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Hedgehog Elicits Signal Transduction by Means of a Large Complex Containing the Kinesin-Related Protein Costal2

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Summary

The *hedgehog* gene of Drosophila melanogaster encodes a secreted protein (HH) that plays a vital role in cell fate and patterning. Here we describe a protein complex that mediates signal transduction from HH. The complex includes the products of at least three genes: *fused* (a protein-serine/threonine kinase), *cubitus interruptus* (a transcription factor), and *costal2* (a kinesin-like protein). The complex binds with great affinity to microtubules in the absence of HH, but binding is reversed by HH. Mutations in the extracatalytic domain of FU abolish both the biological function of the protein and its association with COS2. We conclude that the complex may facilitate signaling from HH by governing access of the *cubitus interruptus* protein to the nucleus.

Introduction

The *hedgehog* gene of Drosophila melanogaster (*hh*) encodes a secreted protein (HH) that plays a vital role in determining cell fate and patterning during the course of development (Mohler, 1988; Hidalgo, 1991; Lee et al., 1992; Tabata et al., 1992; Ingham, 1993, 1995; Heemskerk and DiNardo, 1994; Tabata and Kornberg, 1994; Perrimon, 1995). The vertebrate counterpart *sonic hedgehog* has similar biological roles (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Chang et al., 1994; Roelink et al., 1994; Johnson and Tabin, 1995a; Chiang et al., 1996). HH acts by binding to a cell surface receptor (Marigo et al., 1996a; Stone et al., 1996). The binding, in turn, activates transcription from genes such as *wingless* (*wg*) and *decapentaplegic* (*dpp*), whose products are themselves polypeptide signaling factors that implement the developmental effects of HH (Heberlein et al., 1993; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; Zecca et al., 1995).

The biochemical pathway that mediates induction of transcription by HH has not been elucidated. Recent work has identified one or more candidate receptors for HH, encoded by genes known as *smoothened* (*smo*) and *patched* (*ptc*) (Hooper and Scott, 1989; Nakano et al., 1989; Ingham, 1991; Alcedo et al., 1996; Marigo et al., 1996a; Stone et al., 1996; van den Heuvel and Ingham, 1996). The means by which a signal is transmitted from the putative Ptc/Smo complex to the nucleus is not known. The products of five genes have been implicated in Drosophila: (1) *fused* (*fu*), specifying a putative protein-serine/threonine kinase (FU) for which no substrates have yet been found (Préat et al., 1990; Thérond et al., 1993); (2) *Suppressor of fused* [*Su*(*fu*)], whose product SU(FU) is a pioneer protein without a known function (Pham et al., 1995); (3) *costal2* (*cos2*), newly identified as encoding a distant relative of the kinesin motor proteins (COS2) (Sisson et al., 1997 [this issue of *Cell*]); (4) *cubitus interruptus* (*ci*), whose product CI is a zinc finger protein (Eaton and Kornberg, 1990; Orenic et al., 1990) that apparently activates transcription from *wg*, *ptc*, and *dpp* (Alexandre et al., 1996; Dominguez et al., 1996); the vertebrate counterpart of *ci* is the proto-oncogene *GLI*, also thought to be a transcription factor (Kinzler et al., 1988; Pavletich and Pabo, 1993; Marigo et al., 1996b); and (5) *pka*, which encodes protein kinase A (PKA) (Kalderon and Rubin, 1988).

We have shown previously that signaling from HH leads to the phosphorylation of FU in both Drosophila embryos and cultures of S2 cells (Thérond et al., 1996a). This finding led us to seek other proteins that might interact with FU. The search uncovered a large protein complex that apparently mediates signaling from HH. We first encountered these complexes by purification of a 175 kDa kinesin-like protein that coprecipitates with the FU protein. On further exploration, the 175 kDa protein proved to be the product of *cos2*. The complexes also contain CI and, in the absence of HH signaling, are bound to microtubules in an ATP-sensitive manner. Stimulation of cells by HH releases the complexes from microtubules and induces the phosphorylation of both the FU and COS2 components of the complexes. Mutations in the carboxy-terminal domain of FU that interrupt signaling from HH also disrupt the association with COS2.

These findings provide the first identification of a kinesin-like protein involved in cell signaling. Our results further suggest that both formation of the complexes and their release from microtubules are integral events in signaling from HH. The former would serve to sequester CI in the absence of signaling, the latter to facilitate transfer of CI to the nucleus in response to HH.

[§]These authors contributed equally to this work.
[‖]J. C. S. provided the unpublished nucleotide sequence and antiserum required to identify the kinesin-like protein as the product of *cos2*. He did not perform the experiments reported in this manuscript. His own experiments are described in the accompanying manuscript (Sisson et al., 1997 [this issue of *Cell*]).
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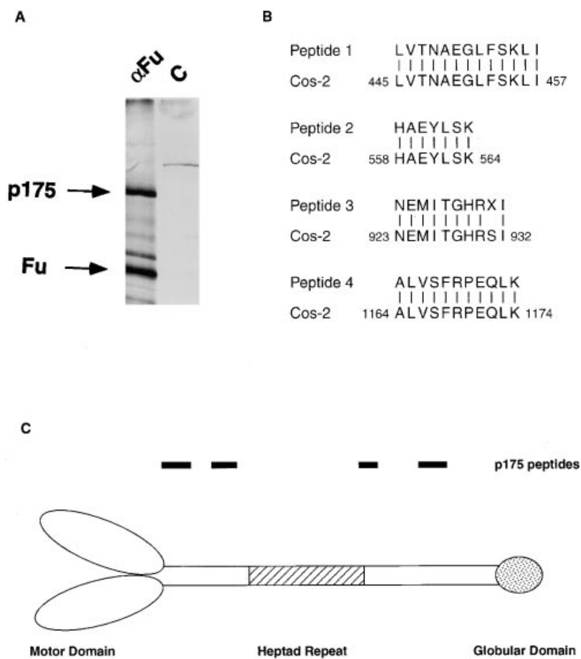

Figure 1. Coprecipitation of FU and p175

(A) Extract from S2 cells. Cells were lysed with NP-40 and immunoprecipitates prepared with either an antiserum against FU or GST protein (C), then fractionated by electrophoresis through polyacrylamide gels. The polyacrylamide gel was stained with silver. The 175 kDa protein MBP-β-galactosidase served as a marker.

