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The Roles of the Fused and Costal2 Proteins in Hedgehog Signaling.

by

Kent Edward Nybakken

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

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I have known Janet since high school, and she has always been a great friend. She has listened to me complain for countless hours about my

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The Roles of the Fused and Costal2 Proteins in Hedgehog Signaling.

Kent Edward Nybakken

Abstract

Hedgehog is a secreted, morphogenetic protein that is involved in patterning numerous tissues and organs in *Drosophila melanogaster*. While the patterning roles and biochemistry of Hedgehog (Hh) itself are well known, the mechanism by which Hh signal is transduced inside cells is not well understood. We have dissected the role that two cytoplasmic proteins, Fused (Fu), a serine/threonine kinase, and Costal2 (Cos2), a kinesin-like molecule, play in transducing the Hedgehog signal using a combination of biochemistry, molecular biology, and genetics. In Chapter 2, we used coimmunoprecipitation and gel filtration chromatography to show that Cos2 binds to Fu. We also demonstrated that Fu and Cos2 bind to another protein involved in Hh signaling, the transcription factor Ci. In addition, we demonstrate that the Fu/Cos2/Ci complex binds to microtubules in the absence of Hh signal, but is released from microtubules in the presence of Hh signal.

In the next two chapters, we used a baculovirus coinfection system to examine the interactions of Fu, Cos2 and Ci in more detail. We have demonstrated that Fu constitutively phosphorylates Cos2 in baculovirus and that the resultant phosphorylation pattern matches that seen in insect cells stimulated by Hh. Hence, Fu phosphorylation of Cos2 is correlated to release of the Fu/Cos2/Ci complex from microtubules. We have also shown that the

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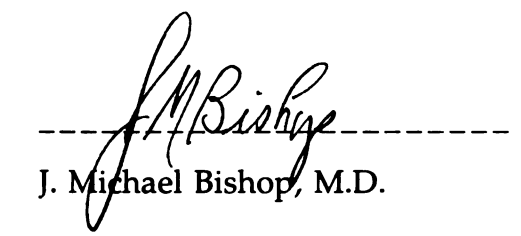
principal site of Fu phosphorylation on Cos2 is Serine 572, located in a region between the microtubule binding domain and the dimerization motifs. This finding implicates this region of Cos2 in regulation of its binding to microtubules. We further found that Fu autophosphorylates in baculovirus and that the autophosphorylation pattern closely matches the phosphorylation pattern found on Fu obtained form cells stimulated by Hh. Finally, we have demonstrated that Fu can phosphorylate Ci in baculovirus, and that Fu kinase activity is also required for normal regulation of Ci in *Drosophila*.. These results imply that Fu may regulate both Cos2 and Ci in response to Hh signaling.



J. Michael Bishop, M.D.

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Chapter 1

Introduction

The Hedgehog Signaling Pathway.

Introduction

There are few biological phenomena that have evoked as much mystery, fascination, and controversy as the development of an adult organism from a single fertilized egg. The concept that the information necessary to generate an elephant from a fertilized egg could be contained within the physical and chemical confines of a structure as seemingly simple as an egg has astonished and entranced scientists and laymen alike for centuries. Investigation of this phenomena forms the basis for the biological discipline of developmental biology. Research in developmental biology has shown that the fertilized egg, or embryo, while not necessarily a simple structure itself, does indeed contain the necessary instructions to convert itself from a single cell into a complex multicellular organism and that this process can involve huge metamorphoses in body plan and tissue structure. Classic examples of the great degree of change involved are the development of butterflies from caterpillars and the growth of huge trees from seeds the size of pebbles. The complexity of the developed structures can vary from relatively simple body plans of some microscopic protozoans and sponges to organs as complex as the primate or cetacean brain.

One of the oldest questions that developmental biologists have sought to answer is how the basic body plan of organisms is laid out: that is, how the structures found along the anterior-posterior and dorsal-ventral axis of an animal are formed. Although this question has been studied in a great variety of organisms, the greatest contribution to our understanding of how the overall body plan of multicellular eukaryotes is laid out has come from the study of the fruitfly, *Drosophila melanogaster*. This is due principally to the fact that genetics has shown itself to be a powerful tool in the analysis of

animal development, and no multicellular eukaryotic organism has a more extensive repertoire of genetic information and tools available than *Drosophila melanogaster*.

