Hedgehog-stimulated Phosphorylation of the Kinesin-related Protein Costal2 Is Mediated by the Serine/Threonine Kinase Fused

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The Hedgehog (Hh) signaling molecule is required for the development of numerous tissues in Drosophila. Within the cell, Hh signal transduction utilizes a large protein complex consisting of the Fused (Fu), Costal2 (Cos2), and Cubitis interruptus (Ci) proteins, but the functional interactions between these proteins are still largely uncharacterized. Using a baculovirus system, we demonstrate that the serine/threonine kinase Fu phosphorylates the kinesin-like protein Cos2 when coexpressed with Cos2. Coexpression of Cos2 and a kinase-inactive version of Fu eliminates the majority of Cos2 phosphorylation. We then show that the primary Fu-induced phosphorylation site of Cos2 is serine 572, whereas serine 931 is phosphorylated to a lesser extent. Mutation of serine 572 to alanine eliminates most, but not all, specific phosphopeptides of Cos2 when coexpressed with Fu. We also demonstrate that the phosphorylation pattern of Cos2 produced by baculovirus coexpression with kinase-dead Fu is almost identical to the phosphorylation pattern of Cos2 isolated from unstimulated S2 cells. Finally, the phosphorylation pattern of Cos2 produced by baculovirus coexpression with wild-type Fu is almost identical to that of Cos2 isolated from S2 cells stimulated by Hh, indicating that phosphorylation of serines 572 and 931 is a genuine Hh signaling event. This study clarifies the unique functions of Fu and Cos2 in Hh signal transduction and identifies only the second known phosphorylation site of a kinesin-like molecule.

The secreted products of the Hedgehog (Hh) family of cell signaling proteins play a key role in patterning a great many tissues in both vertebrates and invertebrates (1–7). Vertebrate Hh homologs and members of its signaling pathway are further involved in the generation of certain types of cancer and several genetic syndromes in humans (8–18). Hence, elucidating the mechanism of Hh signal transduction is of interest not only in the context of development but also with respect to oncogenesis and disease.

Studies have revealed that Hh utilizes very unusual signal propagation and transduction mechanisms. Hh is a secreted polypeptide that is thought to be bound to the cell surface via covalently attached cholesterol and palmitic acid moieties (4, 7, 19–21). Hh binds to the product of the patched (ptc) gene, which encodes a multiple pass transmembrane protein, and this binding appears to limit the extent of Hh signaling (22–24). Loss of ptc causes ectopic expression of Hh target genes, indicating that ptc is a negative regulator of the Hh signaling pathway (25–27). The transmembrane protein encoded by the smoothened (smo) locus is thought to be the positive transducer of the Hh signal, since loss of smo causes a loss of Hh target gene expression, regardless of the presence of Ptc (28, 29). Since Ptc is a negative regulator of the pathway that binds Hh, whereas Smo is a positive regulator, it is thought that Ptc inhibits Smo and that binding of Hh to Ptc inhibits Ptc and thus relieves its inhibition of Smo.

How the Hh signal is transduced after Smo is activated to cause transcription is a question that is only now being clarified. The distal component of the Hh signaling pathway is Cubitis interruptus (Ci), a zinc finger transcription factor (30–34). In the absence of Hh stimulation, Ci is normally cleaved from its full-length 155-kDa form to a smaller N-terminal fragment called Ci75, or CiRep (35). Ci75 lacks the transcriptional activation domains that are found C-terminal to the cleavage region and is localized to the nucleus, where it represses transcription of Hh target genes (30, 35–37). Hh stimulation blocks this cleavage and leads to an accumulation of full-length Ci155, which, possibly dependent on further, uncharacterized, post-translational modifications, can then act as a transcriptional activator (35, 36, 38, 39).

Five cytoplasmic proteins in addition to Ci are known to play a role in transducing the Hh signal: Fused (Fu), Costal2 (Cos2), Suppressor of Fused (Sufu), supernumerary limbs (slimb), and Drosophila protein kinase A. Fu encodes a novel serine/threonine kinase with no particular homologies to other kinases (40, 41). Cos2 encodes a kinesin-like protein (42), whereas Sufu encodes a novel protein of unknown function (43, 44) and slimb encodes a member of the F-box family of proteasomal targeting proteins (45). In the absence of Hh stimulation, Fu and Cos2 are basally phosphorylated and, together with Ci, form a high molecular weight protein complex that binds to microtubules. In the presence of Hh stimulation, Fu and Cos2 are hyperphosphorylated, and the complex only weakly binds to microtubules (42, 46, 47). The kinase activity of
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Fu is required for Hh signal transduction, but the substrate(s) phosphorylated by Fused has not been identified (38, 39, 47).

To ascertain the substrate of Fu, we have utilized a baculovirus coexpression system that recapitulates the binding of the intracellular components of the Hh signaling pathway (48). In this coinfection system, Fu kinase activity appears to be constitutive and does not require Hh stimulation to be activated. Using this system, we have identified the Cos2 protein as a substrate of the Fu kinase and found that serine 572 in Cos2 is the major Fu-induced phosphorylation site, whereas serine 931 is phosphorylated to a lesser extent. We have further shown that coinfection of a kinase-dead Fu with Cos2 prevents almost all Cos2 phosphorylation. Finally, we demonstrate that the phosphorylation pattern of Cos2 isolated from unstimulated S2 cells matches that of Cos2 produced by coinfection with a kinase-dead Fu. Conversely, the phosphorylation pattern of Cos2 isolated from S2 cells stimulated by Hh matches that of Cos2 obtained from a wild-type Fu coinfection in S21 cells. Hence, the hyperphosphorylation of Cos2 that occurs during Hh stimulation is principally due to serine 572 and 931 phosphorylation by Fu and further suggests that Cos2 phosphorylation is the cause of the reduced microtubule binding of the Fu-Cos2-Ci complex seen during Hh stimulation. This represents a unique use of both a kinase and a kinesin in a signal transduction pathway.