(B) Amino acid sequence of peptides from p175. Six tryptic peptides obtained from a similar preparation of p175 as illustrated in Figure 1A were sequenced as described in Experimental Procedures. Four of the six peptides had amino acid sequences found also in COS2, as illustrated. The other two peptides remain unidentified. Peptide 4 had homology to kinesin-like proteins.

(C) Topography of COS2. The diagram illustrates the major structural domains within COS2 and the location of the four peptides described in panel (B). Kinesin-related proteins are usually dimers, and this bias is reflected in the diagram.

Results

The Product of *cos2* Is Associated with FU

It appears from previous work that FU is part of the signaling pathway downstream of the receptor(s) for HH (Forbes et al., 1993; Ingham, 1993; Préat et al., 1993) and that at least a portion of that pathway is represented in S2 cells (Thérond et al., 1996a). We therefore examined S2 cells and embryos for proteins that could be coimmunoprecipitated with FU. A major candidate emerged with a molecular mass of 175 kDa (p175) (Figure 1A). The yields of FU and p175 from S2 cells were superior to those from embryos, so we used the cells as a source to isolate p175 in sufficient amounts to permit microsequencing.

Amino acid sequence was obtained for six peptides from p175. Comparison of the peptides to known protein sequences revealed a motif reminiscent of kinesin-like proteins (Figure 1B), an unprecedented finding for a component of a signaling pathway. Cognizant of unpublished evidence that *cos2* encodes a kinesin-like protein (see accompanying manuscript, Sisson et al, 1997), we examined the sequence of a cDNA for *cos2* and found that four of the six peptides contained a recognizable portion of the COS2 protein (Figures 1B and 1C). Two of the peptides isolated from the preparation of p175 did not match any sequence found in either COS2 or other known proteins. We cannot presently account for these peptides.

We used immunoprecipitation to further authenticate the identity of p175 (Figure 2A). Extracts of S2 cells and S2 cells producing ectopic HH (HH-S2) were precipitated with antiserum against either FU or COS2. The precipitates were then analyzed by fractionation in polyacrylamide gels and by Western blotting. The results with S2 cells showed that the FU antiserum also precipitated a 175 kDa protein that reacted with COS2 antiserum. Conversely, antiserum against COS2 precipitated both FU and p175. We concluded that p175 is indeed COS2, associated either directly or indirectly with FU in S2 cells (see Discussion).

Coprecipitation of FU and COS2 was also observed with extracts from HH-S2 (Figure 2A). We have shown previously that these cells contain both FU and a hyperphosphorylated isoform designated FU-P, which is induced by an autocrine or paracrine stimulus from HH (Thérond et al., 1996a). Both FU and FU-P were apparent in the immunoprecipitates prepared with HH-S2 cells (Figure 2A). Moreover, FU-P predominated over FU when the precipitates were prepared with COS2 antisera, as if FUP might be preferentially associated with COS2 (Figure 2A, and see below). Thus, the apparent complex between FU and COS2 forms in the absence of signaling from HH, but is modified by phosphorylation of the FU component in response to HH (which also elicits phosphorylation of COS2, see below).

FU and COS2 Associate with One Another and with the *cubitus interruptus* Protein in Drosophila Embryos

Although a convenient source of FU and COS2, S2 cells apparently do not contain the complete signaling pathway commanded by HH. They do not produce CI, and they fail to activate the *wg* and *ptc* genes in response to stimulation with HH (D. Casso and T. Kornberg, personal communication). We therefore sought to identify a complex between FU and COS2 in extracts of Drosophila embryos, prepared at a time when HH is active (Ingham, 1993a; Heemskerk and DiNardo, 1994). As before, antiserum against either FU or COS2 precipitated both proteins in roughly equal amounts (Figure 2B). The activity of HH was manifested by the presence of FUP as well as FU.

Knowing that *ci* has also been implicated in signal transduction from HH, we examined the immunoprecipitates for the presence of the CI protein. We found that antiserum against either FU or COS2 precipitated CI as well (Figure 2B). Similar results were obtained with extracts of imaginal discs (data not shown). We conclude that both FU and COS2 are bound to CI in the embryo extracts, although it was not yet apparent whether all three proteins are in a single heteromeric complex.

Multiprotein Complexes Involving FU and COS2 in S2 cells

In an effort to further evaluate protein complexes in the HH signaling pathway, we used fractionation by gel filtration through FPLC columns. We began this analysis with S2 cells because they provide a system in which signaling downstream of HH can be induced at will (Thérond et al., 1996a). Fractionation of extracts from either S2 or HH-S2 cells revealed three populations of FU (Figure 3A). Two of these represented molecular weights much larger than that of FU itself (population A, ca. 40 million Da; and population B, greater than 700,000 Da); the third, population C, had a nominal mass of ca. 200,000 Da. Both populations A and C eluted in regions of limited resolution, so the assigned molecular masses should be viewed as approximations. The relative amount of the three populations varied from one preparation to another: population B was typically most abundant and population A varied the greatest. Since population B was well resolved and was by far the most abundant form, we focused our attention on it. Both FU and FUP were apparent in population B from HH-S2 cells, reflecting the stimulus provided by HH (Thérond et al., 1996a).