Segmentation in *Drosophila* Development

An integral part of the development of the body plan of *Drosophila*, and most other bilaterally symmetric metazoans, is the formation of segments along the anterior-posterior axis. Segments are divisions of the anterior-posterior axis which are intimately involved in the generation of the unique structures that develop along the anterior-posterior axis, such as the limbs, mouthparts, endo- and exo-skeletons, and the internal organs. Segments are important because part of each segment acts as what is called an organizing center. An organizing center is a group if cells that acts on surrounding tissues or cells and causes them to take on a certain cell fate. Studies in both *Drosophila* and vertebrates have shown that the organizing center within each segment patterns most of the tissue in that segment (Lawrence, 1990; Lawrence, 1992; Lawrence et al., 1996; Lawrence and Struhl, 1996; Martinez-Arias and Lawrence, 1985; Pearse and Tabin, 1998; Riddle and Tabin, 1999; Schweitzer and Tabin, 1999). Each segment is further subdivided into the anterior and posterior compartments, and the border between the two compartments is the organizing center in *Drosophila*. The border between the anterior and posterior compartments of a segment is actually the border between a more fundamental unit of anterior/posterior division called the parasegment (Martinez-Arias and Lawrence, 1985). A parasegment contains an anterior and a posterior compartment, but is one compartment out of register with a segment, and has the organizing center, found at the

anterior/posterior compartment border, as its parasegmental border. Parasegments are important because they turn out to be the units of anterior/posterior division that are initially specified during *Drosophila* development. The location of the anterior and posterior compartments in embryos can be ascertained by the presence of belts of pointed cuticular outgrowths called denticles that are only found in the far anterior compartment (see Lawrence, 1992a, for review).

A central event in the development of all metazoans is the delineation of parasegmental and compartmental boundaries. Segmental pattern formation in the *Drosophila* embryo, and particularly the embryonic ectoderm and cuticle, has been extensively studied both during egg formation and after fertilization. After the embryo is fertilized, segmental patterning arises from the sequential activation of three zygotic gene regulatory networks. These three zygotic gene groups were originally characterized in the pioneering studies of the genes affecting Drosophila segmentation conducted by Nusslein-Volhard and Wieschaus (Aszterbaum et al., 1999; Belloni et al., 1996; Boyle et al., 1991; Nusslein-Volhard et al., 1985; Nusslein-Volhard and Wieschaus, 1980). I refer to each of these grouping as "gene regulatory networks" because the interactions between the genes within each group can be very complex (Jackle et al., 1992; Jackle and Sauer, 1993), whereas the interactions between the networks tend to be more straightforward.

The first network, comprised of the gap genes, is involved in specifying the identity of large regions--that is many presumptive segments-- along the anterior-posterior axis. The next network, comprised of the pair-rule genes, specifies the actual parasegments and their boundaries. The gap genes are activated first and then activate the pair rule genes (although there is some

feedback between the two networks that occurs to refine pattern and maintain proper spacing) (Jackle et al., 1992; Jackle and Sauer, 1993).

The pair-rule genes then activate the segment polarity genes, which are involved in maintaining the parasegmental and compartmental borders and, directly or indirectly, in establishing intrasegmental polarity and pattern (St Johnston and Nusslein-Volhard, 1992). Segment polarity genes are named because of the effect mutations in these genes have on the denticle belts of the embryonic epidermis. Denticle belts are brush-like cuticular structures that cover part of every larval segment and are separated from one another by flat, or "naked" cuticle. Mutations in genes which cause these belts to either disappear, leaving only naked cuticle, or fuse in various ways constitute the segment polarity gene mutants. Molecular epistasis studies have shown that the segment polarity genes are genetically downstream of the gap and pair-rule genes. Expression studies of the appropriate cloned genes have demonstrated that the gap and pair-rule genes are turned on before gastrulation and are turned off at the beginning of gastrulation, while the segment polarity genes are first expressed during gastrulation and appear to maintain one another's expression in the embryonic ectoderm. Evidence from epistasis studies, gene expression patterns, and the molecular nature of cloned segment polarity genes indicate that they compose two separate, yet mutually maintaining, signaling pathways in the *Drosophila* embryonic epidermis.

The mechanisms by which the segment polarity genes act and the number of different pathways they appear to function in are still poorly understood, but a combination of genetics and molecular biology is beginning to elucidate these roles. There are at least 12 genes which fit the original definition of segment polarity genes as defined by Nusslein-Volhard and

Wieschaus (Nusslein-Volhard and Wieschaus, 1980). Of these, 11 have now been cloned. Nusslein-Volhard and Wieschaus originally identified six segment polarity genes, but additional genes, such as protein kinase A (PKA) (Jiang and Struhl, 1995; Lane and Kalderon, 1993; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995), *costal2* (Whittle, 1976), and *smo*(Nusslein-Volhard et al, 1984; Alcedo et al, 1997), have been shown to have segment polarity defects when mutated and so have been added to the original tally.