Experimental Procedures

Constructs, Mutagenesis, and Cloning—A full-length Cos2 cDNA was the kind gift of J. Sisson, K. Ho, and M. Scott (Stanford University), and the D6 Fused cDNA was the gift of P. Theron. pKN5 was created as a shuttle vector by excising the KpnI/SacII fragment from pBlueprint KS(−) (Stratagene) and inserting a new multiple cloning site with a central stop codon in frame with all restriction sites (details available upon request). BamHI/SalI-cleaved Cos2 was cloned into the BamHI/NotI sites of pKN5 to give a Cos2 with an in frame stop codon, pKN5Cos2.

Mutagenesis of single and multiple residues of Fu and Cos2 was accomplished using PCR-based site-directed mutagenesis (using Pfu polymerase (Stratagene)) on the full-length Fu cDNA, D6, or, in the case of Cos2, pieces of the Cos2 cDNA encompassing the site to be mutated cloned into pKN5 or pBlueprint KS(−) (Stratagene) and inserting the plasmid into mammalian (293T) cells using Lipofectamin (Gibco BRL). For each virus, a plaque-purified stock was created, and multiplicity of infection ranging from 3.5 to 4.0 for each virus, resuspending the cells, and incubating the cells at 28 °C for 1 h, shaking every 10 min. The infected cells and medium were then plated out on 10 plates, and 12 ml of Grace’s medium was added. All baculoviruses were confirmed to be expressing the appropriate protein by immunoblotting.

Cell Culture and [32P]Orthophosphate Labeling—S2 cells, S2-Hh-N cells, and S2-Hh cells were cultured in supplemented Grace’s medium with or without 5 mM benzamidine-HCl, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After 4 days of infection, cells were lysed and immunoprecipitated as above. Each tryptic map represents the Cos2 immunoprecipitated from 12 10-cm plates. S21 cells were labeled by infecting 12.5 × 106 cells/10-cm tissue culture plate as above and then 24 h later washing the plates with 5 ml of phosphate-free Grace’s medium, followed by the addition of 5 ml of phosphate-free Grace’s medium, supplemented with 10% dialyzed fetal bovine serum, 1× penicillin/streptomycin, and [32P]orthophosphate to 0.4 μCi/ml (PerkinElmer Life Sciences). Plates were then incubated for 1 h at room temperature, followed by a 5-ml wash of phosphate-free Grace’s medium. Lysis and immunoprecipitation were then conducted as above. Immunoprecipitates were run out on 8% SDS-PAGE gels and autoradiographed.

Phosphoamino Acid and Phosphotryptic Peptide Analysis—Radiolabeled Cos2 proteins was excised from SDS-PAGE gels and cut into small pieces. Phosphoamino acid analysis was conducted as described (49). For phosphotryptic peptide analysis, these fragments were placed in tubes and washed twice for 30 min each with 100 μl of 50 mM ammonium bicarbonate, pH 8.0, 50% acetonitrile. The gel fragments were then digested with the fungus humicola (Promega) by rehydration and incubation for 1 h at 55 °C in 50 mM ammonium bicarbonate supplemented with 10 mM dithiothreitol. The dithiothreitol solution was removed, and the fragments were acetylated by incubation for 45 min with 50 mM ammonium bicarbonate plus 55 mM iodoacetamide. The fragments were washed twice for 10 min each with 50 mM ammonium bicarbonate and then twice for 30 min each with 50 mM ammonium bicarbonate plus 50% acetonitrile and dried overnight in a vacuum (Speed-Vac) before the fragments were rehydrated with 150 μl of 50 mM ammonium bicarbonate plus 5 μg/ml modified trypsin (Promega) and incubating for 24–48 h at room temperature. Peptides were then eluted in two 300-μl washes of 60% acetonitrile plus 0.1% trifluoroacetic acid in distilled H2O. Eluates were combined and dried down in a Speed-Vac (Savant). The peptides were washed and dried in a Speed-Vac twice with 500 μl of distilled H2O before resuspension in pH 1.9 buffer. The peptide solution was applied to thin layer chromatography plates, and phosphotryptic peptide analysis was carried out as described by Doyle et al. (49), using pH 1.9 buffer for TLE in a HTLE-7000 apparatus (CBS Scientific) and isobutyric acid buffer for chromatography.

HPLC Analysis and Edman Sequencing of Radiolabeled Proteins for Position and Sequence—Radiolabeled Cos2 protein was excised from 8% SDS-PAGE gels (either unfixed or fixed and Coomassie-stained), cut into pieces, and digested with trypsin or endoproteinase Lys-C as described (50). The trypsin-digested peptides were then subjected to reverse phase HPLC on a microbore C8 column (Vydac, Hesperia, CA), and an aliquot of each collected fraction was scintillation-counted.

