MOLECULAR AND CELLULAR BIOLOGY, Mar. 2002, p. 1555–1566 Vol. 22, No. 3
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.3.1555–1566.2002

The Carboxyl-Terminal Domain of the Protein Kinase Fused Can Function as a Dominant Inhibitor of Hedgehog Signaling

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Received 7 June 2001/Returned for modification 19 July 2001/Accepted 5 November 2001

The secreted protein hedgehog (Hh) plays a critical role in the developmental patterning of multiple tissues. In *Drosophila melanogaster*, a cytosolic multiprotein signaling complex appears necessary for Hh signaling. Genes that encode components of this Hh signaling complex (HSC) were originally identified and characterized based on their genetic interactions with *hh*, as well as with each other. It is only in recent years that the mechanistic functions of these components have begun to be unraveled. Here, we have investigated the relationship between two components of the HSC, the serine/threonine protein kinase Fused (Fu) and the kinesin-related protein Costal2 (Cos2). We have reconstituted a Fu/Cos2 complex in vitro and shown that Fu is able to directly associate with Cos2, forming a complex whose molecular size is similar to a previously described complex found in *Drosophila* cell extracts. We have also determined that the carboxyl-terminal domain of Fu is necessary and sufficient for the direct binding of Fu to Cos2. To validate the physiological relevance of this interaction, we overexpressed the carboxyl-terminal domain of Fu in wild-type flies. These flies exhibit a phenotype similar to that seen in *fu* mutants and consistent with an *hh* loss-of-function phenotype. We conclude that the carboxyl-terminal domain of Fu can function in a dominant negative manner, by preventing endogenous Fu from binding to Cos2. Thus, we provide the first evidence that Hh signaling can be compromised by targeting the HSC for disruption.

The secreted protein hedgehog (Hh) exerts potent tissue patterning activity during the development of a diverse array of organisms (15). During *Drosophila melanogaster* development Hh is responsible for the correct patterning of a variety of embryonic and adult tissues (5, 11, 26, 30, 35, 54). *hh* mutations are embryonically lethal, exhibiting a strong segment polarity phenotype (35). *hh* is also expressed within the posterior (P) compartment of imaginal discs that go on to form the various adult structures (26, 53). Hh then acts on adjacent anterior (A) compartment cells to specify cell fates in a concentration-dependent manner (26, 39, 54). The various concentrations of Hh are translated into different cell fates through a series of poorly understood molecular events that go through, at least in part, components of a large intracellular Hh signaling complex (HSC) (16, 19, 33, 43, 51, 52).

The HSC includes the kinesin-related protein Cos2 (43, 47), the Ser/Thr protein kinase Fu (41, 57), the pioneer protein Suppressor of fused [Su(fu)] (38), and the transcription factor Cubitus interruptus (Ci) (1, 13, 37). Ci exists in at least four different forms: a repressor form (Ci_{75}) (4), a cytosolic default form (Ci_{155}) (4, 32), an active full-length form ($^{act}Ci_{155}$), and an activated labile form (Ci*) (1, 9, 12, 22, 36, 59, 60, 64). It has been hypothesized that A cells interpret their position within the Hh concentration gradient by regulating the conversion between the various forms of Ci, in turn repressing or activating various Ci-dependent target genes (3, 45). This processing of Ci would be regulated by the other components of the HSC. In the absence of Hh, Cos2, Fu, and Ci_{155} are enriched on microtubules, where Ci processing to Ci_{75} has been proposed to occur (4, 43). Consistent with this, a disruption in Ci_{155}-to-Ci_{75} processing is observed in *cos2* or *fu* mutants (2, 29, 47, 61, 63). Upon Hh activation, the complex no longer enriches on microtubules and Ci_{155} processing to Ci_{75} is blocked, resulting in an increased cytosolic concentration of Ci_{155} (4, 9, 43, 62). This allows $^{act}Ci_{155}$ to accumulate in the nucleus (9, 61, 63, 64), without the other members of the HSC (29, 50). Anterior cells that abut the A/P border are exposed to the highest concentration of Hh. These cells cease to produce Ci_{75} and convert their Ci_{155} to Ci*, the most active form of Ci (4, 36). In cells that lack Cos2 or Fu, the conversion of Ci_{155} to Ci* is also lost (2, 28, 36, 61, 63, 64).

fu mutations are embryonically lethal, displaying a segment-polarity defect consistent with *fu* being a positive regulator of Hh signaling (16, 35). This embryonic lethality can be rescued by maternal copies of *fu* mRNA, allowing the mutant embryos to develop into adults. *fu* adults display a variety of phenotypes, including a fusion of longitudinal veins 3 and 4 (LV3 and LV4) (14, 31). Additionally, the wings of *fu* flies have a posterior extension of the double row of marginal bristles, into the intervein region of LV3 and LV4. This posterior extension of double-row bristles is indicative of a loss of *hh*-dependent anterior *en* expression (20, 46, 55).

Two major classes of *fu* alleles have been characterized, class

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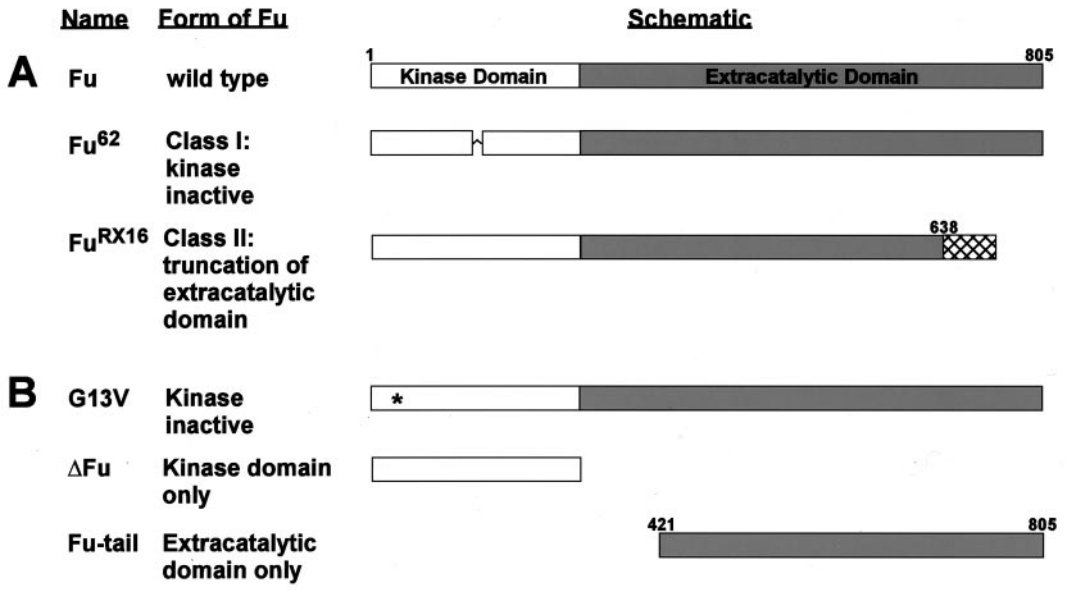


FIG. 1. Various Fu mutants. (A) Schematic diagram of wt, Class I (Fu^{62}) and Class II (Fu^{RX16}) Fu gene products. Class I *fu* alleles encode mutations within the kinase domain that are thought to inactivate Fu kinase activity. Class II *fu* alleles encode frameshift mutations that effectively truncate the carboxyl-terminal domain (extra catalytic domain). Fu^{62} has three amino acids deleted (positions 139 to 141) within the kinase domain as indicated by the symbol ^. Hatch marks indicate the 84 extra amino acids coded as a result of a frameshift mutation in the Fu^{RX16} mutant. (B) Schematic diagram illustrating the various Fu gene mutants used in this study. G13V contains a mutation that replaces a conserved Gly with Val at position 13; such a mutation behaves like a class I allele. The asterisk within the G13V schematic denotes the mutated amino acid. ΔFu contains only the kinase domain of Fu and is similar to various class II mutants. Fu-tail contains only the carboxyl-terminal domain of Fu, aa 421 to 805.

I and II, based on their genetic interactions with *Su(fu)* (42, 56). Class I alleles encode mutations that appear to affect the protein kinase domain, while class II alleles encode frame shift mutations that truncate the carboxyl-terminal domain of Fu (42, 56). Fu isolated from class I mutant flies can associate with Cos2, whereas Fu isolated from class II mutant flies cannot (43). Thus, the kinase activity of Fu is not required for its interaction with Cos2, but the carboxyl-terminal domain of Fu is required. These results suggest that the carboxyl-terminal domain of Fu plays a structural role in maintaining HSC integrity. However, it was also recently shown that the carboxyl-terminal domain of a human homolog of Fused (hFU) is an activator of the human Hh pathway (34). Murone et al. showed that when hFU is transfected into cells it can activate a reporter gene construct containing Gli (the vertebrate homolog of Ci) DNA binding consensus sites. hFU also acted synergistically with GLI2 to activate this reporter gene. hFU did not seem to require an intact kinase domain for function, as constructs expressing the carboxyl-terminal domain of hFU appear sufficient to activate the various assays performed. In light of these results, we decided to investigate, in vitro and in vivo, the role that the carboxyl-terminal domain of *Drosophila* Fu plays in Hh signal transduction.

