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Phosphorylation of the fused protein kinase in response to signaling from hedgehog

(segment polarity genes/protein kinase A)

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ABSTRACT **The hedgehog gene (*hh*) of *Drosophila melanogaster* exerts both short- and long-range effects on cell patterning during development. The product of hedgehog is a secreted protein that apparently acts by triggering an intracellular signaling pathway, but little is known about the details of that pathway. The *Drosophila* gene fused (*fu*) encodes a serine/threonine-protein kinase that genetic experiments have implicated in signaling initiated by hedgehog. Here we report that the fused protein is phosphorylated during the course of *Drosophila* embryogenesis, as a result of hedgehog activity. In cell culture, phosphorylation of fused protein occurs in response to the biologically active form of hedgehog and cannot be blocked by activation of protein kinase A, which is thought to be an antagonist of signaling from hedgehog. These results suggest that fused and protein kinase A function downstream of hedgehog but in parallel pathways that eventually converge distal to fused. The reconstruction of signaling from hedgehog in cell culture should provide further access to the mechanisms by which hedgehog acts.**

The hedgehog gene (*hh*) of *Drosophila melanogaster* plays vital roles in the developmental patterning of diverse tissues (1–3). The product of *hh* (Hh) can exert a direct paracrine effect on nearby cells as well as an indirect effect on more distant cells by means of intermediate effectors (1–3). For example, the imaginal discs that give rise to the head and thoracic structures of *Drosophila* are divided into anterior and posterior compartments (4). Hh is produced in cells of the posterior compartment, immediately adjacent to the compartmental boundary, but it exerts its effects on cells in the anterior compartment, both those in the immediate vicinity of the compartmental boundary and those more remote from it. The former action is direct; the latter is mediated at least in part by induction of the wingless (*wg*) and decapentaplegic (*dpp*) genes, which are themselves polypeptide signaling factors (5–9). In addition, Hh may exert long-range effects in a direct manner, as shown recently for the homologous vertebrate protein Sonic hedgehog (for a review, see ref. 10).

Hh is a secreted polypeptide that cleaves itself into amino-terminal (Hh-N) and carboxyl-terminal (Hh-C) products (see below, Fig. 2*A*) (11). The secretion of Hh is essential for biological activity (12). Hh-N is responsible for the signaling activity of *hh* (12, 13), whereas Hh-C carries the protease activity required for autocleavage and may also be involved in tethering Hh-N to the cell surface—perhaps to restrict its range of direct action (13).

Hh apparently acts by binding to a cell surface receptor and triggering an intracellular signaling pathway. Genetic analysis in *Drosophila* has uncovered five genes whose products function downstream of Hh in one manner or another (2, 3). The fused (*fu*) and cubitus-interruptus genes specify positive elements in the signaling pathways, whereas patch, costal-2, and *DCO* (which encodes the catalytic subunit of protein kinase A, or PKA) specify negative elements. The details of how these various genes act are not known. The eventual result of the signaling in *Drosophila* includes the induction of transcription from genes such as *dpp* and *wg*.

The product of *fu* is a 92-kDa protein (Fu) that has been inferred from its amino acid sequence to be a distinct type of serine/threonine-protein kinase (14, 15). Mutations in the catalytic domain of Fu prevent the induction of *wg* expression by Hh (ref. 16 and P.P.T., unpublished results), indicating that the kinase is indeed functional *in vivo* and participates in transduction of a signal from Hh. Virtually nothing is known, however, about how that transduction might occur. Here we report that Fu becomes phosphorylated during the course of *Drosophila* embryogenesis, as a result of *hh* activity. The phosphorylation can be reconstructed in cell culture, where it requires the biologically active form of *hh* and cannot be blocked by the stimulation of PKA. These results identify a signaling event downstream of Hh and establish an assay that provides access to the mechanisms by which Hh acts.

MATERIALS AND METHODS

Fly Stocks. The Df(3R)EB6/TM6B strain of *Drosophila* (which is deficient in *hh*) has been described elsewhere (17). Homozygous mutant embryos were identified morphologically under a dissecting microscope by a lack of segmentation, apparent at stage 11–12. Wild-type Oregon-R embryos of the same stage were used as controls.