Individual radiolabeled fractions were subjected to covalent Edman degradation on Sequelon AA membranes (Perseptive Biosystems, Cambridge, MA) with a PerkinElmer Life Sciences model 492 protein sequencer. The anilinothiolinolino-amino acids were extracted from the membranes with undiluted trifluoroacetic acid and scintillation-counted.

Edman degradation for sequence was conducted similarly as above, but the HPLC elution gradient was shallower to allow better separation of the peptides, and the anilinothiolinolino-amino acids were eluted using standard conditions instead of undiluted trifluoroacetic acid and sequenced on a PerkinElmer model 492 protein sequencer. Amino acids found at each position were then compared with the known Cos2 sequence, and candidate peptides were identified based on a presence of at least three sequentially matching amino acids.
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RESULTS

The Time Courses of Fu and Cos2 Phosphorylation Are Similar, and Cos2 Is Phosphorylated Predominantly on Serine—We have previously shown that Fu and Cos2 are both phosphorylated in the absence of Hh stimulation and that both become hyperphosphorylated when S2 cells are stimulated by Hh (46). It has further been shown that Fu is hyperphosphorylated at \( \sim 30 \) min after Hh stimulation (47), but the time course of Cos2 phosphorylation has not been characterized. Since Fu and Cos2 bind tightly to one another (46), Cos2 is an obvious candidate for a Fu substrate, but if Cos2 were phosphorylated prior to Fu, this might suggest that Fu was not responsible for the phosphorylation of Cos2. It therefore intrigued us when we found that Cos2 from S2 cells was phosphorylated between 30 and 45 minutes after treatment with Hh-N conditioned media, a profile temporally similar, but somewhat delayed, to that of Fu, which starts to become phosphorylated between 15 and 30 min after Hh-N treatment (Fig 1A) (47). Interestingly, the slower migrating form of Cos2 found in cells treated with Hh-N conditioned medium for 45–60 min was intermediate in electrophoretic mobility between the non-Hh-stimulated form and the chronically Hh-stimulated form found in S2-Hh cells. This may be due to differential kinetics of phosphorylation of the two Fu-induced phosphorylation sites of Cos2 (see below).

Phosphoamino acid analysis of radiolabeled Cos2 from S2 cells, S2 cells expressing Hh constitutively, and S2 cells constitutively expressing Hh-N, further demonstrated that Cos2 was predominantly phosphorylated on serine in all cases (Fig 1B). The fact that Fu is a serine/threonine kinase and the observation that Cos2 appears to be phosphorylated very soon after Fu confirmed that Fu was a strong candidate for the Cos2 kinase and led us to attempt more directed assays of Fused's ability to phosphorylate Cos2.

Costal-2 Is Hyperphosphorylated in a Fu/Cos2 Baculovirus Coexpression System, and This Phosphorylation Is Dependent on Fu Kinase Activity—We first attempted to determine whether Fu phosphorylates Cos2 using radiolabeled Fu and Cos2 immunoprecipitated from S2 cells and embryos, but found that the degree of radiolabeling was insufficient for mapping (data not shown). In vitro kinase assays using immunoprecipitated or in vitro translated Fu and Cos2 were also not successful (data not shown). We therefore looked for an alternate system with which to ascertain if Cos2 is a Fu substrate and to identify the phosphorylation site(s) of Cos2. Baculovirus expression systems, in addition to allowing expression of large quantities of proteins, have been shown to recapitulate normal protein-protein interactions and catalytic events, such as phosphorylation, methylation, and glycosylation (51–59). Furthermore, it has previously been shown that baculovirus-produced Fu and Cos2 maintain their normal physical interaction when coinfected together (48).

When SF21 cells were infected with Fu and Cos2-His\(_6\)-tagged baculoviruses, either alone or in combination, radiolabeled, and precipitated with a Ni\(^{2+}\)-agarose or an anti-Fu antiserum, both
Fu and Cos2 were phosphorylated, and Cos2 from a Fu coinfection had a slower electrophoretic mobility than Cos2 from a Cos2 single infection (Fig. 1C). This slower mobility was similar to that of Cos2 from S2 cells stimulated by Hh and seemed to correlate with an increased level of phosphorylation of the Cos2 from the coinfection as compared with Cos2 from the single infection (Fig. 1C, right two lanes with samples diluted 1:5).

To demonstrate that Fu was actually responsible for the increase in phosphorylation and reduction in electrophoretic mobility of Cos2, we used a baculovirus expressing Fu with a G13V mutation in the kinase domain (FuM) and coexpressed it with Cos2. The Fu G13V mutation has been shown to bind to Cos2 (74) and removes a conserved ATP binding residue that should inactivate Fu kinase activity. When FuM was coexpressed with normal Cos2, the amount of radiolabeled phosphate incorporated into Cos2 was reduced, and it no longer showed reduced electrophoretic mobility (Fig. 1, D and E). To be sure that this result was not an effect of the amount of Fu protein expressed (since we used Fu to immunoprecipitate Cos2), we Coomassie-stained the gel after autoradiography. The amounts of Fu and FuM expressed in coinfections with Cos2 were roughly the same, as were the amounts of Cos2 that were immunoprecipitated with the Fu and FuM (Fig. 1E). Similar results were obtained when the same infections plus a Cos2 single infection were immunoprecipitated with an anti-Cos2 antisemur (Fig. 1F).