Further analysis of the column fractions revealed that COS2 was coeluting with FU in population B (Figure 3B, and data not shown). The coelution was observed with extracts from both S2 and HH-S2 cells. In both instances, the amounts of COS2 and FU appeared to be roughly equivalent, based on results with immunoprecipitation, radioactive labeling and stained gels (data not shown). These results further authenticate the existence of complexes between FU and COS2 in S2 and HH-S2 cells.

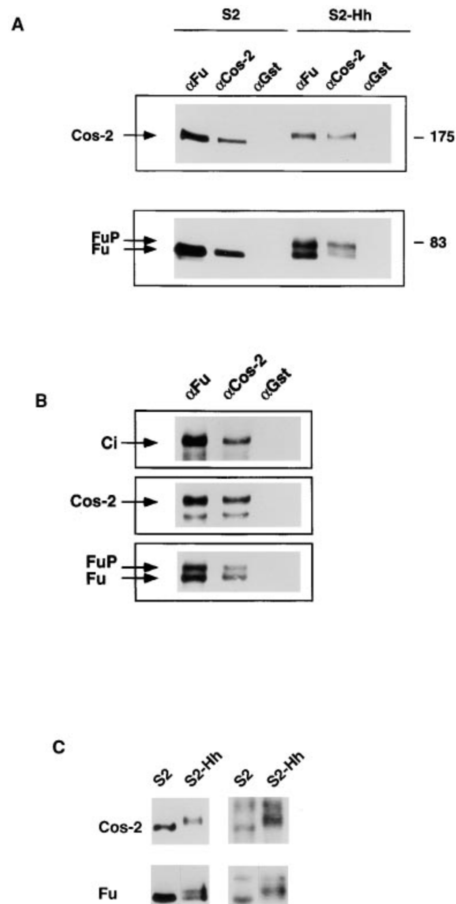

Figure 2. Coprecipitation of FU, COS2, and CI

Extracts of either cultured cells or Drosophila embryos were immunoprecipitated. The precipitates were then examined for the presence of specific proteins by Western blotting.

(A) Coprecipitation of FU and COS2 from extracts of S2 and HH-S2 cells. Extracts were prepared with NP-40 and immunoprecipitated with different antisera, as indicated for individual lanes (Gst, control with antiserum against GST protein). The precipitates were then immunoblotted with antisera against either COS2 (upper) or FU (lower).

(B) Coprecipitation of CI with FU and Cos from embryo extracts. Extracts of Drosophila embryos were prepared as described in Experimental Procedures. Immunoprecipitates were performed as described for panel (A). Western blots were analyzed with antisera against CI (upper), COS2 (middle), and FU (lower).

(C) Coprecipitation of ^{32}P-labeled FU and COS2. S2 and HH-S2 cells were labeled with [^{32}P]orthophosphate as described in Experimental Procedures. Extracts were prepared with NP-40 and immunoprecipitated with FU antiserum. The two lanes on the left portray the results of Western blotting that located the FU and COS2 proteins; the two lanes on the right illustrate labeling with ^{32}P. The additional ^{32}P-labeled band in the COS2 lanes has not been identified, but it has not been apparent in samples analyzed by either Western blotting or labeling with [^{35}S]methionine.

Multiprotein Complexes Involving FU, COS2, and CI in Drosophila Embryos

We next used gel filtration to characterize the complexes involving FU, COS2, and CI in Drosophila embryos, detected previously by immunoprecipitation (see above, Figure 2B). Fractionation of embryo extracts gave a pattern of FU and COS2 reminiscent of but not identical to that obtained with S2 cells (Figure 4A). In particular, FU was spread throughout the range greater than 700,000 Da and was poorly resolved. The broad distribution of FU was probably not an artifact, since the tetrameric kinesin protein known as KRP_{130} (Cole et al., 1994) eluted sharply (Figure 4A). FUP was better resolved, in the range where population B was found with S2 cells, and COS2 appeared to coelute with FUP. Elution of CI overlapped but was not fully coincident with that of FUP and COS2.

Given the relative complexity of these patterns, we used coprecipitation with FU to identify complexes involving FU, COS2, and CI in the eluates. The results revealed a coincident peak of FU, FUP, and COS2 in the region of population B (fractions 32–34 in Figure 4B). The coprecipitation of CI also overlapped this region, but extended in a diffuse manner into a range of higher mass (fractions 23–33), as well. The heterogeneity of elution was most likely due to gradual dissociation of proteins from the complexes during the course of analysis, but it is also possible that the embryos contain a multiplicity of complexes with different compositions. We conclude that Drosophila embryos contain multiprotein complexes involving FU, FUP, COS2, and CI. Among these is a complex that may include all three proteins,

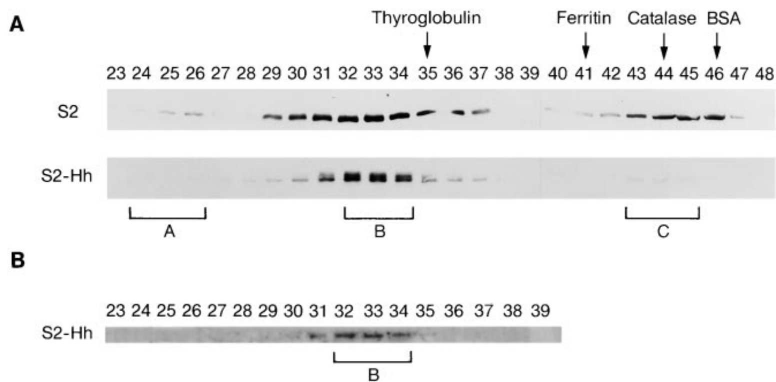

Figure 3. Multiprotein Complexes Involving FU and COS2 in S2 Cells

(A) Identification of large protein complexes containing FU. Extracts of S2 and HH-S2 cells were prepared and fractionated by gel filtration in FPLC columns, as described in Experimental Procedures. The protein in each fraction was then analyzed by electrophoresis through polyacrylamide gels and Western blotting with an antiserum against FU. FU immunoreactivity is present in three different sized peaks (peaks A, B, and C). In the HH-S2 elution profile, FUP is also enriched in peak B. The calculated exclusion volume for the columns was at fraction 22 and is reported to be 4×10^7 daltons for globular proteins. The peaks of elution for various marker proteins are designated in the figure. The results obtained with extracts from S2 and HH-S2 cells are aligned vertically.

