The Fused Protein Kinase Regulates Hedgehog-stimulated Transcriptional Activation in Drosophila Schneider 2 Cells*

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The Drosophila segment polarity gene fused encodes a putative protein-serine/threonine kinase, and plays a critical role in the signal transduction for Hedgehog (Hh)-dependent gene expression. We show that the Drosophila Schneider 2 (S2) cell line has the potential to transduce the Hh-triggered intracellular signals, leading to the activation of target gene expression, when a transcription factor, Cubitus interruptus (Ci), is provided exogenously. Using S2 cells transfected with the Ci-expressing plasmid and a patched promoter reporter construct, we demonstrate that the forced expression of Fused (Fu) stimulates Hh-triggered and Ci-dependent transcriptional activation. The N-terminal kinase domain of Fu is required for this activity, but the C-terminal domain is not. Two kinase-inactive Fu mutants fail to enhance the reporter activation, indicating that the kinase catalytic activity is essential for this function. Negative components of the Hh-signaling pathway, Costal-2 and Suppressor of Fused, strongly antagonize the Fu activity, irrespective of the presence or absence of the Fu C-terminal domain, suggesting an indirect mechanism for the inhibition of Fu by these proteins. Furthermore, mutational analyses of threonine 158 and serine 159, in the activation segment of the Fu protein kinase, indicate that threonine 158 is essential for Fu activity and that phosphorylation of this threonine residue may be involved in the activation of the kinase catalytic activity upon Hh stimulation.

The Hedgehog (Hh) family of secreted factors controls a wide variety of developmental processes in both vertebrates and invertebrates by regulating the proliferation and differentiation of target cells. Drosophila Hh, which was originally identified as a segment polarity gene product, is required for patterning embryonic segment and adult structures such as wing, legs, and eyes (1–3). In mammals, three Hh homologs, Sonic, Desert, and Indian hedgehog, are expressed in a tissue-specific manner and are responsible for morphogenesis of the neural tubes, somites, and other organs (4, 5). The mammalian Hh signaling pathway is also involved in the tumorgenesis of basal cell carcinoma (5, 6).

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1 The abbreviations used are: PKA, protein kinase A; S2, Schneider 2; PCR, polymerase chain reaction; FCS, fetal calf serum; PBS, phosphate-buffered saline; aa, amino acid(s); Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid.

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Fu Function in Hh Target Gene Expression

Fu under the control of the Hh signal (18, 19, 30), although the biochemical basis of this transition is still unclear.

It has been shown that Hh regulates the phosphorylation of downstream signaling proteins, including Fu (31), Cos2 (10), Ci (18, 20, 21), and Smo (32). The phosphorylation of Fu and Smo is induced upon Hh stimulation and appears to represent the active state of these proteins (31–33). Little is known, however, about the biological significance of or the regulatory mechanisms underlying the phosphorylation of these Hh-signaling proteins, except for the PKA-mediated Ci phosphorylation described above. Fu has a typical domain structure for a protein-serine/threonine kinase in its N terminus and has been shown to positively regulate Hh signaling (30, 34, 35). Although the significance of the kinase domain in Hh signaling has been established genetically (36, 37), there is no biochemical evidence indicating that Fu has an intrinsic protein-phosphotransferring activity. Recently, a putative human homolog for Fu was identified (38). The protein kinase domain of human Fu (hFu) shares a high level of homology with that of Drosophila Familia (39), but little homology was found between their C-terminal domains (38).

To elucidate the role of Fu in Hh signaling and Ci activation, it is necessary to analyze the biochemical function of Fu in Ci-dependent transcriptional activation. Previous work indicated that the Hh signal is at least partially transduced in Schneider 2 (S2) cells, because the phosphorylation of Fu is induced by Hh stimulation (31). Here we show that S2 cells have the potential to transduce Hh-triggered intracellular signals to activate target gene transcription when Ci protein is provided exogenously. Using S2 cells transfected with a Ci expression vector and a luciferase-reporter construct, we investigated the mode of action of the Fu protein kinase in Hh signal transduction, and found that forced expression of Fu increased the transcription of the reporter gene. Mutational analysis of the functional domain of Fu suggested that threonine 158 in the activation segment is essential for Fu kinase activity and may be involved in the activating phosphorylation induced by Hh signaling.