Here, we show that Fu associates directly with Cos2 to form a high-affinity complex. The carboxyl-terminal domain of Fu is both necessary and sufficient for this high-affinity association with Cos2. Furthermore, transgenic flies that overexpress the carboxyl-terminal domain of Fu exhibit a phenotype similar to that of known *fu* mutants, consistent with disruption of the Hh pathway. We conclude that the carboxyl-terminal domain of Fu acts in a dominant negative manner to disrupt Hh signaling by preventing the kinase domain of endogenous Fu from accessing its substrates.

MATERIALS AND METHODS

Generation of Fu mutant constructs. See Fig. 1 for schematic details. The kinase-inactive Fu mutant, G13V, was made by replacing glycine 13 with a valine residue, using a PCR-based site-directed mutagenesis kit (Quikchange, Stratagene). ΔFu is a carboxyl-terminal truncated form of Fu containing the first 305 amino acids (aa) (kinase domain) of Fu. ΔFu was subcloned into pBacPak8 by use of a 5′ *Sma*I site on the vector and an internal *Nru*I site in Fu. Fu-tail (aa 421 to 805 of Fu) was subcloned into pFastBacHTb, in frame with a His_6 tag, by use of *Hin*dIII flanking sites. Fu-tail was also subcloned into pAc5.1V5-His in frame with a 5′-Flag epitope and out of frame with the 3′ tandem V5-His_6 epitope by use of *Hin*dIII flanking sites. A slightly larger carboxyl-terminal domain of Fu (aa 270 to 805), which was made by amplifying a portion of the *fu* D6 cDNA by PCR, was used to generate transgenic flies (57). A His_6-tagged version was subcloned into the pUAST vector (7). More-detailed subcloning procedures are available upon request.

Generation and characterization of Cos MAb 5D6. Eight-week-old female BALB/c mice (B&K Universal) were immunized with decreasing doses of antigen (glutathione *S*-transferase [GST]-heptad repeat region of Cos2, kindly provided by M. Scott [47]) over a period of approximately 4 months by standard techniques. Mice having serum antibody titers of approximately 1:400,000 to 1:800,000 were selected for hybridoma development. Three days prior to fusion, mice were administered a prefusion intraperitoneal injection of antigen (5 μg) in phosphate-buffered saline (PBS), and then isolated splenocytes were fused to murine myeloma FO (ATCC no. CRL 1646; American Type Culture Collection). Selected hybridomas were cloned by limiting dilution, and the immunoglobulin subtype was determined for each resulting monoclonal antibody (MAb). Subtypes of selected MAbs were determined with IsoStrip Antibody Subtyping Dipsticks (Boehringer-Mannheim). Antibody 5D6 was determined to be immunoglobulin G1(κ) [IgG1(κ)].

Spinner flasks containing BD Cell MAb Medium (Becton Dickinson Microbiology Systems) plus 10% fetal bovine serum, 2% L-glutamine, and penicillin-

streptomycin were inoculated with hybridoma cell lines at 2×10^5 cells/ml. Cultures were incubated at 37°C in 5% CO_2 for approximately 2 weeks. Hybridoma cells were removed from the culture medium by centrifugation, and MAbs were purified by protein G-Sepharose (Amersham Pharmacia Biotech) affinity chromatography, followed by elution with a glycine buffer, pH 2.5. The pH of the antibodies was adjusted to 7.0 and then dialyzed against PBS.

Cell culture and transfection. Sf21 cells were cultured in Grace's insect medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. *Drosophila* S2 cells were cultured in Schneider's Drosophila medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Transfection of S2 cells was carried out with Lipofectin according to the manufacturer's instructions (Life Technologies, Inc.).

Preparation of baculovirus. Baculoviruses were produced and titered according to the manufacturer's instructions (Life Technologies, Inc.). Infections were carried out with Sf21 cells at a total multiplicity of infection of 4 to 6. Wild-type (wt) baculovirus was used to normalize the coinfections done in this study. The infected Sf21 cells were allowed to incubate postinfection for 44 to 48 h.

Cellular lysates. Sf21 or S2 cells were washed twice with PBS at 4°C and then lysed in Nonidet P-40 (NP-40) buffer (150 mM NaCl, 50 mM HEPES [pH 7.6], 1 mM dithiothreitol, 1 mM EDTA, 1% NP-40, and 1:250 protease inhibitor cocktail [PIC]). PIC contains 1 mM benzamidine, 1 mg of aprotinin per ml, 1 mg of leupeptin per ml, and 1 mg of pepstatin per ml in 100% ethanol. The various cellular lysates were centrifuged at $16,000 \times g$ for 20 min at 4°C. The supernatants were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then analyzed by immunoblotting or subjected to immunoprecipitation analysis.

For gel filtration analysis, the infected Sf21 cells were washed twice with PBS at 4°C and then lysed in a hypertonic lysis buffer (325 mM NaCl, 50 mM Tris-HCl [pH 7.6], 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.0005% NP-40, and 1:250 PIC) using a glass Dounce homogenizer (Kontes). The cellular lysates were centrifuged at $16,000 \times g$ for 20 min at 4°C. The resulting supernatants were then centrifuged at $100,000 \times g$ for 30 min prior to gel filtration analysis.

Immunoprecipitation of cytosolic lysates. The baculovirus-infected Sf21 lysates were normalized according to Cos2 expression, to correct for any variation in protein expression seen in multiple infections. Two micrograms of mouse Flag M2 (Sigma) or 5D6 MAbs per sample was used for each immunoprecipitation. Mouse IgG1 was used as the isotype-matched control for both MAbs. The cellular lysates were immunoprecipitated as previously described (43). The immunoprecipitates were washed five times with 1.25 ml of NP-40 lysis buffer, followed by the addition of 2× Laemmli protein gel loading buffer. The resulting samples were separated by SDS-PAGE and immunoblotted for the appropriate proteins.

Size exclusion chromatography. For gel filtration analysis, baculovirus-infected Sf21 lysates were fractionated by size with a Superose 6 gel filtration column by fast-performance liquid chromatography (Amersham-Pharmacia), equilibrated with 650 mM NaCl, 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 10% glycerol, and 0.001% NP-40.

Purification of a Fu/Cos2 complex. One billion Sf21 cells were coinfected with Cos2 and flag-tagged G13V baculovirus and then lysed 48 h later in hypertonic lysis buffer. The resulting supernatants were made isotonic. One milliliter of anti-Flag (M2) affinity resin (Sigma) was added to the supernatant and shaken for 4 h. The supernatant and resin suspension was applied to a column and washed extensively with ≈100 ml of isotonic buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.6], 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 1:250 PIC). Wash fractions were monitored for UV absorbance at 280 nm on a Spectronic Genesys 5 UV/VIS spectrophotometer until the readings were near background levels. Bound proteins were then eluted with 0.2 mg of Flag peptide per ml in the same buffer. Fractions were collected, resolved by SDS-PAGE, and then immunoblotted with Fu or Cos2 antibodies, or Coomassie blue stained. Fraction 8 contained the peak of Fu and Cos and was injected onto a Superose 6 gel filtration column and fractionated as above.

Construction of *Saccharomyces cerevisiae* vectors and yeast two-hybrid analysis. The directed yeast two-hybrid screen was performed essentially as previously described (24). Briefly, Cos2 and Fu-tail cDNA was subcloned into both pGAD-C1 (GAL4 activation domain) and pGBDU-C1 (GAL4 DNA binding domain) vectors and transformed into the yeast strain PJ69-4A. Each cDNA was also subcloned in its antisense orientation, as a negative control. Yeasts that were able to grow in the histidine and adenine screening plates were interpreted as true and positive clones. A more detailed description of our yeast two-hybrid screening procedure is available upon request.

***Drosophila* strains and generation of transgenic flies.** *Drosophila* strains were maintained as previously described (58). Transgenic flies were made according to the method of Rubin and Spradling (44) with *yw* flies as hosts and maintained at 18°C. *dpp*-GAL4 (48, 49) and *ptc*-GAL4 (21) were kind gifts of the Kornberg laboratory. *ap*-GAL4 (8) was a kind gift from C. Micchelli. Crosses to detect adult wing phenotype were conducted at 29°C. For wing preparations, whole flies were placed in isopropanol overnight. The wings were then dissected and placed into 100% ethanol. The wings were subsequently washed twice in ethanol, mounted in a 6:5 lactic acid-to-ethanol solution, and imaged using an Axiophot microscope with an Axiocam CCD camera and software.