(B) Identification of large protein complexes involving COS2. Western blots from (A) were reprobed with COS2 antisera. Only the blot of HH-S2 extracts is shown but comparable results were seen from the S2 extracts. No COS2 was detected in fractions higher than 39.

but each of the complexes may contain additional, presently unidentified proteins.

Binding of FU, COS2, and CI to Microtubules

Knowing that the complexes contain a kinesin-like protein, we asked whether they might bind to microtubules. Extracts of Drosophila embryos were prepared under conditions that depolymerize microtubules. The monomeric tubulin was then repolymerized and the polymers stabilized by taxol. The repolymerized microtubules were washed and collected by centrifugation. FU, COS2, and CI were enriched in the microtubule fraction (Figure 5). The binding of the three proteins to microtubules resembled that described previously for kinesins and related proteins (Goldstein, 1993): it was extremely strong, resisting disruption by 0.5M KCl; it required polymerization and stabilization of microtubules; and it was impeded by high concentrations of ATP (see Figure 5A). (ATP failed to release complexes already bound to microtubules, in accord with the experience of Sisson et al. [1997].) As the simplest case, we assume that the binding of all three proteins to microtubules was mediated by COS2. If so, these results sustain the view that the bulk of FU, COS2, and CI are indeed bound into a single complex (see Discussion).

Regulated binding of the complexes to the microtubules could be an integral event in signaling from HH, serving to sequester CI in the absence of signal and allowing it access to the nucleus in the presence of signal. Thus, we asked whether signaling from HH might release the complexes from microtubules. The experiments were performed with S2 cells, because these provide a setting in which the effect of HH can presently be examined in a comparative manner. We found that in S2 cells, COS2 and FU coprecipitated with microtubules (Figure 5B). In contrast, binding of COS2 and FU to microtubules was barely detectable in HH-S2 cells. These findings sustain the hypothesis that signaling from HH releases the complexes from microtubules, which would in turn facilitate translocation of CI to the nucleus.

Phosphorylation of COS2 in the Complex with FU

We have shown previously that stimulation of S2 cells with HH elicits phosphorylation of FU to give FUP (Thérond et al., 1996a). It occurred to us that COS2 might also be phosphorylated under these circumstances, particularly since it is associated with FU. The first indication of COS2 phosphorylation came from immunoprecipitations: all of the COS2 that coprecipitated with FU from HH-S2 cells was in an isoform whose electrophoretic mobility was slower than that of the COS2 retrieved with FU from S2 cells (Figure 2C). As before, FUP also appeared only in samples from HH-S2 cells (Figure 2C). Treatment with phosphatase eliminated the more slowly migrating forms of both FU and COS2 (Thérond et al., 1996a and data not shown).

Labeling with [^{32}P]orthophosphate revealed that FU and COS2 were phosphorylated in both S2 and HH-S2 cells (Figure 2C), but the electrophoretic mobilities again revealed an additional phosphorylated isoform of FUP and COS2 in HH-S2 cells. The ostensibly hyperphosphorylated isoforms were also more intensely labeled with ^{32}P (Figure 2C). In all the circumstances described here, the phosphorylations of FU/FUP and COS2 were on serine (data not shown).

It is notable that FUP coeluted with FU in gel filtration of extracts from both HH-S2 cells and Drosophila embryos (Figures 3A, 4A, and 4B), in accord with the assumption that the two isoforms are in the same macromolecular complex. By all indications, COS2 is also in the same complex, irrespective of whether it is hyperphosphorylated or not (see Figures 2 and 4). We conclude that a multiprotein complex involving FU, COS2, and CI is likely to be the site of phosphorylations that occur in response to stimulation by HH.

The Carboxy-Terminal Domain of FU Is Required for Formation of Complexes with COS2

In an effort to explore the functional significance of the interaction between FU and COS2, we used previously characterized mutants of *fu* to locate the binding site for COS2 within the FU protein (Figure 6A) (Préat et al., 1993; Thérond et al., 1996b). The mutants were of two

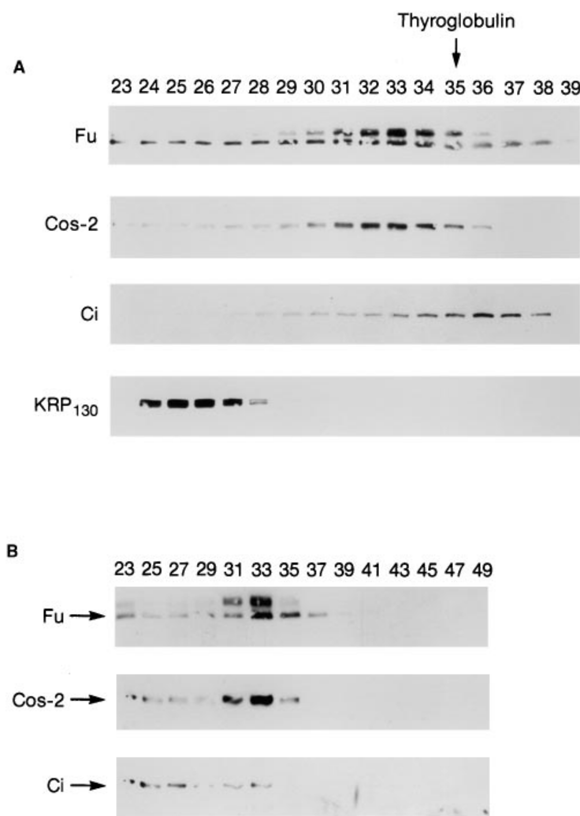

Figure 4. Multiprotein Complexes Involving FU, COS2, and CI in Drosophila Embryos

(A) Fractionation by gel filtration. Extracts of Drosophila embryos were prepared as described in Experimental Procedures and analyzed as in Figure 4. Proteins in the fractions were analyzed by electrophoresis and Western blotting, using antisera against FU, COS2, CI, and KRP_{130} (a tetrameric kinesin protein). The results of the various Western blots, performed on fractions from the same column, are aligned vertically. No immunoreactivity was seen in fractions 40–50, which are therefore not shown.