Expression Vectors. The cDNA for wild-type Hh (18) was modified by the insertion of a termination codon after residue 257 to obtain Hh-N or by replacing the domain upstream of residue 249 with the signal peptide for the easter gene product (19) to obtain Hh-C (see below, Fig. 2*A*). The cDNAs for Hh and Hh-C were expressed by means of the promoter for the *Drosophila* actin-5C gene (20), the cDNA for Hh-N by means of a yeast upstream activating sequence, driven by GAL4 expressed from a co-transfected plasmid.

Cell Culture. The S2 cell line was cultured in S2 medium (Gibco/BRL), supplemented with penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 10% heat-inactivated fetal calf serum (Gemini). Cells were maintained at 25°C under air. S2 cells were transfected using calcium phosphate (21). Stable mixed clones were established under geniticin selection at 1 mg/ml (G418 from Gibco/BRL).

Assay for Hh Activity. Conditioned media were collected from heavily confluent S2 cells (≈2 × 10^7 cells per ml) producing different *hh* constructs. Media were centrifuged twice at 2500 × *g* for 10 min to remove intact cells. Conditioned media were applied for different times on wild-type S2 cells at

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Abbreviations: FuP, phosphorylated isoform of Fu; Hh-C, carboxyl-terminal domain of Hh; Hh-N, amino-terminal domain of Hh; PKA, protein kinase A.

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80% confluency, seeded 4–12 h previously to allow attachment. Cells were then washed in phosphate-buffered saline (PBS) and lysed for analysis, as indicated below.

Preparation of Cellular Extracts and Analysis by Immunoblotting. *Drosophila* embryos were collected at different times after oviposition, dechorionated with 50% bleach, rinsed with water, and homogenized at 4°C by several passes of a Teflon Dounce homogenizer, in a buffer containing 50 mM Hepes buffer (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40 (Sigma), 1.5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 1 mM Na_2VO_4, 1 mM Pefablock (Boehringer Mannheim), 0.01 mM benzamidine-HCl, phenanthroline (1 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin A (10 μg/ml) per ml. Insoluble material was sedimented at 10,000 × *g* for 15 min at 4°C, and the supernatant was collected. The protein concentration of the soluble material was estimated by using a bicinchoninic acid protein assay kit (Pierce). Equal amounts of proteins for each sample were incubated at 100°C for 5 min in the gel-loading buffer (50 mM Tris·HCl, pH 6.8/2% SDS/100 mM dithiothreitol/10% glycerol/0.1% bromphenol blue). Extracts were fractionated by electrophoresis in SDS denaturing polyacrylamide gels (6 or 15% for Fu and Hh analyses, respectively). Proteins were then transferred to nitrocellulose (Schleicher & Schuell) for 1 h at 1.5 mA/cm^2 using a semidry electrotransfer apparatus (E & K Scientific Products). The pattern of proteins was evaluated by staining the filters with Ponceau S solution. The membranes were blocked by incubation for 1 h at room temperature in Tris-buffered saline (5 mM Tris, pH 7.5/135 mM NaCl/5 mM KCl) containing 5% nonfat dry milk, 0.1% Tween 20, followed by a 2-h incubation with either a 1:200 dilution of purified polyclonal antiserum raised against Fu in rabbits (P.P.T., unpublished data) or a 1:3000 dilution of crude polyclonal antiserum raised against Hh (22). The membranes were washed three times with Tris-buffered saline containing 5% nonfat dry milk, 0.1% Tween 20 and 0.5% Nonidet P-40 before incubation with donkey anti-rabbit IgG (coupled to horseradish peroxidase) for 30 min (Amersham) at a 1:5000 dilution in 1:4 diluted wash buffer. The filters were washed as described above and were developed using an enhanced chemiluminescence substrate (ECL system from Amersham) and Fuji x-ray film to reveal the signals.

S2 cells were prepared for analysis by washing once in PBS without Ca^{2+} and Mg^{2+} and then lysed on ice for 20 min in Nonidet P-40 lysis buffer supplemented with proteases and phosphatase inhibitors as above. Insoluble material was sedimented at 10,000 × *g* for 15 min at 4°C, and supernatant was collected. Samples were analyzed by electrophoresis on polyacrylamide gels as described above.