EXPERIMENTAL PROCEDURES

Construction of Expression and Reporter Plasmids—Drosophila Ci and Hh cDNAs were generously provided by Drs. R. A. Holmgren and T. Tabata, respectively. Double-stranded cDNAs for Fu (34), Su(fu) (39), and Cos2 (40) were obtained by the reverse transcriptase-primed polymerase chain reaction (PCR) using polyA+ RNA extracted from S2 cells and primer sets for each molecule. The PCR products were cloned into the pBluescript plasmid, and several independent clones were sequenced to exclude cDNA clones containing PCR-derived mutations. The amino acid sequences encoded by these Cos2 and Su(fu) cDNAs were compared above. The amino acid sequence of the Fu cDNA was obtained from the NCBI protein data base. The amino acid sequences encoded by these Cos2 and Su(fu) cDNAs were inserted into the pBluescript plasmid, and several independent clones were sequenced above lysis buffer containing 1.2 mM ATP, 270 mM MgCl2, 1 mM MgSO4, 0.3% Triton X-100, and 6.5 mM dithiothreitol) (46). For the production of Hh-N-Flag—The S2 cells and S2 transfectants were maintained at 24 °C in DES medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). S2 cells (6 × 10^6 cells/cm2 dish) were co-transfected with 36 µg of pDA-Hh-N-Flag and 4 µg of pAct5C (44) plasmids, using the calcium phosphate co-precipitation method with Bess-buffered saline (45), and transfected cells were selected using hygromycin B (0.3 µg/ml) resistance. The stable transfectants expressing Hh-N-Flag (designated as S2HhNF cells) were propagated to a density of 1 × 10^6 cells/ml, fed with fresh medium containing 10% FCS and 0.3 mg/ml hygromycin B, and harvested at day 6 to obtain the conditioned medium. The S2HhNF-conditioned medium was used as crude Hh-N-Flag to stimulate S2 cells in the luciferase assay. An S2 cell line that was established by transfection with pACTHyg alone was cultured in parallel, and the 6-day-old culture medium was used as a control.

For the experiments in Figs. 1E and 2B, Hh-N-Flag protein was partially purified and concentrated. In brief, 2 ml of anti-FLAG M2 affinity gel (Sigma) was added to 600 ml of the conditioned medium from S2HhNF cells, mixed by gentle rotation for 2 h at 4 °C, and recovered by centrifugation at 1,500 × g for 10 min. The affinity resin was washed twice with 10 ml of phosphate-buffered saline (PBS), and the Hh-N-Flag protein was eluted with 2 × 6 ml of PBS containing 0.01% bovine serum albumin and 100 µg/ml FLAG peptide (Sigma). The eluted fractions were combined and concentrated to 1.8 ml using a Centricon YM-10 ultrafiltration device (Millipore).