Vertebrate homologs of most of the cloned *Drosophila* segment polarity genes have also been identified. For the most part, they seem to function in similar ways to the fly genes, although there are usually several homologs in vertebrates of any given segment polarity gene. Their roles in vertebrate development and in oncogenesis are areas of active investigation. Some of the mechanisms of action of the segment polarity genes have been more clearly elucidated in vertebrates and these instances will be discussed when they serve to enlighten pathway function.

The segment polarity genes were determined to be involved in two separate yet mutually reinforcing signaling pathways through a combination of genetic interaction and expression pattern studies. These studies, which led to the assignment of each of the segment polarity genes to one of the two pathways, are described below, followed by a more detailed description of the identities and interactions of the genes found to be involved in the Hedgehog (Hh) pathway, one of the two segment polarity signaling pathways.

Wingless and Engrailed

The first two segment polarity genes to be cloned were *wingless* (*wg*) and *engrailed* (*en*), and it is from the interactions of these two genes that the

existence of the two segment polarity signaling pathways was determined. The transcripts of both these genes are expressed in stripes in stereotyped positions within the embryonic ectoderm of each segment of the developing embryo. *wg* encodes a secreted molecule that in situ hybridization and immunostaining show to be expressed in the most posterior cells of the anterior compartment (that is, along the anterior face of the parasegmental or compartmental boundary) (Baker, 1988; Baker, 1988; Baker, 1988; Rijsewijk et al., 1987; van den Heuvel et al., 1989). *en* encodes a homeodomain containing DNA binding protein that is expressed throughout the entire posterior compartment (Desplan et al., 1985; DiNardo et al., 1985; Fjose et al., 1985; Kornberg et al., 1985). *en* is found in the nucleus and can act as a transcriptional activator or repressor (Jaynes and O'Farrell, 1988; Jaynes and O'Farrell, 1991).

The initial expression patterns of *wg* and *en* are initiated by the pair-rule genes (DiNardo and O'Farrell, 1987; Howard and Ingham, 1986; Ingham et al., 1988; Limbourg-Bouchon et al., 1991; Martinez-Arias, 1985). However, pair-rule gene expression fades by ~ 3 hours after egg laying (AEL), and subsequently, *wg* and *en* activity are required to maintain the other's expression in the ectoderm. Loss of expression of *wg* eventually leads to a loss of expression of *en* and vice versa (Bejsovec and Wieschaus, 1993; Bejsovec and Wieschaus, 1995; DiNardo and O'Farrell, 1987; Hidalgo, 1991; Ingham, 1993; Ingham and Hidalgo, 1993). The loss of expression of the non-mutated gene in the other's mutant background was not immediate, however, and suggested that it took time for the loss of expression of one to register on the other's transcription. These results, and the two genes' abutting but non-overlapping expression patterns, led several groups to hypothesize that *wg* and *en* are involved in two separate, but mutually

stimulating signaling pathways acting across the parasegmental boundary. In this case, *wg* and *en* expression could be used as benchmarks to determine how mutations in the other segment polarity genes affected the expression of these two genes and hence infer in which of the two pathways a particular segment polarity gene was acting. Several groups then conducted these studies and by cloning the relevant segment polarity genes, checking which part of the segment they were expressed in by in situ hybridization, and examining the effect of mutants on *wg* and *en* expression, were able to assign most of the segment polarity genes into either the *wg* or *en* signaling pathway. Epistasis studies of some of the relevant genes also provided support for these groupings (Forbes et al., 1993).

If the Wg and En proteins were involved in two separate but mutually stimulating signaling pathways, then already it was obvious that several components were missing from each pathway. Wg encoded a secreted signaling factor, but some sort of transcriptional activator had to cause its transcription. En was a transcription factor, but presumably had to cause the expression of a signaling factor other than Wg as En is not expressed in Wg-expressing cells. It was assumed that somewhere amongst the 12 identified segment polarity genes, these two factors would be found and that the other segment polarity genes would be other components of the pathway, such as the receptors of the secreted proteins and the cytoplasmic relay molecules.