Immunoprecipitation and Treatment with Phosphatase. S2 cells or embryos were lysed in Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors as described above. Lysates (2 mg of protein per sample) were precleared by incubation with protein A-Sepharose beads (Sigma) for 1 h at 4°C. After protein A beads were removed by centrifugation, the cleared lysates were incubated with 2.5 μg of purified rabbit anti-Fu antiserum for 1 h at 4°C. The immunocomplexes were precipitated with 50 μl of protein A-Sepharose beads for 1 h at 4°C, washed three times with lysis buffer, and washed once with a buffer provided by the manufacturer of calf intestinal phosphatase (Boehringer Mannheim). Immunocomplexes were then treated with 20 units of calf intestinal phosphatase in 50 μl of buffer for different incubation times at 37°C in the presence or absence of 3 mM Na_2VO_4. Samples were then heated for 5 min at 100°C in SDS loading buffer. Samples were analyzed in a 6% polyacrylamide gel as described above.

Other Procedures. Concentrations of cAMP in cellular extracts were determined as described (23). Activity of PKA in extracts was measured with a kit obtained from Gibco/BRL, using the protocol recommended by the manufacturers. Cells were labeled with [^{35}S]methionine (0.5 mCi/ml; 1 Ci = 37 GBq) in medium prepared without unlabeled methionine or dialyzed against medium lacking methionine. Incorporation of the label was ascertained as described (24).

RESULTS

Phosphorylation of Fu in *Drosophila* Embryos Is Dependent upon *hh*. The activation of protein kinases in signaling pathways is often accompanied by phosphorylation of the enzymes, as either cause or effect. To determine whether Fu might be so modified, we examined the state of the protein in embryonic extracts at various times during development (Fig. 1*A*).

Only one isoform of Fu was apparent during the first 3 h after oviposition, but a second isoform appeared by 4 h, at the time of gastrulation, and persisted for at least 5 h, throughout a period when *hh* and *fu* are known to be required for the expression of *wg* (16, 25, 26). Treatment of the extracts with calf intestinal phosphatase eliminated the second isoform, an effect that could be blocked by the phosphatase inhibitor orthovanadate (Fig. 1*B*). We conclude that the appearance of the second isoform is due to phosphorylation of Fu and have designated this form as FuP.

We next asked whether *hh* had any role in the phosphorylation of Fu. Analysis of extracts from embryos that were completely deficient in *hh* failed to detect FuP (Fig. 1*C*). Thus, the phosphorylation of Fu during the course of embryogenesis is dependent upon the action of *hh*.

Reconstruction of Signaling from Hh to Fu in Cultured Cells. We were able to reconstruct the effect of *hh* on Fu in cultured cells. We prepared vectors to express Hh, Hh-N, and Hh-C (Fig. 2*A*) and transfected these into the S2 line of embryonic *Drosophila* cells, which normally express *fu* but not *hh* (data not shown). Full-length Hh expressed in S2 cells was cleaved into Hh-N and Hh-C (Fig. 2*B*). The Hh-C product was

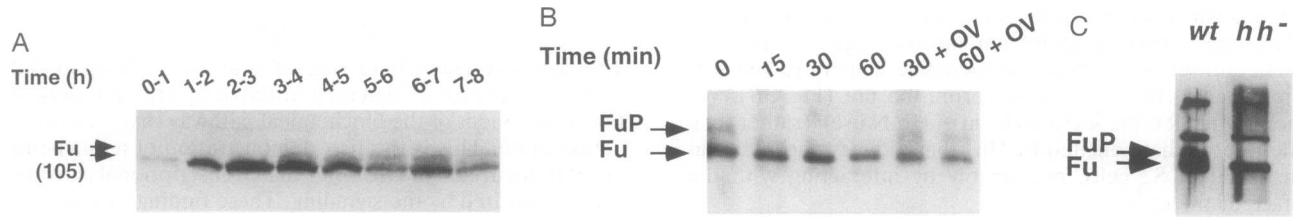


FIG. 1. Modification of fused protein in developing embryos of *D. melanogaster*. (*A*) Phosphorylation of fused protein during embryonic development. Embryonic extracts were fractionated by electrophoresis in polyacrylamide gels, and the 105-kDa fused protein (Fu) located by Western blotting with a polyclonal antiserum specific for that protein. The labeling of lanes denotes hours following oviposition. (*B*) Reversal of modification by phosphatase. Fu was immunoprecipitated from embryonic extracts prepared at 3–6 h after ovoposition and was treated with calf intestinal phosphatase for various periods of time up to 60 min, as indicated for individual lanes. Additional samples were treated with phosphatase for 30 and 60 min in the presence of 3 mM orthovanadate (OV). Samples were then analyzed as in *A*. The additional and apparently phosphorylated isoform of Fu is designated FuP. (*C*) Phosphorylation of Fu requires hedgehog. Extracts were prepared at 6–7 h after oviposition from either wild-type embryos (*wt*) or embryos that were homozygous for a deletion of *hh* (*hh*$^-$). Mutant embryos were identified by microscopy, harvested individually, and lysed directly in loading buffer, using five embryos per lane.