(B) Interactions between FU, COS2, and CI. Odd-numbered fractions from a column similar to that in panel (A) were immunoprecipiated with antiserum against FU, while even-numbered fractions were precipitated with a similar amount of rabbit IgG. The immunoprecipitates were then analyzed by electrophoresis and Western blotting with antiserum against FU, COS2, or CI. The results are aligned vertically. The results for the even-numbered fractions, which showed no immunoreactivity, are not shown.

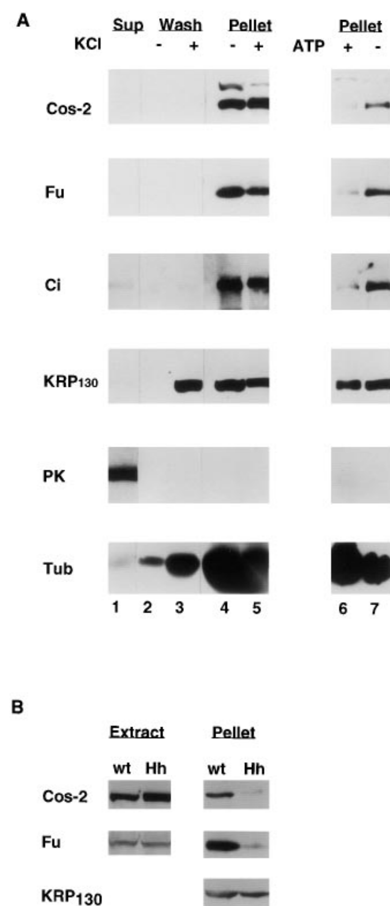

Figure 5. Binding of FU, COS2, and CI to Microtubules

Extracts of Drosophila embryos or cultured cells were treated in a manner to repolymerize and stabilize microtubules, as described in Experimental Procedures. The microtubules were collected by centrifugation and the fractions examined by electrophoresis and Western blotting.

(A) Extracts of embryos. The supernatant remaining after centrifugation of microtubules was collected and saved (lane 1). The microtubule pellets were then washed with polymerization buffer (lanes 2, 4, 6, and 7) or 0.5 M KCl (lanes 3 and 5), followed by another centrifugation. The supernatants (lanes 2 and 3) or the microtubule pellets (lanes 4 and 5) were resuspended in a similar volume of buffer. One sample was polymerized in the absence of Apyrase and AMP-PNP and was supplemented with 5 mM ATP (lane 6). This microtubule pellet and a control pellet (lane 7) polymerized in the absence of ATP were both from an additional experiment. These various samples were then analyzed by electrophoresis and Western blotting, using antisera against FU, COS2, CI, the kinesin protein KRP_{130}, pyruvate kinase, and α-tubulin.

(B) Cultured cells. Extracts and fractionations were performed as described for (A), using either S2 (wt) or HH-S2 (HH) cells. The fractionations were initiated with equivalent volumes of cellular extracts containing similar amounts of total protein. Analysis of KRP_{130} served to validate this normalization.

sorts: Class I, various point mutations within the kinase domain of FU; and Class II, a series of missense mutations that remove portions of the carboxy-terminal extracatalytic domain. The phenotypic effects of the mutations made it necessary to analyse imaginal discs from third instar males that were hemizygous for the mutations (see Experimental Procedures).

We found that COS2 coprecipitated in the usual manner with FU proteins encoded by Class I mutants (Figure 6B). The nature of the mutations is such that the kinase activity of FU is likely to be inactivated (Préat et al., 1993; Thérond et al., 1996b). Thus, functional FU kinase is probably not necessary for FU and COS2 to associate. In contrast, we found no evidence for binding of COS2 to the products of Class II mutants. In one instance (mutant W3), this observation was trivial because no FU was recovered from the extracts, perhaps because the extensively truncated protein was unstable. But the products of two other mutant alleles were recovered in either limited or abundant quantities (RX16 and