Immunofluorescence of *Drosophila* wing imaginal discs. Wing imaginal discs of wandering third instar larvae were dissected. The discs were fixed in PBS–4% formaldehyde (Ted Pella)–0.2% Triton X-100 for 10 min and then washed three times with PBT (PBS plus 0.1% Tween 20). The discs were then incubated with primary antibody in PBT plus 5% donkey serum (Jackson Labs) for 1 h as previously described (4). Anti-Fused antiserum (43) was used at 1:1,000, and MAb 2A1 (anti-Ci) was used at 1:2 (4). Texas Red-conjugated antirabbit (Jackson Labs) and fluorescein isothiocyanate (FITC)-conjugated antirat (Jackson Labs) antibodies were both used at 1:400. Imaging was conducted on a Leica TCS NT confocal microscope (Leica America). Images were processed with Adobe Photoshop.

RESULTS

Fu and Cos2 associate with each other quantitatively. We have previously shown that while Cos2 can associate with kinase-inactive Fu mutants isolated from class I *fu* flies, it is unable to associate with carboxyl-terminal truncated forms of Fu isolated from class II *fu* flies. (43). There are at least two models that might explain these observations: (i) the carboxyl-terminal domain is necessary for regulating an event, or other protein, which allows Fu to bind to Cos2; or (ii) the carboxyl-terminal domain is both sufficient and necessary to bind Fu to Cos2. To test these models, we decided to reconstitute these interactions. Therefore, we made recombinant baculoviruses that express a kinase-inactive Fu (G13V) or a carboxyl-terminal truncation of Fu containing just the kinase domain (ΔFu) (Fig. 1). In flies, G13V is a class I *fu* allele (56), whereas ΔFu is similar to class II *fu* alleles that encode a mutant protein lacking the carboxyl-terminal domain. G13V, ΔFu, and full-length Fu were expressed in Sf21 cells individually, or coexpressed with a Cos2 baculovirus. Using a Cos2 MAb (5D6; see Materials and Methods), we were able to coimmunoprecipitate both Fu and G13V with Cos2 from the appropriate coinfected lysates (Fig. 2A). Under similar conditions we were unable to coimmunoprecipitate ΔFu or GST, expressed by a GST baculovirus (data not shown), with Cos2. Thus, using a baculoviral system, we are able to recapitulate the interactions previously observed in fly imaginal disc lysates between Fu and Cos2; Fu does not require a functional kinase domain to associate with Cos2 but does require the carboxyl-terminal domain.

To establish that the Fu/Cos2 association was direct, we assessed whether the interactions were quantitative under conditions where the two proteins are greatly overexpressed. Lysates from Sf21 cells infected with Fu or Cos2 baculovirus individually, or coinfected, were subjected to size exclusion chromatography (Fig. 2B). Upon size exclusion chromatography of *Drosophila* cellular lysates, Fu is found in three distinct peaks (A, B, and C) (43). Peak A, which contains Fu, Cos2, and Ci, migrates as a large megadalton-sized protein complex. Peak B, which contains the vast majority of Fu and Cos2, migrates as an approximately 700-kDa complex. Peak C is approximately 100 to 200 kDa in size. Fu, from Fu-infected cells, elutes at fraction 44 with a molecular size (100 to 200 kDa) consistent with it being monomeric or dimeric, and similar to that previ-

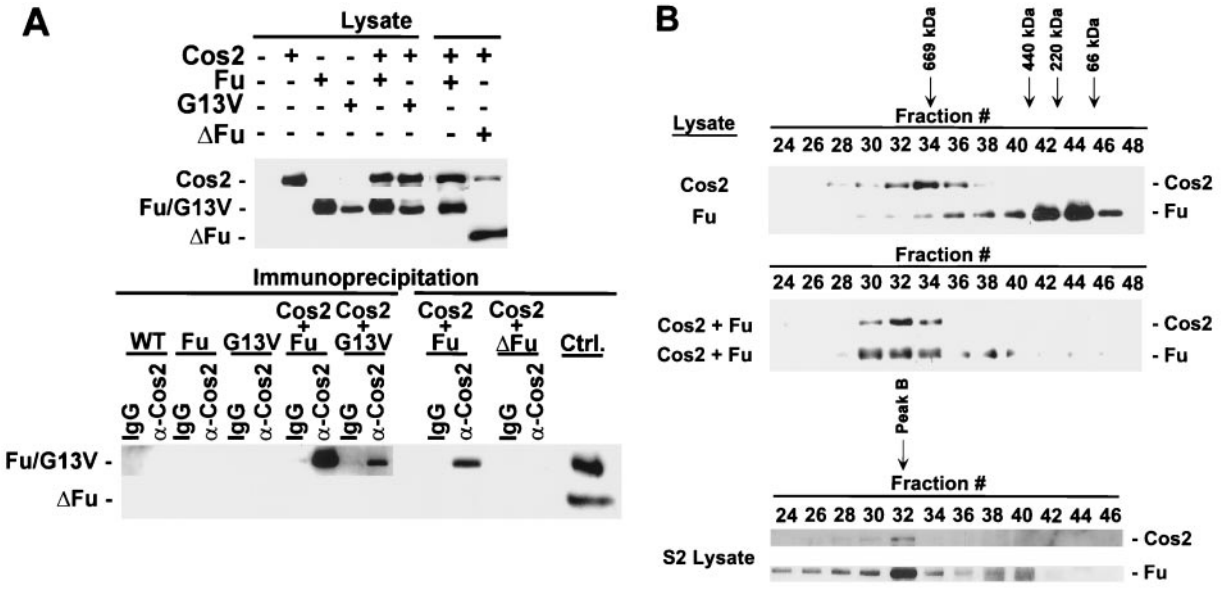


FIG. 2. Cos2 binds Fu quantitatively. (A) Fu and a kinase-inactive mutant of Fu (G13V) associate with Cos2, but ΔFu does not. Immunoblots of various lysates from Sf21 cells infected with baculovirus expressing Cos2, Fu, G13V, ΔFu, or various combinations are shown (top panel). The various lysates were immunoprecipitated with the Cos2 5D6 MAb or an isotype-matched IgG control. The resulting immunoprecipitates were resolved by SDS-PAGE and then immunoblotted for the various Fu mutants (bottom panel). GST served as a negative control in this experiment (data not shown). The immunoblots were probed with 5D6 to verify that Cos2 was immunoprecipitated (data not shown). Lysates containing Fu or ΔFu were used as immunoblotting positive controls in this experiment (Ctrl.) (B) Gel filtration analysis of lysates from Sf21 cells expressing Cos2, Fu, or both. Cos2, when expressed alone, elutes at fraction 34. Fu, expressed alone, elutes at fraction 44 with a molecular size consistent with it being a monomer or dimer (top panel). In lysates from cells coinfected with Fu and Cos2, both proteins comigrate at fraction 32 (middle panel). Various protein standards were used to calibrate the Superose 6 gel filtration column, i.e., thyroglobulin (669 kDa), ferritin (440 kDa), catalase (220 kDa), and bovine serum albumin (66 kDa), which eluted at fractions 34, 41, 43, and 45, respectively. A *Drosophila* S2 cellular lysate fractionated on a Superose 6 column illustrates peak B, where endogenous Fu and Cos2 comigrate (bottom panel). Peak A is a complex that contains Cos2, Fu, and likely other proteins in S2 cells. However, peak A is an unstable complex, whose appearance in S2 cell lysates fractionated by gel filtration, while reproducible, remains variable. The calculated exclusion volume for the column elutes at fraction 22 and is reported to be 4×10^7 daltons for globular proteins.

ously described for peak C. Cos2, from Cos2-infected cells, elutes at fraction 34, comigrating with a 669-kDa protein standard. The anomalous migration of Cos2 (calculated molecular mass, ≈130 kDa) through the gel filtration column is consistent either with it being multimeric, binding some abundant Sf21 cellular proteins or, more likely (see below), with Cos2 migrating through the gel filtration column with a rod-like structure. Such uncharacteristic behavior, upon gel filtration, has been described for other kinesin-related proteins. When extracts from Fu- and Cos2-coinfected cells are analyzed by gel filtration, the peaks of Fu and Cos2 immunoreactivity comigrate at fraction 32. The peak of Fu shifts quantitatively, from 100 to 200 kDa to greater than 669 kDa (from fractions 44 to 32), whereas the peak of Cos2 now elutes two fractions larger than when expressed alone (from fractions 34 to 32). Fu and Cos2 associate with high affinity, as these associations occur in a 650 mM NaCl buffer. These two proteins also appear sufficient to form a complex that migrates at a molecular size similar to that of Fu peak B, from S2 cell extracts (Fig. 2B, bottom panel). These results support the idea that the interaction between Fu and Cos2 is direct and suggest that the previously described peak B is composed primarily of Cos2 and Fu.