When Fu was expressed by baculovirus either alone or in combination with Cos2, it always displayed forms of two different electrophoretic mobilities (Fig. 1, D and E). These two forms appeared to correspond to the unshifted and shifted forms of Fu seen in embryonic and cellular extracts, the latter of which is associated with Hh stimulation (46, 47). FuM, however, appeared to incorporate almost no phosphate into itself, despite its ability to bind Cos2 (Fig. 1, D and E). This result indicated that Fu is capable of autophosphorylating itself in the absence of Hh stimulation in infected Sf21 cells.

**Mapping of Cos2 Phosphorylation Sites**—As Fu/Cos2 coinfections in baculovirus seemed to recreate the hyperphosphorylation of these proteins normally found in response to Hh stimulation in vivo, we used this system to map the Fused-induced phosphorylation sites of Cos2. To do so, we produced large quantities of radiolabeled Cos2 by coinfection of Fu and Cos2 in Sf21 cells followed by radiolabeling of the infected cells. This radiolabeled Cos2 was then isolated on SDS-PAGE gels, digested with either trypsin or Lys-C proteases (trypsin cleaves after arginine or lysine; Lys-C cleaves only after lysine), and analyzed using HPLC and scintillation counting. We used this analysis to compare the peptide elution profiles of radiolabeled, trypsin-digested Cos2 produced from Fu/Cos2 versus FuM/Cos2 coinfections and hence determine which radiolabeled fractions were attributable to Fused kinase activity. Comparing the profiles of these two coinfections demonstrated that Cos2 tryptic HPLC fractions 22, 25, and 27 were only radiolabeled when Fu retained kinase activity, whereas the other fractions were radiolabeled whether or not Fused kinase activity was present (data not shown). We also compared the radiolabeled fraction profiles of Cos2 from a Fu/Cos2 coinfection digested with trypsin with that digested with Lys-C to see if the profiles changed in response to the differing specificities of the proteases. The Lys-C digestion profile was more complex than that of the tryptic profile, but the Lys-C profile also generated radiolabeled fractions 22, 25, and 27 that were specific to Fu activity (data not shown).

To gain further information on the identity of the radiolabeled peptide(s) in each of the Fu-specific fractions, we subected each of these fractions to Edman degradation with stringent wash conditions (see “Experimental Procedures”) to determine the position of the radiolabeled residue relative to the N terminus of the peptide(s). When Edman degradation for position was conducted on radiolabeled fractions 22, 25, and 27 from the tryptic and Lys-C digests of Cos2, we found that, for both digests, the strongest radiolabeled fraction was fraction 27 and the strongest radiolabeled position in fraction 27 was position 5 (Fig. 2, A and B). Fraction 27 from the Lys-C Cos2 digest also revealed a radiolabeled position 9, although the signal for this position was less than that for position 5 (Fig. 2, A and B). In addition, fractions 22, 25, and 27 from the trypsin digest all had radiolabeled position 1, and the trypsin digest of fraction 22 gave a small signal at position 5. The only radiolabeled position that could be found in fraction 22 from the Lys-C digest was in position 4, and no radiolabeled position could be

![Cos2 Peptides, Trypsin](A)

**Fig. 2.** Positioning of radiolabeled phosphoamino acids relative to the N termini of Cos2 phosphopeptides. A, location of radiolabeled residues in the three early eluting fractions of a trypsin digest of radiolabeled Cos2 from a Fu/Cos2 coinfection. The x axis shows the residue number from the N terminus of peptide. Fraction 27 gave a strongly radiolabeled position 5, whereas fraction 22 gave a weakly radiolabeled position 5. Large numbers of counts came off in the first Edman cycle for all fractions. Fraction 25 did not give any other radiolabeled positions. The radiolabeling at position 6 found in both the Lys-C and trypsin digests is probably the “shoulder” of the signal from position 5. B, location of the radiolabeled residue in the three early eluting fractions of a Lys-C digest of a Fu/Cos2 coinfection. Fraction 27 gave a strongly radiolabeled position 5, as found in the trypsin digest and also gave radiolabeled position 9. Fraction 22 only gave a radiolabeled position 4, while fraction 25 gave no radiolabeled position.
found in Lys-C fraction 25 up to 11 amino acids from the N terminus of the peptide. In all cases, the amount of radiolabeling at the other positions in these fractions was much less than that found at position 5 in fraction 27. These data also indicated that the radiolabeled peptide(s) in fraction 27 had a lysine N-terminal to the cleavage site, since radiolabeled position 5 was identified whether the fragments were generated by trypsin or Lys-C digestion. In searching for the Fu-induced phosphorylation site(s) of Cos2, we therefore focused on theoretical tryptic peptides with a N-terminal lysine and a serine 5 amino acids from the N terminus of the peptide.