Cell Culture and Transfection—S2 cells expressing the hyg resistance gene (see above) were used throughout this study for the luciferase assay, as the S2HhNF conditioned medium, which was used for Hh-N stimulation, contained hygromycin B. Cells were plated in a 24-well dish (3.75 × 10^5 cells/well) and transfected with a plasmid mixture, using the calcium phosphate co-precipitation method. Typically, 15 µg of pDA-Flag-Ci and 25 µg of pAct136-Luc plasmids were transfected together with other effector expression constructs. The total amount of plasmid DNA used for each transfection was adjusted to 2.5 µg by adding the vector plasmid (pAct5C0). At 8 h after transfection, the medium was changed to 0.6 ml/well of fresh DES medium with 10% FCS, and 0.15 ml of the S2HhNF conditioned medium or the control conditioned medium. After the cells were incubated at 24 °C for 36 h, they were washed with PBS and lysed in 250 µl of lysis buffer (25 mM Tricine-NaOH (pH 7.8), 0.5 mM EDTA, 0.54 mM Na3PO4, 16.3 mM MgSO4, 0.3% Triton X-100, and 6.5 mM diethiothreitol) (46). For the firefly luciferase assay, 20 µl of the lysate was mixed with 380 µl of the above lysis buffer containing 1.2 mM ATP, 270 µl coenzyme A, and 50 µl luciferin at room temperature, and the luminescence was immediately quantified. Renilla luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). The error bars in the figures indicate standard errors of the mean (S.E.) of two independent transfection experiments.

Immunoprecipitation and Western Blotting—S2 cells were grown in a six-well plate (3 × 10^5 cells/well) and transfected with 20 µg of plasmid DNA as described above. At 12 h after transfection, the medium was changed to fresh DES medium with 10% FCS. The cells were then incubated at 24 °C for 48 h, washed with ice-cold PBS, and lysed in 0.3 ml of Nonidet P-40 lysis buffer (50 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM p-amidinophenylmethylsulfonyl fluoride, 10 µl protease inhibitors/ml aprotinin, and 10 µM leupeptin). The supernatant was recovered after centrifugation at 20,000 × g for 10 min at 4 °C.

For Western blot analysis, cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane filter (Immobilon-P, Millipore). Blotting was performed with monoclonal antibodies specific for Flag (M5, Sigma) and Myc (9E10, Zymed Laboratories Inc.) epitopes or with the mouse anti-Ci
RESULTS

Hh-dependent Transcriptional Activation of a ptc Reporter Gene in S2 Cells—We tested Hh-triggered signal transduction in S2 cells using a reporter construct (ptcΔ136-Luc) that contained a ptc promoter region and firefly luciferase cDNA (18). When the reporter construct alone was transfected into S2 cells, no activation was observed even after Hh-N stimulation (Fig. 1B). We presumed that the inability of S2 cells to respond to Hh might be due to the lack of Ci expression (Fig. 1A), because S2 cells seem to express most of the known Hh-signaling molecules except for Ci (10, 16, 31, 32). We therefore examined the effect of forced expression of Ci on the reporter activity. Transient transfection of S2 cells with an expression plasmid encoding Flag epitope-tagged Ci cDNA resulted in the production of full-length Ci protein (Ci155), which was detected by Western blot analysis using anti-Ci (2A1) and anti-Flag antibodies (Fig. 1A). We found that cotransfection of the ptc reporter with the Ci expression plasmid increased the basal luciferase activity, and that stimulation of the co-transfected cells with Hh-N further enhanced the reporter activity (Fig. 1B). A mutant reporter construct (ptcΔ136-mut) in which the three Ci-binding sites were mutated did not show any response to Ci expression or Hh stimulation, indicating that the induction of the luciferase gene was mediated by the binding of the exogenously expressed Ci protein to the promoter element. An internal control plasmid that expressed Renilla luciferase under the actin 5C promoter produced the same levels of activity irrespective of Ci expression or Hh stimulation (Fig. 1C).

Previous studies showed that the expression of Ci in cultured Drosophila cells resulted in reporter activation without Hh stimulation (18, 20, 49). Indeed, Ci expression increased the basal reporter activity in a dose-dependent manner, but Hh stimulation produced an additional 2–4-fold increase at any Ci dosage tested (Fig. 1D). We also examined the dosage effect of Hh-N on the reporter activation (Fig. 1E). Stimulation of the Ci-transfected S2 cells with Hh-N increased the luciferase expression in a dose-dependent but saturable manner. In the absence of Ci, however, even the highest concentration of Hh-N used in this assay failed to activate the reporter gene, indicating that the ptc reporter activation was strictly dependent on Ci and reflected the extent of Hh signaling. These results demonstrated that the S2 cell line, when supplemented with Ci, has the ability to transduce the Hh signal, leading to transcriptional activation of a target gene.