To verify that Fu and Cos2 associate directly, given their large apparent molecular size upon gel filtration analysis, we purified the Fu/Cos2 complex. Extracts of Sf21 cells, expressing Cos2 and Flag-tagged Fu, were purified over an anti-Flag MAb affinity column and eluted with excess Flag peptide. Fractions from this column were resolved by SDS-PAGE and then either Coomassie blue stained or immunoblotted with antibodies to Fu or Cos2 (Fig. 3A and B). After Coomassie blue staining, two major proteins that migrated at the apparent molecular weights of Fu and Cos2 and comigrated with Fu and Cos2 immunoreactivity were detected. A fraction containing the peak of this purified material (fraction 8) was also analyzed by gel filtration chromatography, to determine if these two proteins alone were sufficient to migrate at the molecular size of peak B. We found that highly purified Fu and Cos2 still comigrate on a sizing column at fraction 32, suggesting that Fu and Cos2 are sufficient to form the bulk of peak B found in extracts of *Drosophila* cells (Fig. 3C, compare to Fig. 2B, bottom panel).

The carboxyl-terminal domain of Fu is necessary and sufficient to bind Cos2. We hypothesized that the region of Fu that binds Cos2 is located in the carboxyl-terminal domain, given the inability of Fu mutants lacking this domain to bind Cos2 (43). To test this hypothesis we constructed a recombinant baculovirus expressing only the carboxyl-terminal domain of Fu (Fu-tail). We then coexpressed Fu-tail with or without Cos2 in Sf21 cells, using ΔFu coinfected with Cos2 as a negative control for Cos2 binding (Fig. 4A, top panel). When Cos2 was immunoprecipitated from extracts of these various infected cells, Fu-tail coimmunoprecipitated with Cos2 whereas ΔFu

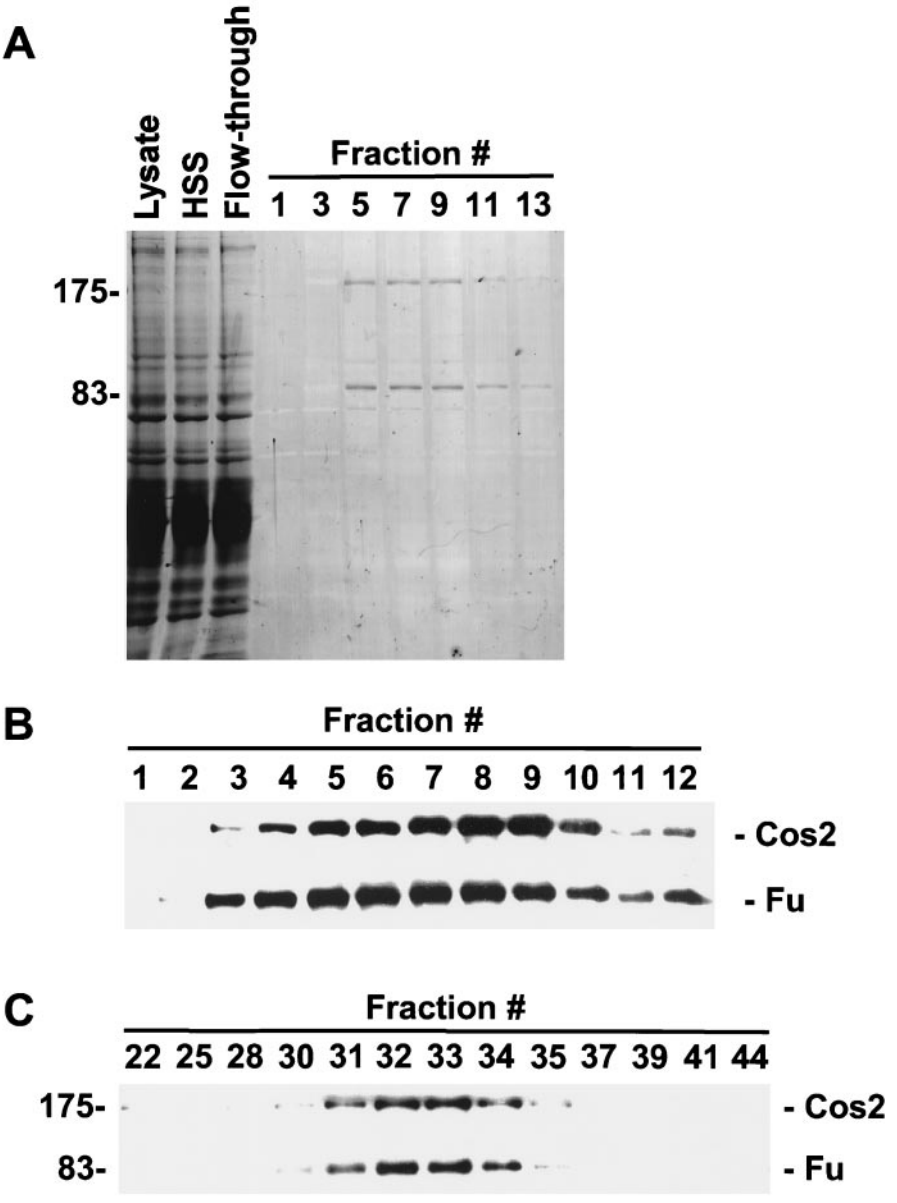
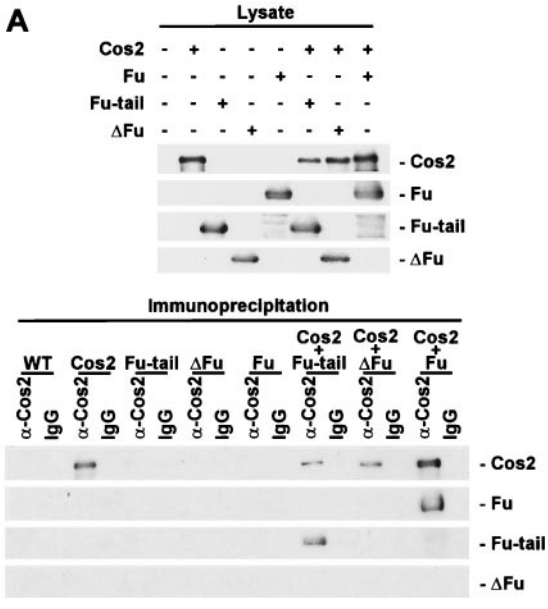
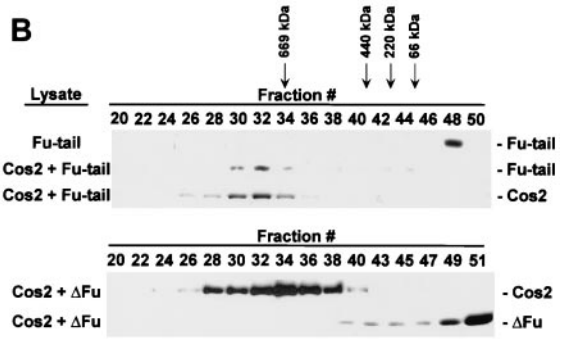


FIG. 3. Purification of a recombinant Fu/Cos2 complex. Lysates of Sf21 cells infected with Cos2 and Flag G13V were purified over an anti-flag MAb (M2) affinity column and washed extensively. Bound protein was eluted with excess Flag peptide. The various fractions were resolved by SDS-PAGE and then Coomassie blue stained (A) or immunoblotted with Fu or Cos2 antibodies (B). (A) Coomassie blue-stained SDS-PAGE showing purification of a Cos2 Fu complex. Starting fractions containing the infected Sf21 lysate (Lysate), the high speed supernatant (HSS), the flowthrough fraction from the Flag M2 affinity resin (Flow-through), and the fractions collected after addition of excess Flag peptide are shown (lanes 1 to 13, every other fraction). Two major proteins that correspond to the apparent molecular weights of Fu and Cos2 are seen in lanes 5 to 9. (B) Immunoblot analysis of the Cos2 Fu purification. Fu and Cos2 immunoreactivity comigrated with the two major Coomassie blue-stained proteins, at molecular weights of 83,000 and 175,000. (C) Fraction 8 off the M2 affinity resin was loaded onto a Superose 6 gel filtration column to determine quantitative association between purified Cos2 and G13V. Both purified proteins comigrated at fraction 32, verifying that Fu and Cos2 are sufficient to form the bulk of peak B seen in S2 lysates.

did not (Fig. 4A, bottom panel). To verify that the interaction between Fu-tail and Cos2 is quantitative, we subjected the various cellular lysates to gel filtration analysis. Fu-tail, when expressed alone, elutes at a molecular size (fraction 48) consistent with its calculated molecular weight. When Fu-tail is coexpressed with Cos2, its peak shifts to fraction 32, where it comigrates with Cos2 (Fig. 4B, top panel). Under these conditions, Cos2 elutes in fraction 32 instead of fraction 34 (see Fig. 2B, top panel). When lysates from ΔFu- and Cos2-coinfected cells are fractionated, ΔFu elutes at a size inconsistent with it being associated with Cos2.