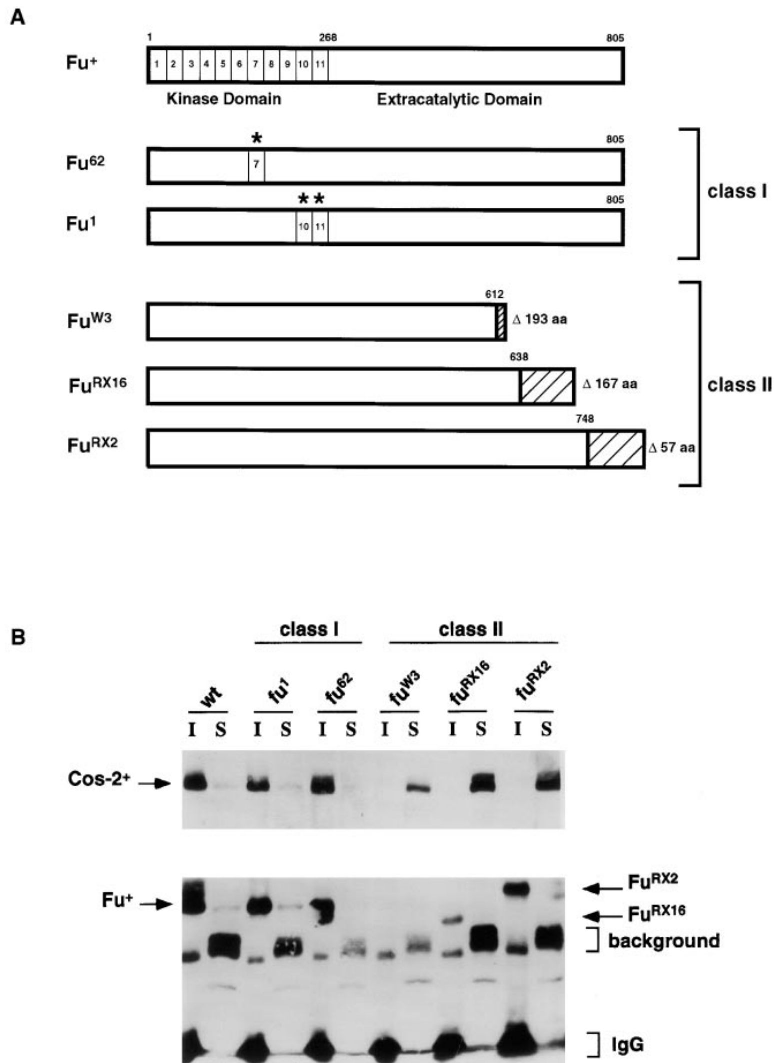

Figure 6. COS2 Binds to the Carboxyterminal Domain of FU

(A) Mutant alleles of FU. The figure illustrates the topography of wild-type FU and five mutant alleles described previously (Thérond et al., 1996b). The catalytic domain of FU can be divided into 11 subdomains found in many kinases (Hanks et al., 1988). Mutations within one or another of these domains have been designated as Class I (Préat et al., 1993); the mutations used here are located as indicated by asterisks. In contrast, Class II mutants involve a series of deletions that create frameshifts as denoted by the cross-hatching and truncate the extracatalytic domain of FU (Thérond et al., 1996b).

(B) Interaction between COS2 and mutant versions of FU. Extracts were prepared from the imaginal discs of third instar larvae, either wild-type or males that were hemizygous for the mutations described in (A). The extracts were analyzed by immunoprecipitation with antiserum against FU, followed by electrophoresis and Western blotting with antiserum against either FU or COS2. Results are illustrated for both the immunoprecipitates (I) and their supernatants (S). An unidentified band ("background") appeared in all of the immunoprecipitates.

RX2, respectively). No COS2 was coprecipitated in either instance; instead, the protein remained in the supernatants of the immunoprecipitates (Figure 6B). Thus, the association with COS2 probably involves the carboxyl terminus of FU (although we do not yet know whether that association is direct or mediated by yet another protein). We conclude that certain mutations in *fu* that interrupt signaling from HH also disrupt formation of the complexes involving FU and COS2, in accord with the view that the complexes themselves play a role in the signaling (see Discussion).

Discussion

The FU, COS2, and CI Proteins Are Associated in Multiprotein Complexes

We have found that at least three of the components in the HH signaling pathway (FU, COS2, and CI) interact with one another in multiprotein complexes (Figure 7). Several independent observations indicate that these associations are not spurious. These include: previous genetic data consistent with interactions among the three proteins; coprecipitation of FU and COS2 by two distinctive antisera against FU (see Results and data not shown), as well as an antiserum against COS2; coelution of the three proteins from gel filtration columns in a coprecipitating form; stability to high salt but not to denaturation by SDS (data not shown); and high affinity binding of all three proteins to microtubules, in a manner that suggests mediation by COS2 (see below). These same points can be taken as indicating that all three proteins are joined in a single complex, although we have yet to demonstrate this decisively.

The population of complexes obtained from embryonic tissue was more heterogeneous than that from S2 cells, presumably because additional components of the signaling pathway are present. In addition, some of the heterogeneity may arise from the stepwise dissociation of participants once they have been released from cells. As defined by coimmunoprecipitation, there is a distinctive complex that contains FU, COS2, and CI (Figure 7). But the heterodisperse behavior of both FU and CI in the fractionation suggests the existence of additional complexes devoid of COS2. It seems likely that there are presently unidentified proteins in one or more of these complexes. One candidate would be SU(FU),

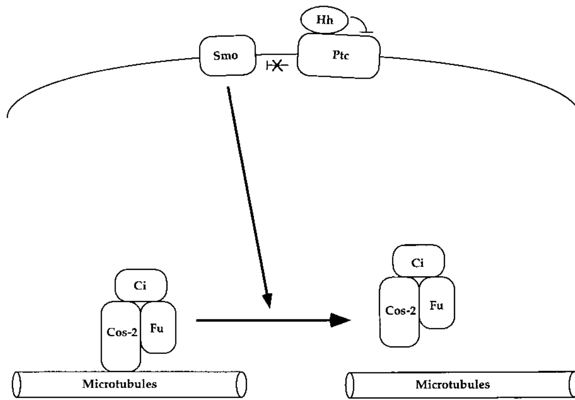

Figure 7. A Protein Machine in the HH Signaling Pathway

Signaling from HH apparently begins when the ligand binds to the receptor Ptc. The binding relieves repression of Smo by Ptc, which in turn initiates signaling downstream of Smo, utilizing the protein complex described in the text. The cartoon is predicated on the hypothesis that the complex among CI, COS2, and FU provides a cytoplasmic tether by which the function of CI is controlled. Signaling from HH leads to release of the complexes, making CI available for translocation to the nucleus. The rendition of how individual proteins contact one another is arbitrary, other than for the likelihood that COS2 binds directly to FU and the reasonable assumption that COS2 binds the entire complex to microtubules (see text).

since mutations in *Su*(*fu*) and *cos2* have similar genetic interactions with *fu* (Préat et al., 1993).