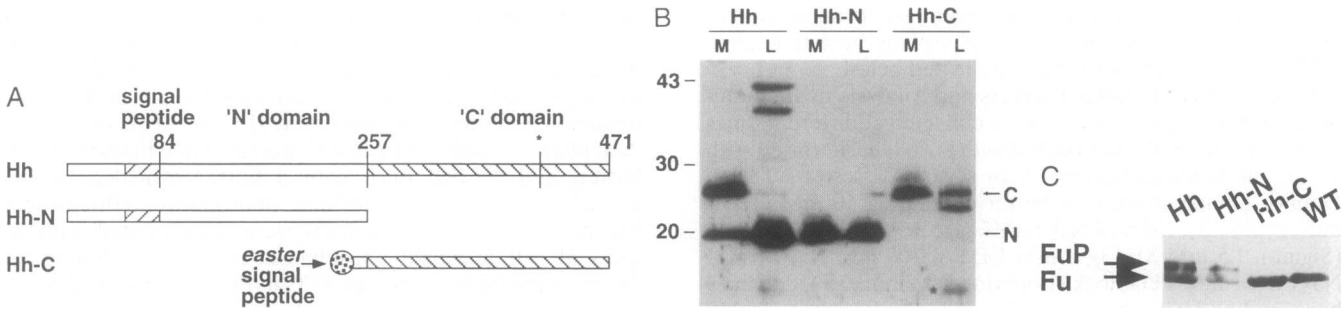


FIG. 2. Phosphorylation of Fu can be induced in cultured cells by hedgehog. (*A*) The structure of Hh proteins expressed in cultured cells. cDNAs were used to express three forms of *hh* proteins. The wild-type protein (Hh) contains a signal peptide that terminates at amino acid residue 84, a major site of autocleavage at residue 257, and a secondary cleavage site whose exact location and significance are not known (approximate location designated with an asterisk). The amino-terminal (Hh-N) and carboxyl-terminal (Hh-C) products of cleavage were also expressed in the forms diagrammed here. A signal sequence from the easter gene was provided to the Hh-C form to obtain secretion of the protein. (*B*) Expression of Hh proteins in cultured cells. The forms of protein diagrammed in panel *A* were expressed in S2 cells. Cellular lysates (L) and media harvested from the cells (M) were then analyzed by electrophoresis and Western blotting for *hh* protein. Loading of the gels was normalized by protein for lysates by number of cells for medium. The locations of Hh-N and Hh-C in the gels are designated by N and C, respectively. The band marked with an asterisk may be the product of the secondary cleavage described in *A*, and the unlabeled band running just in advance of C has not been identified. The positions of various marker proteins in the gels are given to the left in kilodaltons. (*C*) Fused proteins in cells expressing various Hh proteins. Wild-type S2 cells (WT) and S2 cells expressing the various forms of Hh protein described by *A* were analyzed for the presence of Fu and FuP.

secreted from cells efficiently, whereas a disproportionate quantity of Hh-N remained associated with the cells (Fig. 2*B*). In contrast, when Hh-N and Hh-C were engineered for independent expression and secretion, the bulk of both proteins was found in the medium (Fig. 2*B*). Taken together, these results are consistent with the previous suggestion that Hh-C might somehow facilitate the tethering of Hh-N to the cell surface (13).

When we examined the state of Fu phosphorylation, we found that wild-type S2 cells contained only Fu (Fig. 2*C*). Cells expressing either Hh or Hh-N contained FuP as well, whereas cells expressing Hh-C did not (Fig. 2*C*). Expression of Hh in the cl-8 line of imaginal disc cells also elicited phosphorylation of Fu (data not shown).

Phosphorylation of Fu Is Elicited by Secreted Hh-N. To this point, we could not discern whether the phosphorylation of Fu was being induced by secreted or intracellular *hh* protein. We therefore collected growth medium from S2 cells producing Hh-N and applied this in varied dilutions to wild-type S2 cells for 2 h. The results showed that the conditioned medium could induce phosphorylation of Fu in a dose-dependent manner (Fig. 3*A*). Medium conditioned by either wild-type S2 cells or cells producing wild-type Hh had no inductive activity (data not shown). We do not know why the production of wild-type Hh failed to give inductive activity. One explanation is the fact that the cells released relatively little Hh-N into the medium (see Fig. 2*B*); another is that the presence of Hh-C may in some way alter the activity of Hh-N.