After determining the N-terminal cleavage site and radiolabeled residue position, we sought to identify the actual peptide using Edman degradation for amino acid sequence. Conducting Edman degradation on the major radiolabeled fraction, we found several different amino acids in each position, indicating a mix of peptides in the fraction (Fig. 3A). By searching theoretical Cos2 tryptic digest products for amino acids that had matching identity and position (relative to the peptide N terminus) to those amino acids found in the radiolabeled fraction digest, we were able to assign almost all of the amino acids found in the fraction digest to theoretical trypsin digestion products of Cos2 (Fig. 3B). Further, using the criteria that the peptides had to have at least three matching, consecutive amino acids to those found in our fraction sequencing, we narrowed the number of candidate peptides to four. Three of these theoretical peptides have three amino acids in the same position as the residues that we found in our sequencing, but none have a serine in position 5. However, the theoretical tryptic peptide containing amino acids 568–575 has three consecutive and four total amino acids that matched our sequencing results. This peptide also contains a serine at amino acid 572, position 5 relative to the N terminus of the peptide, and a lysine before the cleavage site. A serine was not found in position 5 in our Edman degradation for sequence, which is expected if this serine is phosphorylated; phosphoamino acids are very difficult to release using standard Edman sequencing elution conditions, and this is why harsher conditions are used to elute phosphoamino acids when determining radiolabeled residue position. Thus, serine 572 of Cos2 appeared to be the best candidate for a Fu-induced phosphorylation site in baculovirus.

**Mutation of Serine 572 to Alanine Eliminates Three of the Primary Cos2 Phosphotryptic Peptides**—To confirm that serine 572 was the primary Fu-induced phosphorylation site of Cos2 in baculovirus, we made a baculovirus expressing a Cos2 with a S572A mutation. We then compared phosphotryptic peptide maps of radiolabeled Cos2 S572A with Cos2 from Fu coinfections. There were six major phosphopeptides of differing intensities found in phosphotryptic peptide maps of Cos2 from a Fu/Cos2 coinfection. These phosphopeptides are labeled 1–6 and are shown in the actual map in Fig. 4A and schematically in Fig. 4F. The phosphotryptic peptide map of Cos2 S572A from a Fu/Cos2 S572A coinfection only contained phosphopeptides 1, 3, and 6. Phosphopeptides 2, 4, and 5 were missing from Cos2 S572A coinfections, indicating that these phosphopeptides were most likely tryptic peptide fragments containing serine 572 (Fig. 4B).

Since mutation of serine 572 to alanine only eliminated three of the six major phosphopeptides, we wanted to confirm that the other three phosphopeptides were phosphorylated by Fu. To determine this, we compared the phosphotryptic peptide maps of Cos2 from a FuM/Cos2 coinfection and from a single Cos2 infection with that of Cos2 from the Fu/Cos2 coinfection (Fig. 4, A, C, and D). Phosphotryptic peptide maps of Cos2 from the FuM coinfection and the Cos2 single infection only showed phosphopeptide 1 when compared with Cos2 from a Fu/Cos2 coinfection. However, the radiolabeling of phosphopeptide 1 was much weaker in Cos2 single infections than in FuM/Cos2 coinfections. The fact that only peptide 1 remained while Fu kinase activity was negated in these coinfections indicated that phosphopeptides 2–6 of Cos2 were phosphorylated by Fu, whereas phosphopeptide 1 was phosphorylated by an endogenous Sf21 cell kinase whose activity on Cos2 was at least partially dependent on Fu binding to Cos2. Thus, phosphopeptides 3 and 6 were also attributable to Fu kinase activity, and it was therefore likely that there was one other Fu-induced phosphorylation site on Cos2 (see below).
Verification of Fused-induced Phosphorylation of Serine 572 in S2 Cells

To ascertain the physiological relevance of the sites we identified in baculovirus, we sought to demonstrate that the phosphotryptic peptide map of Cos2 from radiolabeled S2 cells was similar to that of Cos2 from baculovirus coinfections. Fig. 5 shows a comparison of the phosphotryptic peptide maps of Cos2 from S2 cells, S2 cells constitutively expressing Hh, a Fu/Cos2 coinfection, and a FuM/Cos2 coinfection. Cos2 from unstimulated S2 cells contained only one phosphorylated peptide, which, by position relative to the small amount of undigested material above the origin, appeared to correspond to phosphopeptide 1 in the phosphotryptic peptide map of Cos2 from baculovirus coinfections (Fig. 5, compare A and B). Cos2 from S2 cells constitutively expressing Hh contained five phosphopeptides, which closely corresponded to phosphopeptides 1–5 found in the phosphotryptic peptide maps of Cos2 from a Fu coinfection (Fig. 5, compare C and D). Only phosphotryptic peptide 6 was missing from the S2-Hh Cos2 map, indicating that it did not contain a physiologically relevant phosphorylation site. Thus, the major Cos2 phosphopeptides found in Fu/Cos2 baculovirus coinfections match those found in S2 cells constitutively expressing Hh, indicating that the Hh-induced phosphorylation sites of Cos2 match those produced by Fu/Cos2 baculovirus coinfection. Mixing experiments subsequently confirmed that the phosphopeptides found in Cos2 from S2-Hh cells were the same as those found in Cos2 from a Fu/Cos2 coinfection (Fig. 6, A–C). There are one or two other faint phosphopeptides that vary between these two maps, but overall the phosphotryptic peptide maps are remarkably similar, leading us to conclude that the phosphorylation sites of Cos2 identified from baculovirus coexpression of Cos2 and Fu are nearly identical to those found in S2 cells stimulated by Hh and, by extension, to those occurring in vivo.
probably a digestion variant of peptide 5. Which had a lower resolution than previous experiments. Peptide 7 is missing but is most likely merged with peptide 2 in this experiment, numbered as in Figs. 4 and 5. Peptides 1, 2, 4, and 5, which we found in tated with Fu from a Fu/Cos2 coinfection. Phosphotryptic peptides are apparent.