Forced Expression of Fu Enhances Ci-mediated Reporter Activation—Previous genetic studies have implicated Cos2 and Su(fu) in the negative regulation of Hh-triggered gene activation and suggested that Fi plays a positive role (3). To test whether these components could regulate the ptc reporter transcription in the CI-supplemented S2 cells, expression constructs for the N-terminally tagged proteins, Myc-Cos2, HA-Su(fu), or Flag-Fu, were co-transfected into S2 cells together with the Flag-Ci expression plasmid and ptcΔ136-Luc reporter gene. As seen in Fig. 2A, co-expression of Cos2 or Su(fu) resulted in reduction of the Hh-induced reporter activation. The exogenous expression of Fu, in contrast, significantly increased the reporter activity. The effect of Fu expression was observed only in the Hh-stimulated cells, not in the untreated cells, suggesting that the activation of Fu was dependent on Hh. To better characterize the effect of Fu expression, we examined the reporter activity in response to various doses of Hh-N stimulation. At any Hh-N concentration tested, cotransfection of Fu with Ci resulted in a 2–3-fold increase in luciferase activity, compared with Ci transfection alone (Fig. 2B). In the absence of Ci, expression of Fu alone did not increase reporter

Fig. 1. Hh-dependent transcriptional activation of a Ptc reporter gene in S2 cells. A, S2 cells were transfected with pAct5C0 vector (−) or pDA-Flag-Ci plasmid (Ci), and the cell lysates were analyzed by Western blotting using an anti-Ci (2A1) or anti-Flag (M5) monoclonal antibody. B and C, S2 cells (3 × 10^5 cells/35-mm dish) were co-transfected with 2 μg of firefly luciferase reporter plasmid (ptcΔ136-Luc or ptcΔ136-mut), 18 μg of pAct5C0 (−) or pDA-Flag-Ci, and 0.2 μg of Renilla luciferase reporter plasmid (pDA-RL), and then cultured in the presence (20% S2HhNF-conditioned medium) or absence (20% S2-conditioned medium) of Hh-N. Firefly luciferase (B) and Renilla luciferase (C) activities were assayed as described under “Experimental Procedures.” The results are represented as relative luminescence units (RLU). D, S2 cells grown in a 24-well plate (3.75 × 10^5 cells/well) were transfected with the ptcΔ136-Luc reporter plasmid (25 ng) and the indicated amounts of pDA-Flag-Ci plasmid, cultured in the presence (Hh-N) or absence (−) of S2HhNF-conditioned medium for 36 h, and subjected to the firefly luciferase assay. E, S2 cells were transfected with 2.5 μg of pAct5C0 vector, 25 ng of pActΔ136-Luc, and 25 ng of pDA-Flag-Ci (+Ci) or pAct5C0 (−Ci). At 8 h after transfection, the cells were fed with fresh medium, then stimulated with the indicated concentrations (C) of partially purified Hh-N-Flag. After the cells were incubated for 36 h, luciferase activity in the cell lysates was measured.

monoclonal antibody (2A1, kindly provided by Dr. R. A. Holmgren) (47, 48). The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG antibody (Dako), and the immunolabeled bands were detected using the enhanced chemiluminescence detection system.

For immunoprecipitation of Flag-tagged protein, 25 μl of anti-FLAG M2 affinity gel (Sigma) was added to 0.3 ml of cell lysate, incubated for 2 h at 4 °C with gentle rotation, and washed five times with 1 ml of Nonident P-40 lysis buffer for each washing step. Immunoprecipitation of Myc-tagged protein was carried out similarly, using 5 μg of anti-Myc (PL14, MBL) monoclonal antibody and 25 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech). For Western blot analysis of the Myc-tagged protein, biotinylated antibodies for the Flag (M5, Sigma) and Myc (9E10, BAbCO) epitopes were used together with horseradish peroxidase-streptavidin (Roche Molecular Biochemicals).
activity, even at a saturating dosage of Hh-N. Coexpression of Fu and Ci caused no activation of the mutant reporter (ptc136-mut, data not shown). These results indicate that the overexpression of Fu enhanced the Hh-triggered signal transduction by regulating the function of Ci.