Although we highly purified the Fu/Cos2 complex (Fig. 3C), it is still possible that there were numerous copies of both proteins present in this complex, bound together by a cross-bridging protein. Such a putative cross-bridging protein would have to be provided by the Sf21 cells and be present as a minor





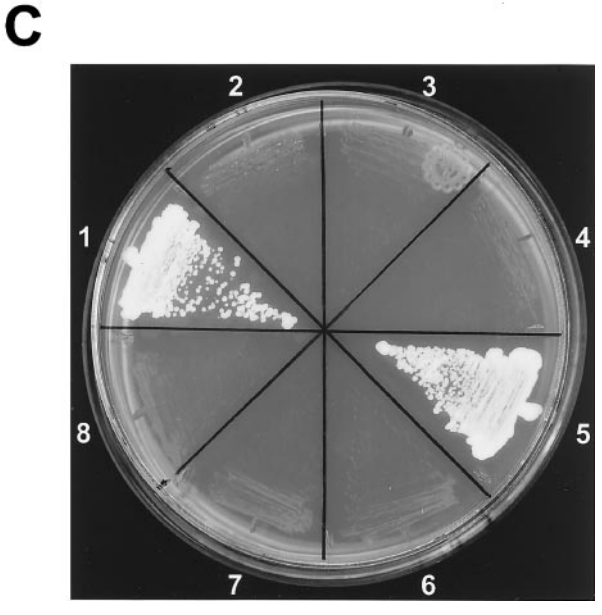


FIG. 4. The carboxyl-terminal domain of Fu tethers Fu to Cos2. (A) Fu-tail associates with Cos2. An immunoblot of Cos2, Fu, Fu-tail, and ΔFu from the various Sf21 lysates is shown (top panel). Fu-tail coimmunoprecipitates with Cos2 from lysates that contain Cos2 and Fu-tail (bottom panel). A lysate containing Cos2 and Fu served as a positive control, while one containing Cos2 and ΔFu served as a negative control for binding in this experiment. A lysate containing Fu was used as an immunoblotting control in the bottom panel (data not shown). (B) Fu-tail quantitatively associates with Cos2. Gel filtration analysis of Sf21 lysates containing Cos2 coexpressed with either Fu-tail (top panel) or ΔFu (bottom panel). Fu-tail elutes at fraction 48 when expressed on its own in Sf21 cells. Cos2 and Fu-tail, from the coinfected Sf21 lysate, comigrate at fraction 32, indicating that Fu-tail can quantitatively associate with Cos2. In a Cos2 and ΔFu coinfection, Cos2 elutes at fraction 34 while ΔFu elutes at fraction 51, verifying that these proteins do not interact significantly, at least not with high affinity. (C) A directed yeast two-hybrid assay demonstrating that Fu-tail directly associates with Cos2. Yeast organisms transformed with various vectors were plated on minimal media without leucine, uracil, or adenine. Yeast transformed with the GAL4 activation domain/Fu-tail and the GAL4 DNA binding domain/Cos2 vectors were able to grow (sector 1). As negative controls, Fu-tail (sectors 2 and 4) and Cos2 (sectors 3 and 4) were also subcloned in their antisense orientation. The reciprocal experiment, in which Fu-tail was subcloned as a fusion protein with the GAL4 DNA binding domain and Cos2 was subcloned as a fusion protein with the GAL4 activation domain (sector 5), was also performed. As before, both Fu-tail (sectors 7 and 8) and Cos2 (sectors 6 and 8) were also subcloned in their antisense orientation.

contaminant in our highly purified Fu/Cos2 complex. To bolster our argument that Fu-tail interacts directly with Cos2, we conducted a directed yeast two-hybrid assay. We expressed either Fu-tail or Cos2 as a GAL4 DNA binding domain fusion protein (bait) and as a GAL4 activation domain fusion protein (prey) (Fig. 4C). We observed that Fu-tail and Cos2 are able to associate sufficiently to permit the growth of yeast under the stringent conditions of our yeast two-hybrid system (Fig. 4C, sectors 1 and 5). This association was independent of whether Cos2 or Fu-tail served as the bait fusion protein or if both Cos2 and Fu-tail were subcloned in their antisense orientation. Thus, Fu-tail is necessary and sufficient to recapitulate the high affinity quantitative association seen with Cos2 and full-length Fu.

The carboxyl-terminal domain of Fu can compete with endogenous Fu for Cos2 binding. We hypothesized that overexpression of Fu-tail in vivo might allow it to act as a dominant inhibitor of the Hh pathway, by associating with Cos2 to compromise the interaction between Cos2 and endogenous full-length Fu. To test this hypothesis we coexpressed Cos2 and Fu with increasing amounts of Fu-tail in Sf21 cells (Fig. 5A, top panel). These lysates were then immunoprecipitated using the 5D6 antibody and subjected to immunoblot analysis. Cos2 and full-length Fu are able to associate tightly in the absence of Fu-tail (Fig. 5A, bottom panel). As Fu-tail expression is increased, the association between Cos2 and Fu is concomitantly reduced. At the highest levels of Fu-tail expression, Cos2 associated predominantly with Fu-tail, instead of full-length Fu, in a manner consistent with our hypothesis.