FIG. 6. Mixing experiment. A, PTP map of Cos2 immunoprecipitated with Fu from a Fu/Cos2 coinfection. Phosphotryptic peptides are numbered as in Figs. 4 and 5. Peptides 1, 2, 4, and 5, which we found in our separate PTP analysis, were found in this experiment. Peptide 3 is missing but is most likely merged with peptide 2 in this experiment, which had a lower resolution than previous experiments. Peptide 7 is probably a digestion variant of peptide 5. B, PTP map of Cos2 immunoprecipitated from S2-Hh cells. Phosphopeptides 1, 2, 4, 5, and 7 are apparent. C, PTP map of an equal mix of A and B. The overlap of the two maps demonstrates that the Cos2 phosphotryptic peptides from baculovirus coinfections and Hh-stimulated S2 cells are the same.

Localization of a Second Fused-induced Phosphorylation Site in Cos2—As noted above, fraction 27 of the Lys-C, but not trypsin, digest contained a radiolabeled position 9 whose pro-duction in coinfections required Fu kinase activity (Fig. 2, A and B). None of the candidate peptides shown in Fig. 3B, however, have more than 8 amino acids. Further, no derivat-ives of these peptides with C-terminal extensions, as might be caused by incomplete protease cleavage, would have a serine in position 9. We therefore looked for theoretical Lys-C peptides of Cos2 that had a serine at position 9. Six theoretical peptides fulfill these criteria. Two of these peptides are 70 amino acids or greater in size and were discounted as unlikely to travel through the column in the early eluting fractions. Two others had arginines between the lysine and the serine that should have given a radiolabeled residue 3 in a tryptic digest, a position we never found phosphorylated in any fractions. One had no arginines between the lysine and the serine and so should have given a radiolabeled position 9 in the tryptic digest, which we did not observe. This left one peptide, containing amino acids 923–942 with a serine at amino acid 931, as the best candidate for the Cos2 peptide with a phosphoserine in position 9. Since this peptide has an arginine at amino acid 930, this peptide, with a phosphoserine at 931, could also account for some of the radiolabel that eluted at position 1 during the Edman degradation for position of the Cos2 tryptic fractions (Fig. 2A). In addition, one of the Cos2 peptides we had obtained in our original sequencing of Cos2 (46) was peptide 923–942, of which we sequenced 10 amino acids. More importantly, serine 931 was not identified in this original sequencing, whereas the arginine immediately N-terminal to serine 931 and the isoleucine immediately C-terminal to serine 931 were both identified. Since phosphoamino acids cannot be identified using standard sequencing methods, these data also indicated that serine 931 might be phosphorylated. We therefore used a mutant Cos2 baculovirus (which we had made for other experiments) with serines 931 and 935 and threonines 927 and 932 all mutated to alanines to test if peptide 923–942 was phosphorylated. Phosphotryptic peptide analysis of this Cos2 mutant coinfected with Fu demonstrated that phosphopeptide 3 was missing when these serine and threonine residues were mutated (Fig. 4E). This indicated that serine 931 was probably the second Fu-induced phosphorylation site on Cos2. It is still possible that serine 935 in this region could be phosphorylated in addition to serine 931, but we believe this to be unlikely given the low levels of radiolabeling of the phosphopeptide containing these two serines and the fact that Edman degradation for position revealed a phosphoamino acid eluting at position 1 from tryptic digests. Again, serine 931 phosphorylation appears to be a physiologically relevant phosphorylation event, since phosphopeptide 3 is found in the S2-Hh Cos2 phosphotryptic peptide map (Fig. 5C).

DISCUSSION

Cos2 Is a Substrate of the Fused Serine/Threonine Kinase— Previously, we have shown that Fu and Cos2 are tightly bound together in a high molecular weight protein complex, but the role of Fu in the complex was indeterminate (46). We have shown here that Fu phosphorylates the kinesin-like protein Cos2 in a baculovirus coexpression system and that the phosphorylation pattern of baculovirus-produced Cos2 and Cos2 isolated from Hh-stimulated S2 cells is almost identical. We have mapped two Fu-induced phosphorylation sites in Cos2: serine 572 in a region of the protein between the microtubule binding domain and the heptad repeats and serine 931 in the C-terminal region of Cos2.

Several observations indicate that the Fu-induced phosphorylations of Cos2 that we observe in baculovirus coinfections are physiologically relevant Hh signaling events. First, the electrophoretic mobility shift of Cos2 seen in embryos and cells that have been stimulated by Hh appears to match that seen in Cos2 taken from cells coinfected with Fu and Cos2. The fact that Fu and Cos2 produced by baculovirus coinfection can still coimmunoprecipitate also indicates that the normal physical interaction between these two proteins is preserved. Most convincing, however, is the fact that both the positions and relative intensities of the Cos2 phosphotryptic peptides isolated from S2-Hh cells and from Fu/Cos2 baculovirus coinfections corre-spond almost exactly, an observation confirmed by mixing ex-periments. This indicates that the Hh-independent Fu-induced phosphorylation of Cos2 observed in baculovirus mimics the Hh-induced phosphorylation of Cos2 found in Drosophila cells. That these coinfections can mimic both the on and off states of Hh signaling is further supported by the observation that the phosphotryptic peptide map of Cos2 from a FuM/Cos2 coinfec-tion closely resembles the map of Cos2 from unstimulated S2 cells. These data strongly suggest that the phosphorylation state of Cos2 obtained from a Fu coinfection mimics that induced by Hh signaling in S2 cells. It should be noted that it is still formally possible that Fu does not phosphorylate Cos2 directly but rather acts through an intermediate kinase that is itself activated by Fu. We cannot discriminate this possibility using the baculovirus system, but we believe it unlikely for the following reasons: 1) the only other kinase known to be involved in Hh signal transduction, Drosophila protein kinase A, does not appear to phosphorylate Cos2 (data not shown) or Fu (47), 2) reductions of Fu baculovirus titer relative to Cos2...
baculovirus titer result in reduced phosphorylation of Cos2, consistent with a stoichiometric phosphorylation of Cos2 by Fu (data not shown), and 3) numerous kinase inhibitors have no effect on the Hh-induced hyperphosphorylation of Fu or Cos2 (data not shown).