The Kinase Catalytic Activity Is Essential for Fu Function in Ci Activation—The Fu protein consists of an N-terminal protein kinase domain and a C-terminal domain with no obvious homology to known protein motifs (31, 37). To characterize the roles of these domains in the Hh-triggered ptc reporter activation, we constructed several mutant Fu proteins (Fig. 3A). The KA3 mutant carried alanine substitutions of three lysine residues, the putative ATP-binding lysine residue (Lys-33) and two neighboring lysine residues (Lys-28 and Lys-37). The DANA mutant had two alanine substitutions of Asp-125 and Asn-130, both of which are invariably conserved among members of the protein kinase superfamily and are involved in the phosphotransfer reaction (50). Two C-terminally truncated mutants, ΔC1 and ΔC2, lacked aa 523–805 and 306–805, respectively, whereas ΔKD was an N-terminally truncated mutant lacking just the kinase catalytic domain (aa 1–255). All the Fu mutants carried an N-terminal Flag tag, and their expression in transfected S2 cells was confirmed by Western blot analysis using an anti-Flag antibody (see Fig. 4B). As seen in Fig. 3B, the two catalytically inactive (kinase-dead) mutants, KA3 and DANA, failed to enhance the Hh-triggered transcriptional activation of the reporter gene. An additional Fu mutant in which only Lys-33 was replaced with a methionine residue was also inactive (data not shown). Likewise, expression of the ΔKD mutant did not increase the reporter activity. On the other hand, ΔC1 and ΔC2 had an effect on reporter activation similar to wild-type Fu. The positive effect of these C-terminal deletion mutants on reporter activation was still dependent on the kinase catalytic function, because versions of the mutations that included an inactivating mutation in the kinase domain (ΔC1/KA3, ΔC1/DANA, ΔC2/KA3, and ΔC2/DANA) were all inactive. Together, these results indicate that the Fu kinase domain plays a primary role in the Hh-dependent activation of target genes in S2 cells and that its catalytic activity is essential for this function.

Su(fu) and Cos2 Inhibit the Fu Function for Reporter Activation in S2 Cells—Genetic and biochemical studies have demonstrated that three Hh signaling components, Fu, Su(fu), and Cos2, associate with Ci to form a multiprotein complex (10, 12, 14, 15) and regulate Ci function in Hh-dependent gene expression. Several lines of evidence suggest that Su(fu) and Cos2 inhibit Ci function by tethering it in the cytoplasm and/or by promoting its proteolytic processing in conjunction with PKA, whereas Fu promotes Ci activity (15, 18, 19, 25, 26, 30, 51). The modes of action of these molecules within the protein complex, however, are poorly understood. To assess the roles of Fu in Hh-triggered Ci activation with respect to the function of Cos2 and Su(fu), we examined the effect of Su(fu) and Cos2 on the Fu-dependent activation of the ptc reporter gene. Cotransfection of an expression plasmid encoding Su(fu) or Cos2 with the Fu construct resulted in strong inhibition of the ptc reporter activation (Fig. 4A). The expression of unrelated proteins such
The above result, together with the reciprocal immunoprecipitation using an anti-Flag antibody (right panels), indicates that the C terminus of Fu (aa 523–805) associates physically with Cos2, as suggested previously (10). In contrast, we were not able to detect a physical association between HA-Su(fu) and any of the Flag-Fu constructs, despite reasonably good expression of the transiently transfected HA-Su(fu) in the S2 cells (data not shown). We then examined the effect of Su(fu) and Cos2 on the reporter activation mediated by ΔC1 and ΔC2. The expression of Cos2 inhibited the reporter activation induced by any of the three Fu constructs with a similar dose dependence (Fig. 4D), indicating that direct association is not required for Cos2 to inhibit Fu function. Su(fu) also showed dose-dependent inhibition against the three Fu constructs, but ΔC2 was less sensitive to Su(fu) than the others were (Fig. 4E), suggesting that Su(fu) negatively regulates Fu function at least in part through the region deleted in ΔC2 (aa 306–522) in the C-terminal domain.