Hedgehog

The cloning of the *hedgehog* (*hh*) gene provided the reciprocal secreted signaling molecule to Wg. *hh* was isolated in the Nusslein-Volhard and Wieschaus screens as a mutant that gave an embryo covered with denticles;

hence the name hedgehog (Belloni et al., 1996; Nusslein-Volhard and Wieschaus, 1980). When it was cloned, *hh* was found to be a secreted signaling factor expressed in the same cells as *en* in the embryo and the imaginal discs (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993). *hh* mutants lose most *wg* expression in the embryonic epidermis (Hidalgo and Ingham, 1990; Ingham, 1993; Ingham and Hidalgo, 1993) and overexpressing *hh* in embryos causes an anterior expansion of the area of *wg* transcription although not ubiquitous expression (Hidalgo, 1991; Ingham, 1993; Ingham and Hidalgo, 1993). Both of these results confirmed that *wg* transcription is under *hh* control. Expression of *patched* (*ptc*), another segment polarity gene described below, is also under *hh* control since loss of *hh* causes a loss of *ptc* expression in most of the embryonic epidermis and overexpression of *hh* causes expansion of the region of *ptc* expression in the embryonic epidermis (Forbes et al., 1993; Hidalgo and Ingham, 1990; Ingham, 1993).

In the imaginal discs, clonal analysis has shown that loss of *hh* in the posterior compartment of the wing disc causes an ectopic expression of the secreted signaling molecule *dpp*. Furthermore, ectopic expression of *hh* in clones in the anterior compartment causes pattern duplications in the wing and leg imaginal discs and induces the expression of *dpp* and the accumulation of *Ci* around the clone (see below), similarly to what is seen at the anterior-posterior compartment border (Basler and Struhl, 1994; Motzny and Holmgren, 1995; Slusarski et al., 1995).

As signaling molecules go, Hh affects a remarkably broad range of patterning events in the fly. Hh is expressed in and affects the development of a number of *Drosophila* tissues, including the ventral embryonic ectoderm (where most of the initial characterization of its interactions with other

segment polarity genes took place, see references listed below), the dorsal embryonic epidermis (Bokor and DiNardo, 1996; Heemskerk and DiNardo, 1994), the head (Amin et al., 1999; Gallitano-Mendel and Finkelstein, 1997), the CNS (Matsuzaki and Saigo, 1996; Patel et al., 1989), the legs (Basler and Struhl, 1994; Zecca et al., 1995), the eye (Heberlein et al., 1995; Ma et al., 1993), the wings (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Zecca et al., 1995), the gonads (Chen and Baker, 1997), and the gut (Bilder and Scott, 1998).

Hh was found to have a signal sequence, but, like Wg, had no particular homology to known secreted signaling factors (Basler and Struhl, 1994; Lee et al., 1992; Tabata et al., 1992; Tashiro et al., 1993). Further investigation revealed that Hh itself has a very interesting biochemical character in that it appears to undergo a proteolytic maturation process followed by attachment to cell membranes via a cholesterol linkage. Full-length Hh undergoes an autocatalytic cleavage (Lee et al., 1994), during which a cholesterol moiety is covalently attached to the N terminal fragment of the Hh molecule. The N terminal fragment of the Hh molecule is the only portion which retains signaling function while the C terminal fragment, which is related to serine proteases and the self-splicing peptide inteins, catalyzes the autocleavage (Porter et al., 1996a; Porter et al., 1995; Porter et al., 1996b). The cholesterol moiety is thought to bind the Hh-N fragment to the cell membrane and limit its range of action (Porter et al., 1996a; Porter et al., 1996b). Hh-N produced alone, and hence without its cholesterol moiety, is a more effective inducer of target genes and acts at a greater range (Porter et al., 1996), implying that it may have more freedom to move throughout tissues. Expression of the Hh-N fragment has also been shown to rescue *hh* mutant flies, so it is not clear why a cell membrane binding linkage would be necessary (Porter et al., 1996a).

Hh, in spite of the restricted localization a cholesterol linkage would imply, seems to be able to act both locally and at relatively large distances. It can act locally in the embryonic epidermis and wing imaginal disc where it induces the TGF-ß class signaling molecule Dpp, and in the fly eye, where it also induces *dpp* expression (Lee et al., 1992; Strigini and Cohen, 1997; Tabata et al., 1992; Tabata and Kornberg, 1994). Yet Hh also seems to be able to act over multiple cell diameters to affect pattern in the fly wing. In some cases, the long range effects are caused by Dpp, as is seen in the effects of Hh in the dorsal embryonic epidermis (Basler and Struhl, 1994; Heemskerk and DiNardo, 1994), and in other cases Dpp is not involved (Chen and Struhl, 1996; Mullor et al., 1997).

As only the signal transduction mechanism of Hh is examined in this thesis, only segment polarity genes involved in Hh signaling will be discussed further.