To verify that Fu-tail could compete with full-length Fu, for

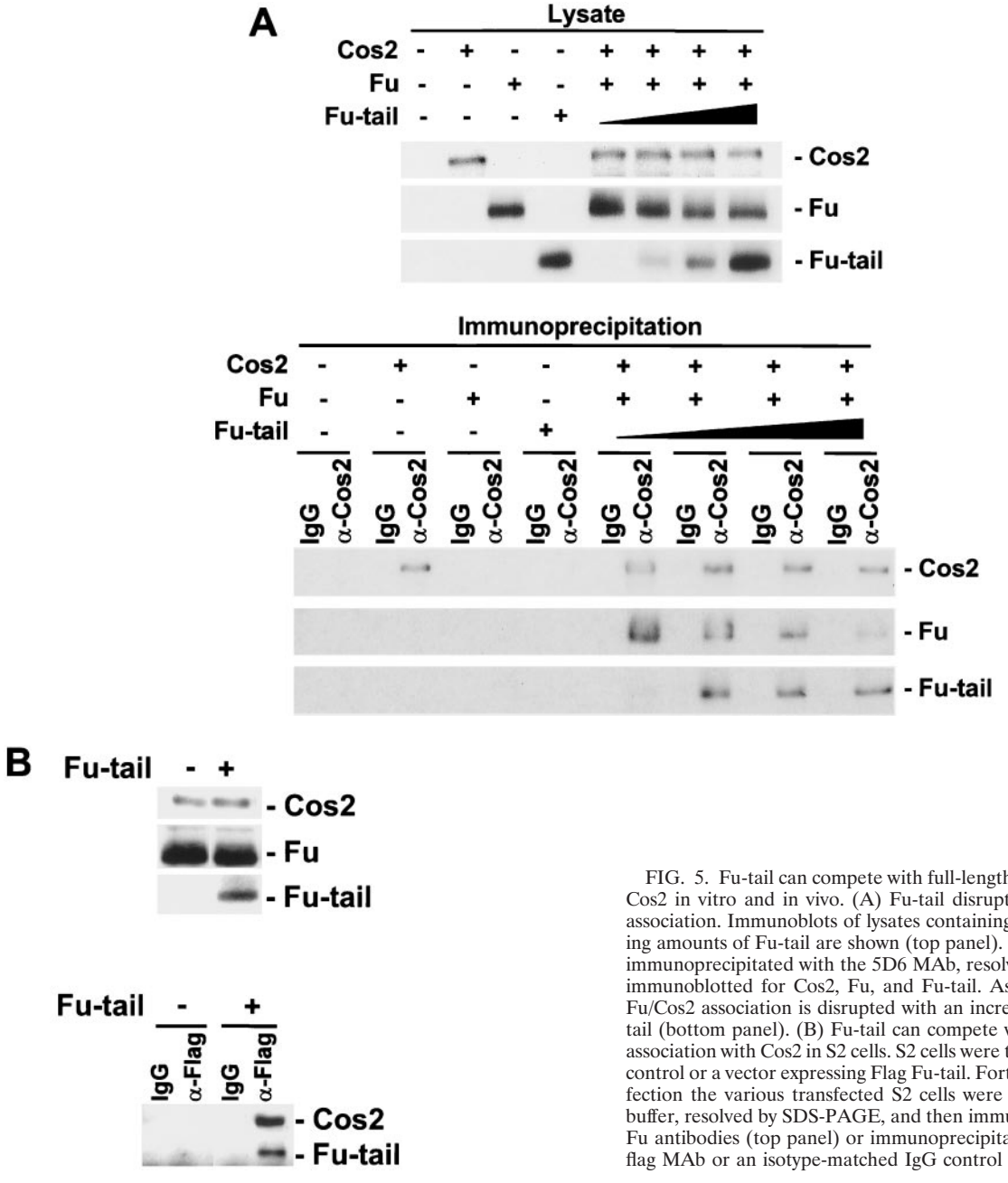


FIG. 5. Fu-tail can compete with full-length Fu for association with Cos2 in vitro and in vivo. (A) Fu-tail disrupts a full-length Fu/Cos2 association. Immunoblots of lysates containing Cos2, Fu, and increasing amounts of Fu-tail are shown (top panel). These lysates were then immunoprecipitated with the 5D6 MAb, resolved by SDS-PAGE, and immunoblotted for Cos2, Fu, and Fu-tail. As shown, the full-length Fu/Cos2 association is disrupted with an increasing expression of Fu-tail (bottom panel). (B) Fu-tail can compete with endogenous Fu for association with Cos2 in S2 cells. S2 cells were transfected with a vector control or a vector expressing Flag Fu-tail. Forty-eight hours posttransfection the various transfected S2 cells were lysed in an NP-40 lysis buffer, resolved by SDS-PAGE, and then immunoblotted with Cos2 or Fu antibodies (top panel) or immunoprecipitated with either an anti-flag MAb or an isotype-matched IgG control (bottom panel).

Cos2, under conditions where more physiologically relevant levels of Fu and Cos2 are present, we expressed Flag-tagged Fu-tail in S2 cells. Lysates from transfected cells were separated by SDS-PAGE and imunoblotted to verify the normalized expression of Fu-tail and Cos2 (Fig. 5B, top panel). The lysates were then immunoprecipitated with an anti-Flag MAb and subjected to immunoblot analysis. Cos2 specifically coimmunoprecipitated with Fu-tail. These results suggest that Fu-tail can specifically associate with endogenous Cos2 (Fig. 5B, bottom panel). This association does not appear to be Hh sensitive (data not shown). Furthermore, our results suggest that Fu-tail can disrupt an association between Cos2 and full-length Fu, since the majority of Cos2 is normally complexed with Fu (Fig. 2B) (43). Thus, Fu-tail can compete with endogenous Fu for Cos2.

Fu-tail can disrupt the Hh signaling pathway. To define the role of the carboxyl-terminal domain of Fu in Hh signaling in vivo, we generated transgenic flies expressing *fu-tail* or wt *fu*. A *fu* mutant adult fly, which displays a classic *fu* LV3-LV4 fusion wing phenotype, can be rescued by expressing full-length UAST-*fu* under the control of either the *dpp*-GAL4 or the *ptc*-GAL4 driver. These results suggested that these domains of expression delineate where Fu is required in the wing imaginal disc (2). Therefore, we expressed UAST-*fu-tail*, or UAST-*fu*, driven by *dpp*-GAL4 or *ptc*-GAL4 in otherwise wt flies, to assess whether Fu-tail could act as a dominant inhibitor of the

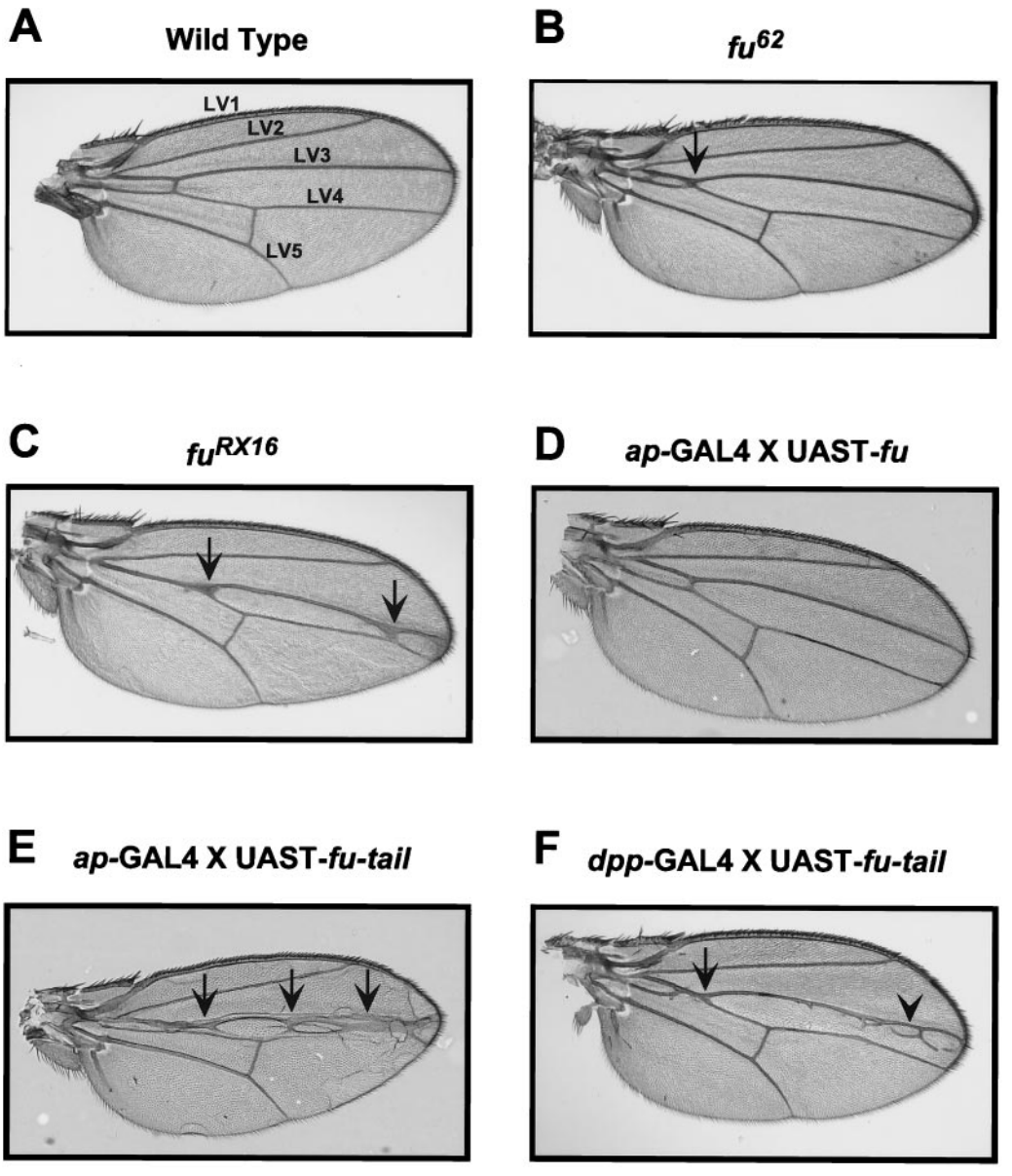


FIG. 6. Fu-tail expression in vivo results in a loss-of-function phenotype. Overexpression of Fu-tail in wing imaginal discs yields a phenotype similar to that seen with *fu* loss-of-function mutations. (A) A wt *Drosophila* wing blade. There is continuous separation between longitudinal wing veins 3 and 4 (LV3 and LV4). (B) A wing from a fu^{62} class I homozygous adult fly (Fig. 1). Proximal fusions between LV3 and LV4 (arrow) as well as narrowing of the distance between these veins distally can be seen. (C) A wing from a fu^{RX16} class II homozygous adult fly. Fusion between LV3 and LV4 can be seen as well as narrowing of this region (arrows). (D) A wing from an *ap*-GAL4 × UAST-*fu* fly. The wing blade appears normal and has no fusion of LV3 and LV4. (E) A wing from an *ap*-GAL4 × UAST-*fu-tail* fly. The wing has severe proximal and distal fusions of LV3 and LV4 (arrows). (F) A wing from a *dpp*-GAL4 × UAST-*fu-tail* fly. The wing has proximal fusions of LV3 and LV4 (arrow) as well as some distal ectopic veins (arrowhead).