Thr-158 Is Essential for Fu Function and Likely to Be Involved in the Activating Phosphorylation of Fu—The above results demonstrate that Fu plays a positive role in Hh signaling through its kinase catalytic activity. This leads to a hypothesis that Hh stimulation might activate the protein kinase activity of Fu, as seen in cases of many protein kinases involved in growth factor/cytokine signal transduction. If so, the Fu catalytic activity might be regulated by the phosphorylation of amino acid residues in the activation loop (also called the activation segment) in kinase subdomain VIII (50, 53). This activating phosphorylation could be performed by an upstream protein kinase or in an autokatalytic fashion. To examine this hypothesis, we replaced Thr-158 and Ser-159 with alanine, individually or in combination (Fig. 5A), because these residues lie in the position corresponding to the activating phosphorylation site(s) for many protein kinases. As shown in Fig. 5B, mutation of Ser-159 of Fu (TA mutant) had no effect on the ability to augment the Hh signal, whereas substitution of Thr-158 (AS) resulted in the complete loss of the activity. The doubly mutated Fu (AA) was also inactive. Similar results were obtained with the ΔC1 and ΔC2 constructs containing the same point mutations. These results clearly indicate that Thr-158 is essential for Fu function in activating the ptc reporter but Ser-159 is not, and suggest that phosphorylation of Thr-158 may be involved in the activation of the kinase catalytic activity of Fu by Hh stimulation.

To test this possibility further, we mutated Thr-158 and
Ser-159 to acidic amino acid residues, aspartate and glutamate, singly or in combination, in an attempt to mimic phosphorylated amino acid residues (Fig. 5A). As seen in Fig. 5C, mutants with a single Asp or Glu substitution at either position showed activity that was similar to or slightly weaker than the activity seen with wild-type Fu. In contrast, the DE and ED mutants were twice as active as wild-type Fu when stimulated with Hh-N. Interestingly, expression of DE or ED increased the reporter activity even in the absence of Hh-N stimulation, suggesting that these mutants are constitutively active without the Hh signal, although Hh stimulation was still required to activate the Ci-mediated reporter expression to its maximum level. Furthermore, the introduction of the DE or ED mutation into the ΔC1 and ΔC2 constructs also resulted in increased basal and induced activity (Fig. 5D).

Combining the DE and KA3 mutations resulted in a complete loss of the transcription-enhancing activity of Fu (data not shown), indicating that the effect of the DE mutation was still dependent on the kinase catalytic activity of Fu. Collectively, these results strongly suggest that the kinase catalytic activity of Fu is regulated through its phosphorylation at Thr-158, at least in part. However, the co-expression of either Cos2 or Su(fu) completely inhibited the reporter activation caused by any Fu mutant (data not shown), suggesting that the constitutively active Fu does not predominate over the inhibitory action of Cos2 and Su(fu) in Ci-mediated gene expression.

**DISCUSSION**

In this paper we describe our investigations into the function of Fu in the Hh-triggered Ci-dependent activation of a ptc-luciferase reporter construct in S2 cells. We found that S2 cells could transduce the Hh signal for the transcriptional activation of the ptc gene only when supplemented with the essential transcription factor Ci (Fig. 1). Moreover, the ptc reporter activation is strictly dependent on Hh signaling. These features suggest that S2 cells will be very helpful in further investigations of the Hh-dependent regulation of Ci activity.