Patched and Smoothened

The *patched* (*ptc*) gene was originally characterized as one of the segment polarity genes that had an effect on cuticular phenotype in the mutant that was opposite that of *hh* mutants (Boyle et al., 1991; Martinez-Arias et al., 1988). Whereas *hh* mutants give a great increase in the number of denticles in the embryonic cuticle, *ptc* mutants reduce the size and change the shape of the denticle belts in a mutant cuticle (Boyle et al., 1991; Hidalgo and Ingham, 1990; Hooper and Scott, 1989; Ingham, 1991; Ingham, 1993; Ingham et al., 1991; Nakano et al., 1989). Later genetic analysis showed that *ptc* and *hh* had opposing effects on each other's activity and that *ptc* is epistatic to *hh* (Forbes et al., 1993; Ingham et al., 1991). *wg* transcription in *ptc*

mutants is independent of Hh, whereas in wild type flies, wg transcription is dependent on *hh* (Ingham, 1993). It was also found that *ptc* transcription itself depends on hh expression (Hidalgo and Ingham, 1990; Ingham et al., 1991). These data indicated that *ptc* is a negative regulator of Hh signaling, but is itself a transcriptional target of Hh activity.

Upon cloning, *ptc* was found to encode a large, multiple-pass transmembrane protein with at least twelve membrane spanning domains, (depending on which algorithm is used to determine hydrophobicity) (Hooper and Scott, 1989; Nakano et al., 1989). Ptc was thought to have no identifiable homologies until recently when the gene encoding the Niemann-Pick type C disease gene was cloned and its products shown to have homology to a part of the ptc protein. Ptc appears to have a sterol sensing domain of the type found in proteins involved in lipid and cholesterol metabolism (Carstea et al., 1997). Vertebrate homologs of ptc have also been cloned, with most vertebrates having two ptc homologs (Concordet et al., 1996; Goodrich et al., 1996; Goodrich et al., 1999; Hahn et al., 1996; Johnson et al., 1996; Motoyama et al., 1998; Motoyama et al., 1998). Overexpression of ptc by heat shock promoter in flies has no effect on cuticle development, but more concentrated overexpression by the UAS-Gal4 system results in a loss of wing vein patterning in both the anterior and posterior compartments of the wing (Brand and Perrimon, 1993; Ingham et al., 1991; Johnson et al., 1995; Sampedro and Guerrero, 1991).

The *smoothened* (*smo*) locus is another gene identified in the Nusslein-Volhard screens and is named because of a vast reduction in the numbers of denticles and the size of the denticle belts in *smo* mutants. Smo is epistatic to Ptc with respect to wg and en expression in the embryonic cuticle (Alcedo et al., 1996). *smo* encodes a transmembrane protein with

seven membrane spanning domains and homology to G-protein coupled receptors, but little else is known of its function (Alcedo et al., 1996; van den Heuvel and Ingham, 1996).

The Ptc and Smo proteins were, for a long time, the leading candidates for a Hh receptor, but for many years after the cloning of both, no evidence of their interaction in *Drosophila* could be found. In 1996, Stone et al demonstrated that the mouse and rat homologs of Hh and ptc, Shh and vPtc respectively, could bind to one another and that vPtc and vSmo could bind to one another, but that Shh could not bind to vSmo (Stone et al., 1996). There is still no published evidence of their physical interaction in flies, although Chen and Struhl have shown genetically that *ptc* seems to be able to titer *hh* in a dose dependent fashion and that *ptc* activity is needed to prevent *smo* activity (Chen and Struhl, 1996).

Cubitis interruptus

Cubitis interruptus (*Ci*) is a segment polarity gene originally discovered in the 1930's and reisolated in the Nusslein-Volhard screens (Nusslein-Volhard et al., 1985; Nusslein-Volhard and Wieschaus, 1980; Tiniakov, 1933). It causes an apparent mirror-image duplication of the denticle belts and a reduction in the size of the embryo (Nusslein-Volhard and Wieschaus, 1980; Orenic et al., 1987). There are several alleles of *ci* with differing phenotypes that have been isolated, including Ci^D, a dominant allele, and the locus called *Cell*, also dominant, which was found to be allelic to *Ci* (Orenic et al., 1987). *Ci* eliminates wg expression when mutant, indicating it is a positive regulator of the pathway, and has been shown to be epistatic to *ptc* (Forbes et al., 1993; Hidalgo and Ingham, 1990). It is also required for *ptc* and *wg* transcription in

the embryonic epidermis and for *ptc* and *dpp* transcription in the wing disc (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997; Hidalgo and Ingham, 1990; Methot and Basler, 1999).