Hh pathway in vivo. Additionally, we expressed *fu-tail* using the *apterous* promoter because this promoter, unlike *dpp*-GAL4 or *ptc*-GAL4, is not Hh sensitive. Regardless of the promoter used to drive expression, when *fu-tail* is expressed in wing imaginal discs, we observe a fusion of LV3 and LV4, a phenotype identical to *fu* loss-of-function mutations (Fig. 6 and data not shown) (16, 31). This phenotype is indicative of a loss of tissue at the A/P border of the wing imaginal disc and consistent with decreased Hh signaling (14). Moreover, overexpression of Fu-tail also results in the posterior extension of the wing margin double-row bristles (Fig. 7E and F). In *fu* mutants, the double row of bristles extends as far as LV4, consistent with a loss of anterior *en* expression (Fig. 7B and C, compare to Fig. 7A) (6, 46). Therefore, expression of Fu-tail disrupts anterior *en* expression, as determined by the loss of the single-row bristles and subsequent expansion of the double-row bristles. These findings are distinct from those seen for flies overexpressing full-length Fu, which do not exhibit any apparent wing

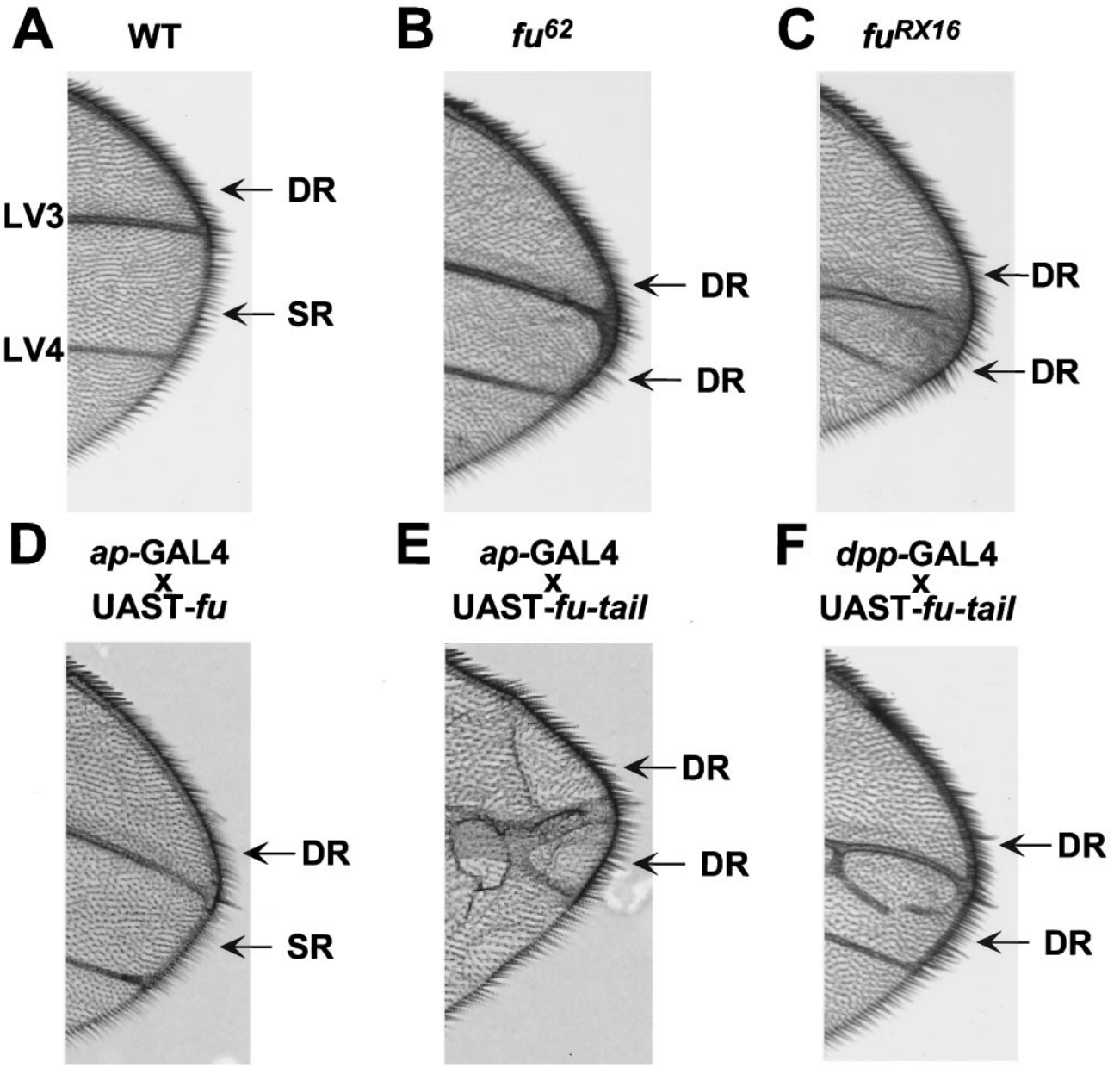


FIG. 7. Fu-tail disrupts anterior *en* expression. (A) The wing margins, from wt flies, have a double row (DR) of bristles from LV2 to LV3, and a single row (SR) of fine bristles in the intervein region between LV3 and LV4. The SR bristles are indicative of Hh-dependent anterior *en* expression (see text). In class I (B) and class II (C) *fu* alleles, DR bristles are found to extend beyond vein 3 to vein 4. (D) This extension of DR bristles is not observed when wt Fu is overexpressed using *ap*-GAL4, but it is clearly seen when Fu-tail is overexpressed with either *ap*-GAL4 (E) or *dpp*-GAL4 (F) drivers.

phenotype (Fig. 6 and 7; data not shown) (2), even though wt *fu* is overexpressed to the same degree as *fu-tail* (Fig. 8).

Wild-type discs have increased Ci_{155} levels in a tight band close, but not adjacent, to the A/P border, reflecting this area's increased exposure to Hh (2, 4, 32, 36, 61, 63). However, Ci_{155} levels noticeably decrease in the region directly adjacent to the A/P border, as previously described. These cells contains Ci*, which is responsible for anterior *en* transcription late in wing disk development (6, 36). To verify that overexpression of Fu-tail had resulted in changes in Ci processing to its various forms, we performed indirect immunofluorescence for Ci in wing imaginal discs expressing either wt *fu* or *fu-tail*. Ci staining in discs overexpressing full-length Fu is not significantly different from that observed for wt (Fig. 8B and data not shown). However, in disc regions where Fu-tail is overexpressed, Ci_{155} levels are not reduced at the A/P border, compared to either wt discs or more ventral regions of the A/P border (Fig. 8E). These results suggest that Fu-tail may block the ability of Ci to be processed into its most active labile form, Ci*. We find that in anterior regions distant from the A/P border, Ci_{155} does not accumulate, despite the overexpression of Fu-tail (Fig. 8D and E). These results suggest that overexpression of Fu-tail may