**Functional Domains of Fu in Hh-triggered Ci Activation**

The data presented here demonstrate that Fu stimulates Ci-mediated transcription of the ptc reporter gene in S2 cells (Fig. 2). This is the first report showing Fu function in Hh-dependent gene activation in cultured *Drosophila* cells. The stimulatory effect of Fu on the Hh signal was absolutely dependent on the exogenously expressed Ci protein, indicating that Fu requires Ci to exert its function.

Deletion of the Fu C-terminal domain did not alter its activity, whereas deletion of the kinase domain resulted in the complete loss of the activity. Furthermore, none of the kinase-dead mutants (KA3 and DANA in the full-length, ΔC1, and ΔC2 constructs) showed any effect on Hh-dependent reporter activation (Fig. 3). These results demonstrate that the kinase catalytic activity of Fu is essential for its stimulatory effect on Hh signaling in S2 cells. We showed a physical interaction between Fu and Cos2 in S2 cells using co-immunoprecipitation-Western analysis (Fig. 4). The full-length Fu and AKD mutant bound Cos2, and ΔC1 did not, indicating that the C-terminal region (523–805) is critical for the interaction with Cos2, as suggested previously (10, 14). However, the expression of Cos2 strongly inhibited the Fu-mediated reporter activation, irrespective of the existence of this C-terminal region. This result implies that the C-terminal domain is not essential for the negative regulation of Fu function by Cos2. In contrast, Su(fu) may antagonize Fu function partly via the region (aa 306–436)
in the C-terminal domain, because ΔC2 was less sensitive to Su(fu) than ΔC1, although we could not detect a physical association between Fu and Su(fu) in S2 cells.

Our results demonstrated that the Fu C-terminal domain is dispensable for reporter activation in S2 cells, but genetic studies have unequivocally shown that the C-terminal domain is essential for Fu function, i.e. the class II fu mutants, most of which have a C-terminal deletion like ΔC1 and ΔC2, show the same phenotype as the kinase-inactive class I and null mutants in the wild-type background (36). Subsequent analyses of numerous fu alleles have suggested that the Fu C-terminal domain is involved in regulating the N-terminal catalytic domain (31). In contrast, other studies have suggested that, in the absence of the Hh signal, the Fu C-terminal domain regulates Ci stability and/or the formation of the Ci75 repressor, independent of its kinase catalytic function (26, 51). Recently, Murray and co-workers (38) identified a putative human homolog of Fu and tested its function in the gene activation mediated by the Gli family transcription factors. The expression of human Fu stimulated Glis2-mediated transcription in mammalian C3H10T1/2 cells. In contrast to our results with Drosophila Fu, neither the kinase catalytic activity of human Fu nor the kinase domain itself appeared to be required for Glis2 activation, but the C-terminal domain seemed to be responsible (38). Although the reason for this apparent discrepancy between Drosophila Fu and human Fu is not obvious, we note that the analysis of hFu function was performed completely in the absence of Hh stimulation, and therefore the authors might have observed a kinase-independent function of the C-terminal domain in cells that were not responding to the Hh signal. Taken together, these results and ours suggest that the Fu C-terminal domain supports the integrity of the Hh signaling complexes containing Fu, Cos2, Su(fu), and Ci (10, 14, 51), and also plays a role in regulating Ci function that is nearly independent of its kinase activity in cells that do not receive the Hh signal.