Ci encodes a zinc finger DNA binding protein related to a homologous mammalian protein called GLI, a nuclear transcription factor of the Kruppel family (Eaton and Kornberg, 1990; Kinzler et al., 1987; Kinzler et al., 1988; Orenic et al., 1990; Ruppert et al., 1988; Ruppert et al., 1990). Ci can activate transcription of reporter genes in a heterologous yeast system and in transiently transfected S2 cells (Alexandre et al., 1996; Von Ohlen and Hooper, 1997; Von Ohlen et al., 1997). Expression of fragments of Ci has demonstrated that the DNA binding domains and putative transcriptional activation domains are required for Ci induced transcription of *ptc*, *dpp*, and *en*. Studies of the *wg* and *ptc* promoters have also shown that they are activated by normal Ci and require the GLI/Ci binding sites found in their promoters for this activation (Alexandre et al., 1996; Von Ohlen and Hooper, 1997; Von Ohlen et al., 1997). Evidence that Ci can bind these sites in responsive promoters has been obtained by showing that Ci can bind oligos containing the GLI/Ci bonding site in vitro (Von Ohlen et al., 1997). When functioning as an activator, Ci works with the dCBP (the Drosophila CREB binding protein homolog) general transcriptional coactivator (Akimaru et al., 1997). Ci overexpression in the posterior compartment of the wing imaginal disc causes repression of en transcription, indicating that Ci can function as both an activator and a repressor (Dominguez et al., 1996). Interestingly, Ci RNA levels are constant throughout the compartments they are expressed in, but the protein levels vary, being higher near the compartment boundary in the anterior wing disc, for instance (Motzny and Holmgren, 1995). These levels are not affected by *ptc* or *hh* overexpression.

As *ci* transcription is not affected by *ptc* or *hh* overexpression, yet its protein levels are affected, it was suspected that *ci* might be regulated post-transcriptionally. Aza-Blanc et al showed that full length Ci in the absence of Hh stimulation is normally cleaved by an unknown protease into an N and a C terminal fragment, and that the N terminal fragment has transcriptional repressor activity (Aza-Blanc et al., 1997). They further showed that the C terminus contains a cytoplasmic tethering domain which is removed from the N terminus by the cleavage event. The N terminal fragment is called Ci75 and appears to be able to directly repress transcription of *hh* (Aza-Blanc et al., 1997; Dominguez et al., 1996). Uncleaved, full length Ci accumulates adjacent to sources of Hh protein while the repressor form is more evenly distributed (Aza-Blanc et al., 1997; Dominguez et al., 1996; Motzny and Holmgren, 1995; Slusarski et al., 1995).

The protease that cleaves Ci is unknown. The cleavage site has been narrowed to between amino acids 611 to 760, but also has yet to be identified (Methot and Basler, 1999). Mutations in a gene called *supernumerary limbs* (*slimb*) has been found to induce effects similar to those of Ci overexpression. This gene encodes a protein with homology to E3 ubiquitin ligases found in the ubiquitin mediated degradation pathway, implying that the proteolytic activity of the proteasome could be involved in Ci cleavage (Jiang and Struhl, 1998). Recent data have also implicated the proteasome as a potential mediator of Ci cleavage by showing that an proteasome inhibitor, lactacystin, can reduce production of the Ci75 form of Ci in Cl8 cells (Chen et al., 1999).

While most evidence points to Ci as a transcription factor, it has been difficult to show definitively that Ci is present in the nucleus, where one would expect to find some significant portion of a transcription factor. Endogenously produced full length Ci155 has never been convincingly

shown to have nuclear staining, although a recent report claimed to document an increase in immunostaining of nuclear speckles with a Ci155 antibody in response to Hh-N treatment of Cl8 cells (Chen et al., 1999). Whether this is true nuclear staining of Ci or some sort of background effect of Hh-N treatment is not clear, but it is possible that Ci localization in the nucleus is limited to a very few sites and it will not be possible to get broad nuclear immunostaining of Ci. Arguing against this, however, are more results from Chen et al, wherein they treat Cl8 cells with leptomycin, a drug that blocks nuclear export, and find a broad nuclear staining with Ci antisera. Along similar lines, it had already been shown that Ci contains a cytoplasmic tethering sequence that is required for localization of Ci in the cytoplasm and that Ci75, which lacks this tethering sequence, transfected into S2 cells will almost totally localize to the nucleus (Aza-Blanc et al., 1997).

Exactly how Ci is processed and how it eventually controls transcription are questions that promise to have interesting answers.

Protein Kinase A (PKA)

Another factor involved in Hh signaling, and one which was not isolated in the Nusslein-Volhard and Wieschaus screens, is the *Drosophila* cAMP regulated serine/threonine kinase protein kinase A (PKA). PKA is a negative regulator of the pathway. Loss of PKA in clones of anterior compartment cells in the wing imaginal disc leads to ectopic expression in the clone of *dpp*, *ptc*, and, in leg discs, *wg*, all of which are targets of Hh signaling. These PKA negative clones in the anterior compartment of wing or leg discs lead to pattern duplications, similar to those found in ectopically expressing clones of Hh and Ci in the anterior compartment (Jiang and Struhl, 1995; Li et al., 1995; Ohlmeyer and Kalderon, 1997; Pan and Rubin, 1995). Expression of a

PKA dominant negative in anterior clones can also induce these pattern duplications and can partially suppress a *hh* mutant background (Li et al., 1995; Pan and Rubin, 1995). These results are interpreted as a loss of negative regulation because *hh* is not required for *ptc* expression in the PKA null clones (Jiang and Struhl, 1995; Pan and Rubin, 1995).