To demonstrate that the active factor in conditioned medium was Hh-N, we incubated conditioned medium with either preimmune serum or antiserum directed against Hh. The specific antiserum blocked the inductive activity of the medium, whereas the preimmune serum did not (Fig. 3*B*). We conclude that it is the secreted form of Hh-N itself (rather than some intermediate elicited by Hh) that induces phosphorylation of Fu in S2 cells, presumably by interacting with the surface of the cells.

Kinetics and Stability of Fu Phosphorylation in Response to Hh-N. Phosphorylation of Fu could be detected within 30 min after the application of conditioned medium to cells (Fig. 3*C*). The quantity of FuP reached more than 50% of maximum by 50 min but continued to accumulate for at least 4 h in the presence of the conditioned medium (Fig. 3*D*). After removal of the conditioned medium, FuP persisted for several hours (Fig. 3 *E* and *F*). We conclude that Hh-N can signal from the exterior of cells to elicit relatively stable phosphorylation of Fu.

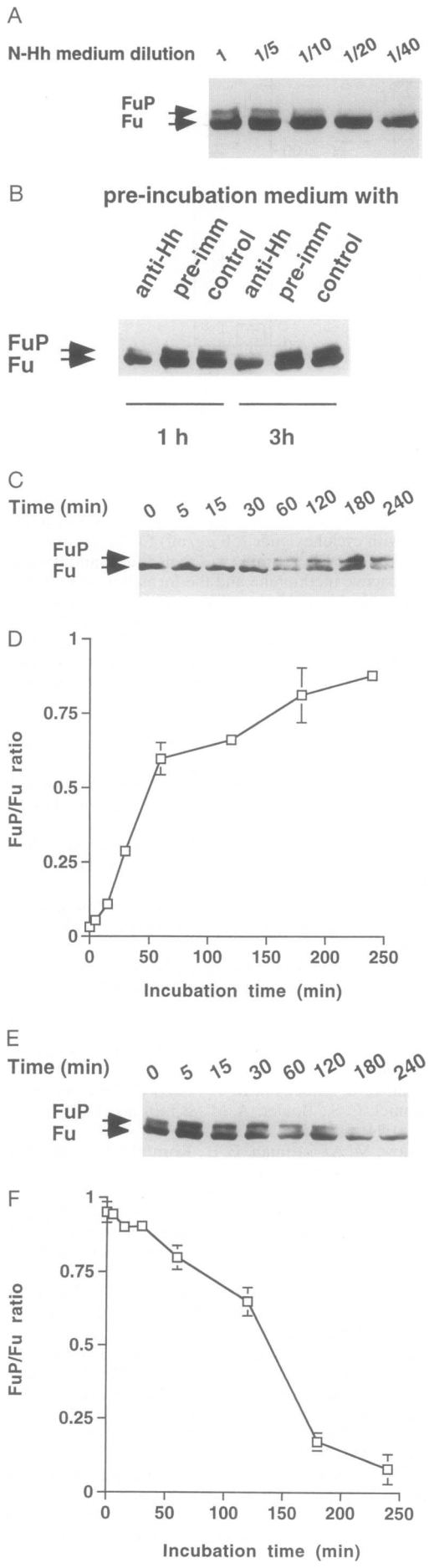
Phosphorylation of Fu Does Not Require New Protein Synthesis. The substantial delay between application of Hh-N to cells and the appearance of Fu-P raised the possibility that phosphorylation of Fu required the synthesis of new protein. We therefore asked whether the induction of Fu-P could be prevented by inhibition of protein synthesis. Fig. 4*A* shows that inhibition of protein synthesis to 98% by cycloheximide had no effect on the appearance of Fu-P in cells treated with Hh-N. Similar results were obtained with inhibition of protein synthesis by puromycin and inhibition of RNA synthesis by actinomycin D (data not shown). We conclude that the machinery responsible for phosphorylation of Fu preexists the application of Hh-N to cells.