Fu Function in Hh Target Gene Expression

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Our studies suggest that the Hh signal is mediated at least in part through the activation of the catalytic activity of the Fu protein kinase. In S2 cells, the ΔC2 mutant is as active as wild-type Fu and is still dependent on Hh stimulation, suggesting that an upstream signaling event acts on the kinase domain and activates its catalytic activity. A common mechanism for the regulated activation of protein-serine/threonine kinases is the phosphorylation of one or more Ser/Thr residues in the activation segment (50, 53). Analysis of alanine substitution mutants of Thr-158 and Ser-159 indicated that Thr-158 is essential for Fu activity and suggested that Fu kinase catalytic activity may be regulated by phosphorylation at Thr-158 (Fig. 5). This hypothesis is further supported by the fact that the introduction of acidic amino acid residues into this position augmented both the basal and Hh-induced Fu activities in our reporter assay. Although Ser-159 is unlikely to be essential as an site for activating phosphorylation, the acidic substitution of both Thr-158 and Ser-159 was required for the generation of constitutively active mutants. A single carboxyl residue may not fully mimic the dianionic phosphothreonine residue, and an additional negative charge may be required to generate an active conformation (53). The ptc reporter was activated by co-transfection with the DE or ED mutants, even in the absence of Hh-N, but the activity was further enhanced upon Hh-N stimulation. This result suggests that some additional modification, presumably the phosphorylation of other sites, may be required for the full activation of the Fu protein kinase, or that some other Fu-independent events, regulated by Cos2, Su(fu), or PKA, may cooperate with Fu to activate Ci. Interestingly, the activating segment including Thr-158 and Ser-159 is one of the most conserved regions (75% amino acid identity) between the Drosophila and human Fu amino acid sequences, suggesting that a similar phosphorylation-dependent mechanism is conserved in the activation of human Fu.

Possible Fu Function for Ci Regulation in Hh Signal Transduction—The data presented in this paper suggest that the Fu protein kinase that is activated upon Hh stimulation phosphorylates some target protein(s), leading to transcriptional activation by Ci. Recent studies have demonstrated that the Hh signal controls Ci function via at least two posttranslational mechanisms: the proteolytic conversion from the full-length Ci155 to a repressor, Ci75, which is triggered by phosphorylation by PKA, and the active translocation and accumulation of Ci155 into the nucleus, which is regulated by Su(fu) and Cos2 (15, 18, 19, 25, 26). Several lines of evidence suggest that Fu, in conjunction with Su(fu), is involved in Hh-dependent nuclear transport (19, 26). If so, a possible target of phosphorylation by Fu could be Su(fu), and Fu would oppose the action of Su(fu), perhaps by promoting its dissociation from the Ci155 complex (30, 37). Similarly, Fu may phosphorylate Cos2 and antagonize its negative role in Ci regulation (3). However, the fact that co-expression of Cos2 or Su(fu) strongly inhibits Fu (Fig. 4) may suggest that the catalytic phosphorylation by Fu cannot completely account for the mechanism of inactivation of these proteins, and suggests that the release of Ci from negative regulation by these molecules may be a prerequisite for Fu-mediated activation of Ci. There appears to be another Hh-dependent mechanism for Ci regulation, which stimulates its transition (also called maturation or activation) from the relatively stable, inactive form of Ci155 into a short-lived, transcriptional activator. Therefore, an alternative possibility is that Fu is involved in this “activation” step of Ci155, which may be independent of its translocation into the nucleus (18, 30). In this scenario, Fu may directly phosphorylate Ci155 to modulate its interaction with other transcriptional machinery, or indirectly regulate the Ci activation via the phosphorylation of an unidentified regulator of Ci.

In summary, we propose that the kinase catalytic domain of Fu is activated by Hh-dependent phosphorylation at least on Thr-158, and in turn phosphorylates target proteins to transduce the Hh signal for Ci activation. This activating phosphorylation of Fu could be performed either by an upstream protein kinase or in an autocatalytic fashion. A biochemical protein kinase assay is necessary to unequivocally elucidate the function and regulatory mechanism for Fu, but our attempts to measure the protein-phosphotransferring activity of Fu have been unsuccessful, as previously described for both Drosophila and human Fu (10, 31, 38). We expect that the development of constitutively active Fu mutants could help to solve this difficulty and would be useful for elucidating the mechanisms of Hh signal transduction leading to specific gene activation.

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Fu Function in Hh Target Gene Expression

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