It is not entirely clear how PKA fits into the Hh signaling pathway. A constitutively active PKA does not rescue ptc mutants and, conversely, ptc does not affect PKA activity (Jiang and Struhl, 1995; Li et al., 1995). Since constitutively active PKA doesn't affect the hh signaling pathway and can rescue PKA mutants, it seems that PKA activity is required for some basal inhibitory function, presumably involving phosphorylation of some component(s) of the Hh pathway (Li et al., 1995; Ohlmeyer and Kalderon, 1997). Most cloned segment polarity genes have consensus sites for PKA phosphorylation, but only Ci has been shown to be directly phosphorylated in vitro by PKA (Price and Kalderon, 1999). It has also been shown for Ci that mutation of the four PKA consensus sites of Ci greatly increased its activity in a reporter transfection assay and greatly reduced the amount of Ci75 produced from Ci in transfections (Chen et al., 1998). Similar results have been shown for the vertebrate Gli3 (Dai et al., 1999). In the Ci study, however, coexpression of PKA with a Ci mutant for all four possible PKA sites actually increased transcription from a reporter construct, so PKA's role is clearly not completely defined.

Fused

Of all the segment polarity genes, *fused* has the oldest lineage. The *fused* mutant was originally isolated by Bridges and Morgan in 1916 as a

spontaneous mutant in an adult fly that showed a fusion of the longitudinal wing veins 3 and 4-- hence the name, *fused* (Morgan, 1916). *fused* mutants were subsequently shown to exhibit numerous phenotypes, including a mirror image duplication of the denticle belts in homozygous larvae and female sterility in homozygous viable *fused* alleles (Gergen and Wieschaus, 1986; Martinez-Arias, 1985; Smith, 1966). Clonal studies using gynandromorphs showed that *fused* function was only required in the anterior compartment at the anterior-posterior compartment border of the wing (Fausto-Sterling, 1971; Fausto-Sterling, 1971; Fausto-Sterling, 1978; Wurst, 1979) *fused* was reisolated as a segment polarity mutant in the Nusslein-Volhard and Wieschaus screens, where its mirror image duplication of the denticle belts was interpreted as a loss of compartmental identity (Limbourg-Bouchon et al., 1991; Nusslein-Volhard and Wieschaus, 1980). Loss of *fused* function leads to an eventual loss of *wg* and *en* expression in the embryo and fused was shown to be epistatic to *patched* , indicating it functioned in the Hh signaling pathway (Forbes et al., 1993).

When the *fused* (*fu*)gene was cloned it was found to encode a putative serine-threonine kinase of 805 amino acids with the N terminal third of the protein containing the kinase domain and the C terminus having no homologies to known proteins (Preat et al., 1990; Therond et al., 1993). Its kinase domain has the greatest homology to a yeast serine/threonine kinase called SNF1, but this homology is not especially strong and Fu can not be placed in any particular kinase subfamily. Until this time, Fu has not been shown to have kinase activity, although it has been shown that the kinase domain is required for rescue of a *fu* mutant background (Therond et al., 1996). A large collection of Fu mutants has been collected over the years and a number of these have been sequenced and the molecular nature of their

lesion characterized. It has been found that Fu mutants fall into three classes; those that are large deficiencies and are lethal quite early; those that are point mutants or small, in-frame deletions in the kinase domain; or those that are missense mutations causing truncations of the Fu protein from the C terminus (Therond et al., 1996). The latter two categories contain alleles that form an allelic series with respect to time of lethality and severity of adult and embryonic phenotypes (Limbourg-Bouchon et al., 1991; Therond et al., 1996).