Phosphorylation of Fu Is Not Affected by Activation of Protein Kinase A. The inactivation of PKA in *Drosophila* can produce phenotypes similar to those obtained from ectopic expression of *hh* (2, 3). One interpretation of these results is that PKA directly inhibits the action of Hh. We explored this possibility by cotreating wild-type S2 cells with medium containing Hh-N and forskolin. The efficacy of the treatment with forskolin was documented by demonstrating substantial elevation of cAMP and activation of PKA (Fig. 4 *C* and *D*). Demonstrable activation of PKA had no effect on the appearance of Fu-P in response to Hh-N (Fig. 4*B*). Similar results were obtained with S2 cells expressing Hh-N constitutively (data not shown). We conclude that the antagonism between PKA and Hh is probably implemented downstream of Fu, perhaps by the convergence of two signaling pathways—one utilizing Hh, the other PKA.

DISCUSSION

The data presented here suggest that Fu is phosphorylated during the course of signaling initiated by Hh and, thus, may be a component of the biochemical pathway that mediates the action of *hh*. This is the first demonstration of a biochemical event downstream of Hh, other than transcriptional activations that are elicited by the signaling. These findings are in accord with previous genetic analyses that also placed Fu downstream of Hh (25, 27). It remains possible, however, that the phosphorylation of Fu results from "cross-talk" between two pathways. Further analysis will be required to resolve this issue.

We have two reasons to believe that the induced modification of Fu in S2 cells is physiological in nature. First, the modification is elicited by Hh-N, which is responsible for transducing the biological activity of *hh* (12, 13). Second, the modification can be inhibited by the action of the patch protein



(unpublished work), which is generally regarded as a negative regulator downstream of Hh (28). Nevertheless, we presently have no evidence that the phosphorylation of Fu in S2 cells is chemically identical to that in the embryo. It would also be desirable to ask whether signaling from Hh induces the kinase activity of Fu, but to date, there is no *in vitro* assay for this activity. The ability of Hh-N to induce phosphorylation of Fu provides a potential explanation for how the short-range effects of *hh* might be mediated. We have yet to show, however, that FuP can be generated in cells that are responding phenotypically to Hh (such as cells in the anterior compartment of imaginal discs).

In preliminary studies, we have found that the phosphorylation of Fu to create FuP occurs on both serine and threonine (K. Nybakken, D. Robbins, P.P.T., and J.M.B., unpublished results). We do not know the identity of the responsible protein kinase, but it appears that this kinase must preexist in cells before the activation of signaling by Hh, since inhibition of protein synthesis with cycloheximide has no effect on the appearance of FuP. Thus, Fu must be part of a dynamic cascade designed to either transduce or moderate signals from Hh. Since mutations in the catalytic domain of Fu block signaling from Hh (ref. 16 and P.P.T., unpublished results), we presume that the kinase activity of Fu is essential to that signaling.

Genetic analysis has indicated that PKA antagonizes the action of Hh during signaling (2, 3), and pharmacological agents that activate PKA have also been shown to block the effects of the vertebrate homologue, Sonic hedgehog (29, 30). We found that pharmacological activation of PKA had no effect on phosphorylation of Fu in response to Hh. These results suggest that the antagonism between Hh and PKA may be implemented through parallel pathways that converge downstream of Fu, rather than in a more direct manner.

The results reported here conform to previous conclusions that Hh-N is secreted and then acts on a cell surface receptor (2). It appears that S2 cells must make this receptor. We found no evidence for down-regulation of the receptor, because the FuP that results from activation of signaling is sustained for at least several hours in the continued presence of Hh-N and decays only slowly subsequent to withdrawal of the ligand.

It is notable that expression of wild-type Hh in S2 cells did not engender detectable Hh activity in the growth medium. Similarly, biochemical fractionations indicated that the release of Hh-N into the medium from cells expressing Hh was comparably restricted. These observations conform to the view that when produced by autoproteolysis, much of Hh-N remains