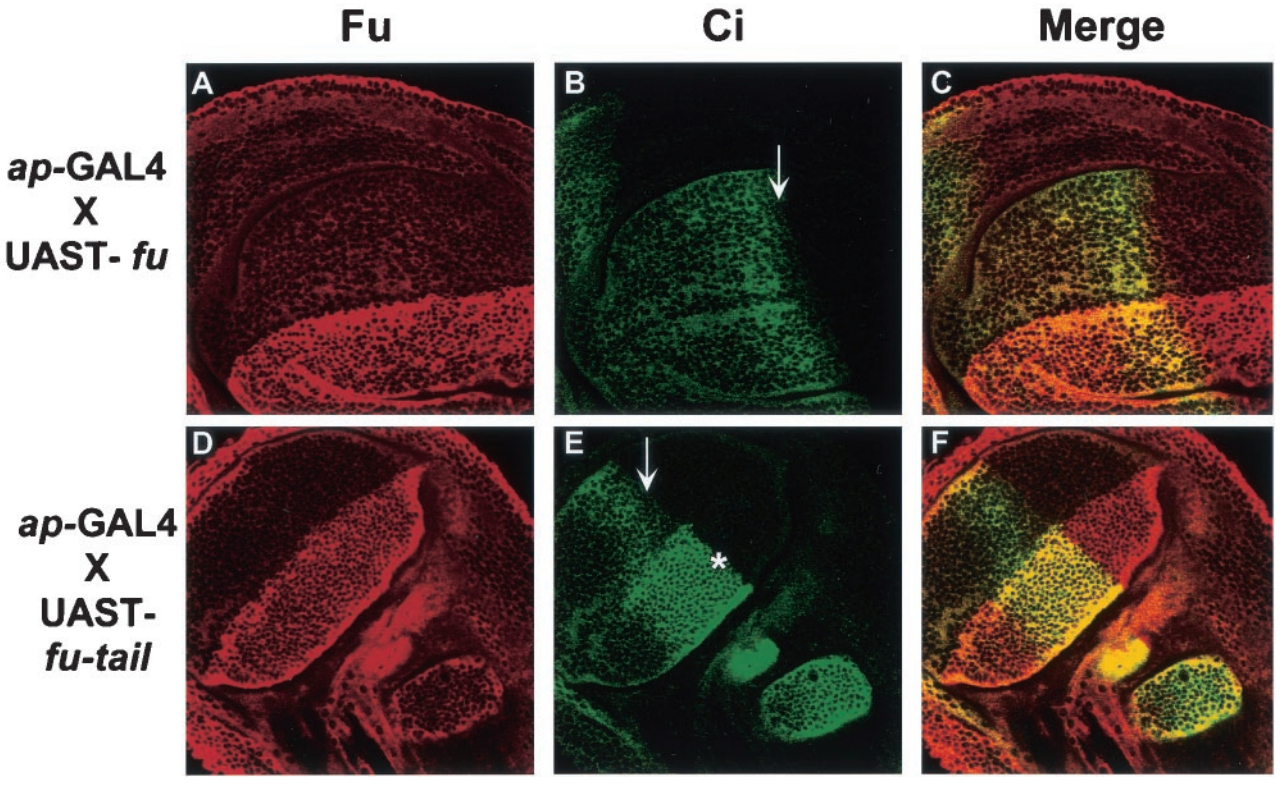


FIG. 8. Fu-tail expression in vivo affects Ci_{155} processing. Staining for Fu, Ci, or both (merge) in third-instar wing imaginal discs isolated from transgenic *Drosophila* expressing UAST-*fu-tail* or UAST-*fu* driven by *ap*-GAL4. (A and D) Fu immunofluorescence, with an antiserum that recognizes both endogenous Fu and expressed Fu-tail. (B and E) Ci immunofluorescence with the 2A1 MAb that detects full-length Ci. (C and F) Composite images for top and bottom rows, respectively. Overexpression of Fu (A) does not appear to affect the staining of Ci (B) in the anterodorsal compartment of the wing pouch. Flies overexpressing Fu are phenotypically wt. The accumulation of full-length Ci at the A/P border can clearly be seen. In addition, Ci_{155} levels decrease slightly at a region immediately adjacent to the A/P border (arrows in panels B and E). This region contains the most active form of Ci, which has the ability to upregulate anterior *en* expression. Fu-tail is expressed across the entire dorsal compartment of the imaginal disc as indicated by the increased Fu staining (D). In the dorsal compartment, where Fu-tail is expressed, Ci_{155} levels do not decrease in the region immediately adjacent to the A/P border (*) in discs expressing Fu-tail (E), in contrast to the reduced staining of Ci in the ventral region of the same disc (arrow in E), where Fu-tail is not expressed.

not be sufficient to disrupt the proteolytic processing of Ci_{155} to Ci_{75}. Thus, overexpression of the carboxyl-terminal domain of Fu disrupts the HSC in a specific manner that perturbs formation of Ci* but not Ci_{75}.

DISCUSSION

In this study we show that Fu associates directly with Cos2 to form a high-affinity complex, which appears to comigrate with the peak of an endogenous *Drosophila* Fu/Cos2 complex (Peak B). We also show that the carboxyl-terminal domain of Fu is both necessary and sufficient to form this high-affinity association with Cos2. Finally, we test the physiological relevance of this association by demonstrating that overexpression of Fu-tail, in wing imaginal discs, disrupts Hh signaling. These results provide evidence that a targeted disruption of the Fu-Cos2 interaction effectively prevents Hh signaling, demonstrating the physiological importance of the HSC. We propose that Fu-tail is acting in a dominant negative manner in these discs, disrupting the interaction between Cos2 and endogenous Fu, for the following reasons: (i) Fu-tail is sufficient to bind to Cos2, (ii) Fu-tail is able to compete with endogenous wt Fu for binding to Cos2, (iii) overexpression of Fu-tail in a wt background results in phenotypes similar to loss of function fu mutants, and (iv) overexpression of wt Fu yields no phenotype.

Earlier experiments showed that class II *fu* alleles encode carboxyl-terminal truncations of Fu that do not bind Cos2, while class I alleles expressed point mutations or in-frame deletions in the kinase domain that do bind Cos2 (43). Those experiments, however, did not determine if the carboxyl-terminal domain was sufficient for binding. Based on the results presented here, we suggest that the main function of the carboxyl-terminal domain of Fu is to target the kinase domain to Cos2. Cos2 could then act as a substrate of Fu or could in turn present another substrate to Fu. This model would explain the similar phenotypes that result from either class I or class II mutations, at least in the presence of Su(fu) (40, 43). Class I mutant Fu would be unable to phosphorylate its substrate, and class II mutant Fu would be unable to locate its substrate. A similar model, based on experiments that used a recombinant *fu* construct lacking a kinase domain to decrease the severity of a *fu* class II/Su(fu)$^{-/-}$ phenotype, has been previously suggested (58). Alternatively, Fu-tail may also have some other function in vivo that results in a *fu* phenotype.

It has been proposed, based on the distribution of the var-

ious forms of Ci in either *fu* or *cos2* mutants (2, 3, 28, 36, 47, 61, 63), that both Fu and Cos2 regulate Ci processing to either Ci_{75} or Ci*. However, our results indicate that while the expression of Fu-tail in wing imaginal discs blocks the conversion of Ci_{155} to Ci*, it does not appear to block the processing of Ci_{155} to Ci_{75}. Thus, our Ci immunostaining pattern is similar to that described for *fu* class I mutant discs, which display an expanded stripe of Ci_{155} accumulation close to the A/P border (2, 56, 64), whereas *fu* class II mutant imaginal discs appear to accumulate Ci_{155} throughout most of, if not the entire, anterior compartment. We propose the following model to account for our results. When Fu-tail is overexpressed in wing imaginal discs, it displaces endogenous Fu from binding to Cos2. If the kinase domain of Fu is not bound to Cos2, Ci conversion to Ci* is blocked. Absence of Ci* results in a loss of anterior *en* expression and in the reduction or lack of *ptc* upregulation (2, 36, 64). The increased region of Ci_{155} stabilization, observed in wing imaginal discs expressing Fu-tail, would result as a secondary consequence of Hh being able to diffuse farther into the anterior regions of the imaginal disc, as previously suggested (2). However, overexpression of Fu-tail did not appear to affect Ci conversion to Ci_{75} at more anterior regions of the wing imaginal discs, even though wt Fu would also be separated from Cos2 in this part of the disc. These results suggest that the carboxyl-terminal domain of Fu has two functions. In the absence of Hh, the carboxyl-terminal domain of Fu acts to stabilize the HSC, in part with Cos2, and efficiently process Ci to Ci_{75}, as has been recently suggested (27). At high levels of Hh signaling, the carboxyl-terminal domain of Fu is required to target its kinase domain to Cos2. Cos2 could then act as a substrate of Fu or could in turn present another substrate to Fu.

We propose, based on our data and those of others (2, 34, 43, 56), that Fu-tail plays a structural role in maintaining a functional HSC. Given the role that components of the human Hh pathway play in a variety of cancers (10, 18, 23, 25, 65), functional inhibitors of this pathway will be therapeutically beneficial. Therefore, it would be of interest to determine whether disrupting a human FU/COS2 complex with a human FU-tail would inhibit the human Hh signaling pathway. However, while we present a model whereby Fu-tail acts as a dominant inhibitor of Hh signaling, it has been previously suggested that hFU-tail can activate the mammalian Hh pathway (34). This study also showed that hFU and the mammalian Ci homolog GLI2 synergize to activate a Gli-reporter assay, an assay that appeared not to require a functional kinase domain. In contrast, during the revision of the manuscript for this report, another group has shown that the kinase activity of *Drosophila* Fu is required for Ci-dependent transcriptional activity (17). The reasons for these apparent discrepancies between hFu and *Drosophila* Fu remain unknown.

ACKNOWLEDGMENTS

We thank the members of the Robbins' laboratory, A. Capobianco and members of his laboratory, M. Wathelet, and Y. Sanchez (University of Cincinnati) for helpful discussions. We thank the members of the Sanchez laboratory for their help in our yeast two-hybrid work. We thank P. Aza-Blanc and T. Kornberg (University of California, San Francisco) for assistance with confocal microscopy and K. Hill (University of California, San Francisco) for assistance in generating the transgenic flies. We also thank J. M. Bishop (University of California, San Francisco), in whose laboratory part of this work was done.

This work was supported by National Institutes of Health grant CA82628-01 (to D.J.R.) and NCI National Institutes of Health training grant 5T32 ES07250 (to M.A.). D.J.R. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences.

M.A. and K.E.N. contributed equally to this paper.

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