Binding to Microtubules

The structural resemblance of COS2 to kinesins is mirrored in the ability of the complexes to bind microtubules. The binding is exceedingly stable, removing the bulk of COS2 and FU from embryo extracts (see Figure 5). The small fraction of CI that does not bind microtubules may represent complexes devoid of COS2 (see Figure 7).

We doubt that the binding to microtubules is spurious. First, the affinity is exceedingly high, greater even than that of another kinesin-related protein (KRP_{130}). Second, soluble proteins such as pyruvate kinase are not trapped among the bound proteins. Third, the binding can be impeded by high concentrations of ATP, as anticipated if the binding were mediated by COS2 (displacement of ADP by ATP greatly reduces the affinity of kinesins for microtubules; see Vale, 1996). ATP reduced the binding of COS2 complexes to microtubules if included at the time that tubulin was first polymerized (see above), but failed to displace the complexes once the binding had occurred (D. J. R., unpublished data). We attribute this difference to the high effective concentration of microtubules following polymerization, but the matter deserves further exploration. And fourth, there is some intracellular colocalization between tubulin and COS2 (Sisson et al., 1997). It is important to note, however, that binding by means of COS2 does not assure movement of the complexes along microtubules. Indeed, there is reason to doubt that such movement will occur: the amino acid sequence of the putative "motor domain" of COS2 is substantially diverged from that of true kinesins (Sisson et al., 1997).

Despite the prominence of their binding to microtubules, the complexes are stable in the absence of such binding: they were readily detectable by both immunoprecipitation and gel filtration, utilizing extracts prepared under conditions that depolymerize microtubules (see Experimental Procedures); and they remain intact following release from microtubules in vivo, in response to HH.

Phosphorylation of Proteins in the Complexes

Stimulation of cells with HH leads to additional serine phosphorylation of both FU and COS2. The protein kinase(s) responsible for these phosphorylations have not been identified. The HH-induced phosphorylation of FU to FUP appears only 30 minutes following induction (Thérond et al., 1996a), suggesting that it represents a feedback device rather than an event in initial signal transduction. This leads in turn to the possibility that FU is not phosphorylating itself, even though the phosphorylation can be abolished by mutations in the catalytic domain of FU (see Figure 6B). To date, our efforts to explore this issue with kinase reactions in vitro have been unsuccessful. Similarly, FU is apparently not directly responsible for the phosphorylation of COS2, which occurs even when inactivating mutations are present in the kinase domain of FU (see Figure 6B). Phosphorylation of COS2 following exposure of S2 cells to soluble HH is also delayed by 15–30 minutes (data not shown), so the functional significance of this phosphorylation may be similar to that of FU.

Functional Significance of the Complexes Involving HH Signaling Proteins

The end result of signaling through the HH pathway is activation of transcription from several genes, presumably mediated at least in part by CI (Orenic et al., 1990; Alexandre et al., 1996; Dominguez et al., 1996). Inclusion of CI in the complexes with COS2 and FU may be the means by which the function of this transcription factor is governed. The binding of the complexes to microtubules suggests several possible mechanisms.

First, COS2 might serve to transport CI into the nucleus in response to signaling. This seems unlikely, given the genetic definition of COS2 as an inhibitor of HH signaling (Forbes et al., 1993; Capdevila and Guerrero, 1994). In addition, there is no evidence as yet that COS2 itself can move along microtubules.

Second, inclusion of CI in the complex might represent a means by which to control its stability. This possibility gains credence from the observation that the quantity of CI rises sharply in response to HH, or in the absence of negative regulators such as Pka and Ptc (Johnson et al., 1995b; Dominguez et al., 1996).

Third, the binding to microtubules might serve to retain CI in the cytoplasm until signaling releases it for translocation to the nucleus (Figure 7). We prefer this explanation because of our demonstration that stimulation of S2 cells with HH apparently causes release of the complexes from microtubules. There are precedents for this sort of control, including the cytoplasmic tethering of the Dorsal and NFκB transcription factors to the Cactus and IκB proteins, respectively (Siebenlist et

al., 1994). It is notable that CI has yet to be found decisively in the nucleus, even in cells that are ostensibly stimulated by HH.

Our results provide two different forms of evidence that the complexes among FU, COS2, and CI are functionally significant. First, certain mutations in FU that block response to HH also disrupt the complexes. Second, stimulation of cells by HH is accompanied by release of the complexes from microtubules. Both forms of evidence are correlative in nature and, thus, not decisive. But together, they provide an indication of how signaling downstream of HH might be mediated.

Experimental Procedures

Reagents

Buffer A: 50 mM β-glycerophosphate (pH 7.6), 1.5 mM EGTA, 0.1 mM Na_2VO_4, 1 mM DTT, 10 mM NaF, plus PIC; buffer B: 20 mM Tris (pH 7.5), 20 mM p-nitrophenol phosphate, 1 mM EGTA, 50 mM NaF, 50 μM Na_2VO_4, 5 mM benzamidine; buffer C: 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1 mM EGTA, 50 mM NaF; buffer CX: buffer C supplemented with 1 mM Na_2VO_4, 1 mM Pefabloc (Boehringer Mannheim); buffer D: 50 mM β-glycerophosphate (pH 7.6), 150 mM NaCl, 1.5 mM EGTA, 0.1 mM Na_2VO_4, 1 mM DTT, 10 mM NaF; protease inhibitor cocktail (PIC): 0.01 mM benzamidine-HCl, phenantroline (1 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin A (10 μg/ml). All buffers contained 0.1–1.0 × PIC. The 1% NP-40 buffer corresponded to buffer A supplemented with 1% NP-40 and 150 mM NaCl.