FIG. 3. Secreted Hh-N induces the phosphorylation of Fu. (*A*) Induction of phosphorylation by conditioned medium from cells producing Hh-N. Growth medium was harvested after 24 h from S2 cells producing Hh-N, diluted to the indicated extents, and applied for 2 h to wild-type S2 cells. The cells were then analyzed for fused proteins (Fu/FuP). (*B*) Inactivation of inductive activity by antisera against Hh. Medium was conditioned by cells expressing Hh-N, as in *A*, and diluted 5-fold. Separate samples were then applied directly to wild-type S2 cells (control lanes) or incubated for 1 and 3 h with either preimmune serum or polyclonal antiserum directed against Hh prior to application to S2 cells. The antisera were used at a final concentration of 5%. After 2 h, the cells were analyzed for Fu/FuP. (*C*) Time course of phosphorylation induced by extracellular Hh-N. Medium was conditioned by cells expressing Hh-N, as in *A*, and applied without dilution to wild-type S2 cells. At various times thereafter, cells were analyzed for the presence of Fu/FuP. (*D*) Quantified time course of induction. The data illustrated in *C* were quantified by laser densitometry. The error bars indicate the range of results from two separate experiments. (*E*) The decay of FuP. Wild-type S2 cells were exposed for 18 h to conditioned medium containing Hh-N. The conditioned medium was then removed and the cells were washed twice with PBS and then placed in fresh growth medium. At various times thereafter, the cells were analyzed for the presence of Fu/FuP. (*F*) Quantification by laser densitometry of FuP decay. The error bars indicate the range of results from two separate experiments.

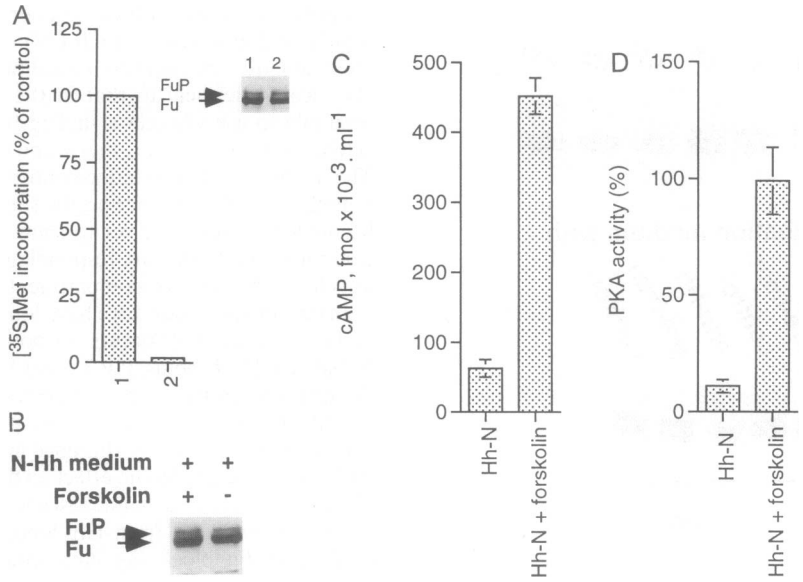


FIG. 4. Phosphorylation of Fu does not require new protein synthesis and is not inhibited by activation of protein kinase A. (*A*) Inhibition of protein synthesis has no effect on formation of FuP. Wild-type S2 cells were incubated with cycloheximide (50 μg/ml) for 90 min (bar 2) or with regular medium (bar 1). Dialyzed conditioned medium containing Hh-N, [^{35}S]methionine, and cycloheximide was then added, and the incubation continued for 60 min. Extracts of the cells were then analyzed for incorporation of radioactive methionine and the formation of FuP (see *Inset*). (*B*) Activation of PKA does not impede formation of FuP. Wild-type S2 cells were incubated with regular medium or medium containing 100 μM forskolin (Calbiochem) for 30 min. Medium containing Hh-N and forskolin was then added and the incubation was continued for 45 min. Extracts of cells were then analyzed for formation of FuP. (*C*) Increase in cAMP in response to forskolin. Extracts of S2 cells manipulated as for *B* were assayed for content of cAMP. All measurements were made in triplicate. The error bars show the range of results among two independent experiments. (*D*) Activation of PKA by forskolin. Extracts of S2 cells manipulated as for panel *B* were assayed for PKA activity. All measurements were made in triplicate. The error bars show the range of results in two independent experiments.

tethered to membranes, possibly through some action of Hh-C (13). The tethering would serve to restrict the range of direct action by Hh. Alternatively, Hh-C may modify Hh-N in some manner that affects activity.

Genetic analysis has identified two genes that appear to function downstream of *fu*, costal-2 and cubitus-interruptus (16, 27). The products of these genes are candidates as potential substrates for the Fu kinase. The ability to detect a prompt biochemical response to Hh in cultured cells should provide further access to the signaling pathway(s) that mediate the action of *hh*. Moreover, the similarities between *hh* and its vertebrate counterparts are so great that any description of how Hh signals in *Drosophila* is likely to have broader implications.

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