Further supporting our mapping data is a recent study by Giet et al. (60), who show that the *Xenopus laevis* kinesin-like protein, XIEg5, physically associates with and is phosphorylated by an Aurora family kinase, pEG2. pEG2 and XIEg5 are both required for mitotic spindle assembly in *Xenopus* egg extracts, and disruption of either protein prevents mitotic spindle assembly. The site of pEG2 phosphorylation of XIEg5 was roughly mapped using fragments of XIEg5 and demonstrated to be in the "stalk domain," a region of XIEg5 between the motor domain and the tail domain (60). XIEg5 and Cos2 are similarly structured kinesin-related proteins (both having an N-terminal motor domain and a short C-terminal domain), and the area of Cos2 analogous to the "stalk domain" is where serine 572 of Cos2 is located. The fact that both Eg5 and Cos2 are phosphorylated in the same general region suggests that this region could be important in the regulation of a diverse array of kinesin motor protein functions. However, any similarity in phosphorylation site usage of the homologous Cos2 and Eg5 regions is probably only functional, since we have not been able to find any common motifs between Cos2 and Eg5 in this region.

The human version of Eg5, which is also involved in centrosome migration and spindle assembly, is the only other kinesin-like molecule in which a phosphorylation site has been mapped (61). In that study, human Eg5 (HsEg5) was shown to be phosphorylated by p34<sup>cdc2</sup> on threonine 927 in the C-terminal tail domain of the protein, and this phosphorylation was shown to modulate binding of HsEg5 to dynactin and to the microtubule spindle in dividing cells. In this case, however, the phosphorylation appeared to increase an already extant binding between HsEg5 and dynactin, the increase in which was sufficient to cause spindle localization (61, 62). Unlike HsEg5, Cos2 has not been found to be involved in cell division (42) and has little phosphothreonine, even in the Hh-stimulated state. Serine 931 of Cos2, like threonine 927 of HsEg5, is located in the tail region of the protein, and it is possible that it functions in a roughly similar manner to the C-terminal phosphorylation site of Hs Eg5 in regulating attachment of Cos2 to a cargo (but see below).

**Regulation of Cos2 Activity by Fu Phosphorylation**—Previoulsy, we demonstrated that the Hh-induced hyperphosphorylation of Cos2 correlated with a reduction in the binding of the Fu-Cos2-Ci complex to microtubules (42, 46). Here, we provide evidence that Fu is the kinase that phosphorylates Cos2 in response to Hh stimulation, suggesting that the Fu-induced phosphorylation of Cos2 is responsible for the reduction in microtubule binding of the Fu-Cos2-Ci complex. Our work does not answer the question of how this phosphorylation might control binding. It is possible that the Fu-induced phosphorylation of Cos2 does not regulate binding of Cos2 to microtubules but rather regulates Cos2 movement. In this case, Cos2 might normally be immobile and bound to microtubules. Hh signaling and subsequent Fu and Cos2 phosphorylation would then trigger Cos2 to move. The reduction in Cos2 binding to microtubules in response to Hh stimulation would then reflect a reduced affinity of Cos2 for microtubules while it is in the process of moving along microtubules and not an elimination of binding per se. Studies of classic kinesin function have demonstrated that a region just C-terminal to the microtubule binding/motor domain, called the neck region, is important in creating movement in addition to determining the direction of motion (63, 64). Serine 572 is distant from the analogous Cos2 region but is still on the motor side of the heptad repeats, and phosphorylation of this site could act through intervening sequence to regulate the motor domain and modulate movement and/or direction. This possibility can be addressed using baculovirus-produced Fu-Cos2 complexes once assays for Cos2 motor activity become available.

How might a simple binary system consisting of microtubule-bound and unbound states of the Fu-Cos2-Ci complex work in the context of what is now known about Hh signaling? In the absence of Hh signaling, binding of the complex to microtubules is constitutive, and this leads to processing of Ci, possibly by the proteasome (35, 36). Some sort of processing protein or complex, such as the proteasome, might move down the microtubules (or in some other way be targeted) to the microtubule-bound Fu-Cos2-Ci complex, where the processing protein could then cleave Ci. Fu phosphorylation of Cos2 stimulated by Hh would release this complex from or cause it to move along microtubules, which would prevent the processing protein from acting on Ci. Disruption of microtubule binding of the complex through any means would then emulate Hh signaling.