Production of Antisera Against FU

A fragment containing nucleotides from position 2230 to 2455 of the *fu* gene (Thérond et al., 1993) was amplified by the polymerase chain reaction and cloned into the pGEX 4T-2 vector (Pharmacia). The final product was a fusion protein (GST-4H) of the GST protein and residues 419–493 of FU. E. coli DH5α cells carrying different constructs were used to produce GST alone and GST-4H as previously described (Smith and Johnson, 1988). The cells were harvested and the GST proteins isolated as previously described (Crowley et al., 1996). The proteins were fractionated by SDS–PAGE, and the resulting gel-purified protein was used to inoculate rabbits for polyclonal antisera production (BABCO). The antibodies were then affinity purified using standard procedures (Harlow and Lane, 1988).

[^{32}P]Orthophosphate Labeling

S2 cells and HH-S2 cells were cultured in supplemented Schneider cell medium as previously described (Thérond et al., 1996a). Confluent dishes of cells were then washed once with 5 ml of phosphate-free Schneider medium supplemented with dialyzed fetal calf serum. [^{32}P]orthophosphate (0.5 mCi/ml; ICN 64014) was added to this phosphate-free medium, and cells were incubated at room temperature for 14 hours. The cells were then washed once with 5 ml of phosphate-free S2 medium and lysed in 0.5 ml of lysis buffer C. Insoluble material was sedimented at 10,000 × g for 10 min at 4°C. Supernatants were then treated for immunoprecipitation as described below.

Purification of p175

Cellular extracts from sixty plates (24 cm^2) of confluent S2 cells were prepared and immunoprecipitated as described below using 1 μg of purified FU antibody per mg of extracts. Immunoprecipitates were separated by SDS–PAGE. The Coomassie-stained band running next to the 175 kDa prestained marker (New England Biolabs) was excised and used for sequence determination. Protein sequencing of the gel slice was essentially as previously described, except 0.05% Tween-20 was used instead of 0.1% (Wang et al., 1996).

Preparation of Tissue Extracts for Immunoprecipitation

The mutant alleles of *fu* used in this study were previously described (Busson et al., 1988; Préat et al., 1993). Viable *fu* alleles were kept in stock with the FM3 X-chromosome balancer. Wild-type Oregon-R strain was used as a control.

To obtain FU mutant tissue, we dissected imaginal discs from hemizygous 3rd instar FU mutant male larvae. Third instar male larvae were used to prevent contamination by FM3 balancer males, which die before the third instar. Imaginal discs from 15 male larvae of the same genotype were dissected and pooled together. Embryos and imaginal discs were homogenized at 4°C by several passes of a Teflon dounce homogenizer in lysis buffer CX.

S2 cells were prepared for analysis by washing twice in lysis buffer CX without NP-40 and were then lysed on ice for 30 min in buffer CX. Insoluble material from each tissue was sedimented by centrifugation at 10,000 × g for 15 min at 4°C and the supernatant used for immunoprecipitation.

Immunoprecipitation

Lysates were precleared by incubation with protein A–Sepharose beads (Sigma) for 1 hour or overnight at 4°C. After removal of the protein-A beads by centrifugation, the cleared lysates were incubated with purified rabbit anti-FU or unpurified rat COS2 antisera (Sisson et al., 1997) for 2–4 hours at 4°C. The immune complexes were collected by incubation with protein A–Sepharose beads for 1 hour at 4°C, followed by centrifugation. The immunoprecipitates were then washed three times for 10 min each with lysis buffer C supplemented to 0.5 M NaCl and were fractionated by SDS–PAGE.

Analysis by Immunoblotting

Immunoblotting analysis was performed as previously described (Thérond et al., 1996a). Purified polyclonal antibodies against FU were used at 1–0.5 μg/ml. CI rat monoclonal antibodies, kindly provided by R. Holmgren (Northwestern University), was used at a dilution of 1:3 (Motzny and Holmgren, 1995). COS2 rat polyclonal serum was diluted at 1:250 to 1:500. Eg-5 (which recognizes KRP_{130} in Drosophila) rabbit-purified polyclonal serum, kindly provided by C. Walczak (University of California, San Francisco), was used at 0.2 μg/ml (Cole et al., 1994). Pyruvate kinase polyclonal serum, kindly provided by A. Foster-Barber (University of California, San Francisco), was diluted 1:1000. Donkey anti-rabbit and anti-rat IgG coupled to horseradish peroxidase were used at a 1:5000 dilution.

Gel Filtration Chromatography

Confluent S2 cells and HH-expressing S2 cells were washed once in PBS and once in hypotonic buffer D. These cells were then lysed in a dounce homogenizer, followed by a 10,000 × g centrifugation. Embryos were homogenized at 4°C in buffer D and lysed as for the Schneider cells. The supernatants were centrifuged at 100,000 × g, at 4°C, for 60 minutes. These supernatants were then supplemented to 150 mM NaCl and 0.001% NP-40. The samples were then loaded on to a Superose 6 gel filtration column, on a Pharmacia FPLC system, that had been equilibrated with lysis buffer plus 150 mM NaCl and 0.001% NP-40. Four protein standards of known Stokes radius were used to calibrate the column: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (220 kDa), and BSA (66 kDa).

Microtubule Binding Assay

Microtubule binding proteins were isolated in BRB80 (pH 6.8) as described previously (Kellogg et al., 1989), with the following modifications: (1) 100,000 × g Drosophila embryonic supernatant (from 4–6 hour aged embryos) was additionally supplemented with 0.5 mM 5′-Adenylylimidodiphosphate (AMP-PNP) and Apyrase, unless otherwise stated; and (2) polymerized microtubules were collected by centrifugation (48,000 × g) through a 25% sucrose cushion instead of the 10% sucrose cushion previously reported.

Extracts and fractionation of the S2 and HH-S2 cells were performed as described above. Extracts from the two cell types were normalized to protein concentration with BRB80. Equal volumes of both types of lysates were subsequently compared, for both the starting extract and the microtubule pellet.

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