HSC to form a cytoplasmic tetrameric complex with Fu, Ci, and Cos2.

In response to Hh, we show that full-length Ci leaves these cytoplasmic complexes and translocates to the nucleus, where it acts as a positive regulator of the Hh pathway.

A Trimeric Signaling Complex—There appear to be multiple associations among the various components of the trimeric HSC. These multiple associations may account for the stability of this complex on microtubules (17, 22), as well as provide an explanation for the elaborate genetic interactions observed among the various genes that encode this signaling complex (10, 25, 33, 34). We propose that the interactions between Fu, Ci, and Cos2 in this trimeric signaling complex are direct and do not require participation of any endogenous Sf21 proteins, for the following reasons: 1) Fu, Ci, and Cos2 are highly over-expressed by their respective baculoviruses and should therefore be present in large stoichiometric excess over any HSC component endogenous to Sf21 cells; 2) baculovirus-expressed Cos2 does not bind to GST-Su(fu), consistent with the lack of endogenous Sf21 Fu or Sf21 Ci (see Fig. 2C); and finally 3) Fu isolated from baculovirus-infected Sf21 cells migrates through a gel filtration column with a molecular size inconsistent with the presence of additional members of the HSC.

A Tetrameric Signaling Complex—If a trimeric HSC is sufficient to elaborate Hh signaling, then what role might Su(fu)
display in a tetrameric complex? We speculated that Su(fu) might interact with the HSC because of the genetic interactions among Su(fu), fu, ci, and cos2 (33, 35). For example, alleles of fu that encode mutations in their protein kinase domain display no phenotype in an Su(fu) homozygous background. However, mutations that delete the carboxyl terminus of Fu result in a cos2 phenotype in an Su(fu) background. Additionally, cos2 heterozygotes have a more severe cos2 phenotype in an Su(fu) background than in a wild type background. We have now biochemically demonstrated that Su(fu) interacts with an Fu, Ci, and Cos2 complex to form a tetrameric signaling complex (see Fig. 2). It appears that Su(fu) binds directly to Fu and Ci, but not to Cos2, and that the bulk of these interactions are stable in the presence and absence of Hh. However, this Su(fu)-containing complex does not appear to be enriched on microtubules. Thus, this tetrameric complex may represent a stable intermediate in the progression of Ci from the cytoskeleton to the nucleus. The equilibrium between these various complexes may change in response to Hh, as may occur when Ci sheds the other members of the HSC prior to nuclear translocation (see below).

A number of vertebrate homologs of Su(fu) have recently been identified (32, 36–38) that can act as negative regulators of the mammalian Ci homolog Gli1. These groups suggest that Su(fu) sequesters Gli1 in the cytoplasm, thereby limiting its ability to act as a transcription factor. It has also been proposed that Cos2 may act by sequestering Ci in the cytoplasm (17, 22, 57). These groups suggest that Cos2 may act by sequestering Ci in the cytoplasm (17, 22, 57). This might explain why no change in Ci localization has been detected in either cos2 or Su(fu) mutants, because both gene products would have to be deficient to observe a change in localization of Ci (17).