**A Second Fu-induced Phosphorylation Site on Cos2**—We have provided evidence that there is a second Fu-induced phosphorylation site at serine 931 of Cos2 (Figs. 3B and 4E). As noted above, this phosphorylation site falls into roughly the same region of kinesin-related molecules as the phosphorylation site of HsEg5, which is important in binding to the dynein-interacting protein, dynactin. However, there are no obvious sequence motifs shared by HsEg5 and Cos2 in this region, making it unlikely that there is any sort of common "cargo binding" motif shared by these two kinesin-like molecules. Rather, each kinesin-like protein may have a cargo-binding domain unique to its particular cargo. In addition, there are no common serine-containing motifs in the region of HsEg5 that is homologous to the Cos2 serine 931 region, so it does not appear that Cos2 and Eg5 share a conserved serine 931-like phosphorylation motif. Given the evidence that Fu kinase activity is required both for Ci processing and activation of full-length Ci (38, 39), it is possible that phosphorylation of Cos2 serine 931 could be involved in Ci processing independent of Cos2 microtubule binding. Loss of Fused kinase activity or loss of the serine 931 phosphorylation site could reduce Ci binding to Cos2, which could, in turn, reduce Ci processing by preventing the processing enzyme from interacting with Ci. This increase in full-length but apparently inactive Ci in fu mutants has already been noted (38, 39).

**A Model of the Intracellular portion of the Hh Signaling Pathway**—The results presented in this paper, in combination with previous data, allow a more detailed model of the effects of Hh stimulation on Fu and Cos2 interactions to be elaborated. In the model presented in Fig. 7, Cos2 dimerizes with itself, presumably through its heptad repeats, while one Fu molecule is associated with each Cos2 molecule and one molecule of Ci is bound to the C terminus of the Cos2 homodimer (the molar ratio of Cos2 to Fu and Ci is not known and is purely hypothetical in this model). A Su(Fu) molecule also interacts with the complex, and we illustrate it here as interacting with Fu, Cos2, and Ci, although the strength of its interactions with each is not clear (48). The Ci molecule is phosphorylated on several sites by protein kinase A, which primes Ci for processing (39, 65). In the absence of Hh stimulation, the Fu-Cos2-Ci complex is attached to microtubules. Fu kinase is inactive because either an inhibitory factor bound to Fu that prevents its activation by trans-autophosphorylation or a kinase that activates Fu

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2 K. Nybakken, unpublished data.
is inactive. While this complex is bound to microtubules, Ci is processed to generate the repressive Ci75 form of Ci, which then makes its way to the nucleus to repress Hh target genes.

When Hh stimulation occurs, Fu is activated by release of the inhibitory factor X and subsequent trans-autophosphorylation of Fu. Phosphorylation of Fu then stimulates the Fu molecules to phosphorylate Cos2 on serine 572 and serine 931. Phosphorylation of Cos2 on one or both sites could then cause Cos2 to either release from microtubules or move along them. Release of the complex from microtubules or movement along microtubules would then prevent Ci from being processed and lead to transcriptional activation by Ci. It is possible that the movement/release of the complex depends on phosphorylation of both sites, but we favor a model in which serine 572 controls microtubule affinity due to the fact that serine 572 is closer to the Cos2 motor domain and appears to make up a greater proportion of the Fu-induced phosphorylation of Cos2. Phosphorylation of serine 931 might then contribute to the fine control of microtubule association. Alternatively, serine 931 could be involved in Ci processing, as mentioned above; not all Ci binds to the Fu-Cos2 complex (46), and it is possible that serine 931 phosphorylation could regulate Ci affinity for the Fu-Cos2 complex.

This model theorizes that serine 572 is phosphorylated before serine 931, but the actual order in which these serines are phosphorylated is not known. It is possible that the two serines are phosphorylated simultaneously, but we believe this to be unlikely, since Cos2 from cells stimulated by Hh for 45–60 min has an electrophoretic mobility between that of Cos2 from unstimulated cells and that from chronically Hh-stimulated cells. We have shown that chronically Hh-stimulated cells are phosphorylated on both serines, whereas naive S2 cells are not phosphorylated on either site. It therefore seems plausible that the intermediate mobility form of Cos2 seen when S2 cells are Hh-stimulated for 45–60 min is due to phosphorylation of a single serine.

We have demonstrated that the unique kinase Fu phosphorylates the kinesin-like protein, Cos2, and that these phosphorylation events are very likely required to mediate the Hh signal. The discovery that Fused phosphorylates Cos2 is an interesting finding for several reasons. First, although kinesins have been shown to be phosphorylated (61, 62, 69–73), there are very few examples wherein the actual kinase has been identified (60, 61, 73). Second, this is only the second example that we are aware of in which the kinase and the substrate kinesin have been shown to bind together. This intimate relationship of kinesin and kinesin-kinase may function to ensure that phosphorylation of Cos2 occurs in a stoichiometric fashion instead of a catalytic manner, as in the example of HaEg5 and p34cdc2 (61). Third, the HaEg5 kinesin phosphorylation site, the only one previously mapped, was found in the “tail” domain of HaEg5, whereas we identify, for the first time, a phosphorylation site relevant to signaling that occurs in the coiled regions near the heptad repeats of Cos2. Finally, previous studies of kinesin phosphorylation have focused on the role that the phosphorylation plays in regulating either the cell cycle or kinesin movement, whereas we demonstrate a novel role for kinesin phosphorylation in transducing extracellular signals.

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