Fu has been shown to become phosphorylated in response to Hh signaling in S2 cells and embryos, indicating that this phosphorylation is an activation step in the Hh signaling pathway (Therond et al., 1996). *fu* has also been shown to regulate the amount of Ci found in imaginal discs and in the embryonic epidermis. *fu* mutants accumulate more Ci in the anterior compartment, both at the border, as is normal, and farther anterior in the compartment, where Ci does not normally accumulate (Alves et al., 1998; Motzny and Holmgren, 1995; Ohlmeyer and Kalderon, 1998). This increase has also been documented by immunoblotting (Ohlmeyer and Kalderon, 1998; Slusarski et al., 1995). As Ci and Fu are both thought of as positive regulators of the Hh signaling pathway, it is rather surprising that a loss of *fu* function would cause an increase in the accumulation of Ci, similar to that which occurs at the compartment border in response to Hh stimulation. At the moment, this phenomena is unexplained, although several groups have suggested that this is indicative of a need for at least two "maturation" events, Fu activity being necessary for one of them, to occur before Ci can function as a transcription factor (Ohlmeyer and Kalderon, 1998). Fu function, while being required for signal transduction through Ci, could also be responsible for its degradation and so loss of function of Fu could abrogate signal transduction but increase Ci levels.

Costal2

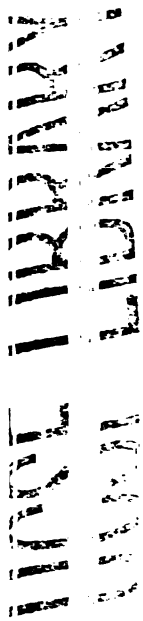
costal2 (*cos2*)was originally described as a mutant that causes duplications of Drosophila appendages, including the wings, legs, and antennae (Grau and Simpson, 1987; Whittle, 1976). *cos2* mutant embryos die with a mirror image duplication of the denticle belts and expanded regions of expression of *ptc* and *wg*, two transcriptional targets of the Hh signaling pathway (Forbes et al., 1993; Grau and Simpson, 1987). In imaginal discs, *cos2* mutants induce ectopic dpp expression and a consequent overgrowth of the disc (Capdevila et al., 1994; Sisson et al., 1997). These data indicated that *cos2* is a negative regulator of the Hh signaling pathway. It has also been demonstrated that *cos2* is epistatic to *fu* and that *fu* mutant males heterozygous for a *cos2* mutant have a partially suppressed *fu* mutant phenotype, again indicating *cos2* is downstream of *fu* (Preat et al., 1993).

cos2 has been cloned and found to encode a 1201 amino acid kinesin-like protein (Sisson et al., 1997). Its sequence is rather divergent from other kinesins, however, and it is not known if it can function as a microtubule motor or if it is restricted simply to microtubule binding.

Suppressor of Fused

Suppressor of fused (*Su(fu)*) is a gene that was isolated in a screen for autosomal semidominant mutants that could suppress the *fu* wing vein phenotype (Preat, 1992). *Su(fu)* alleles have an unusual set of interactions with *fu* alleles and these interactions define two classes of *fu* alleles. Class I *fu* alleles are almost completely suppressed by one mutant copy of *Su(fu)*, while

Class II *fu* mutants in combination with one mutated copy of *Su(fu)* show a *cos2* like phenotype (Preat et al., 1993). Additionally, adding extra doses of *Su(fu)* in a fused mutant background exacerbates the *fused* phenotype. Yet *Su(fu)* homozygous mutants alone have no obvious gross morphological defects and present only a very minor phenotype--the loss of the campaniform sensillae in the wing veins (Ohlmeyer and Kalderon, 1998; Preat, 1992; Preat et al., 1993). *Su(fu)* mutants do, however, seem to cause an reduction in the amount of Ci155, indicating that *Su(fu)* may play a role in the processing of Ci (Ohlmeyer and Kalderon, 1998). The role of *Su(fu)* became no clearer upon its cloning: *Su(fu)* encodes a novel protein whose only known motif is a PEST domain (Pham et al., 1995). Recent two-hybrid studies have suggested that Fused, Ci, and Su(fu) can interact physically, but this has yet to be demonstrated in vivo (Monnier et al., 1998).



Mechanisms of Hh Signal Transduction

All of the aforementioned data have given us a rough framework on which to model the mechanism by which a Hh signal is transduced from the cell surface to the nucleus. If the Hh, Ptc, and Smo proteins all interact in flies as they do in vertebrates, then the Hh signaling cascade begins with the binding of Hh to the transmembrane protein Ptc. This binding of Hh to Ptc relieves the inhibition of Smo by Ptc. Smo is a positive effector of the Hh signaling pathway, and relief of its inhibition by Ptc somehow results in Hh signaling being activated. Hh signaling then causes the cytoplasmic kinase Fu to be phosphorylated. Finally, Hh signaling causes accumulation of Ci155. The Zn finger transcription factor Ci is normally cleaved into a smaller repressor form called Ci75, which represses transcription of Hh target genes.

Hh signaling prevents this cleavage, resulting in the accumulation of full length Ci155 form which can activate transcription of Hh target genes. PKA phosphorylates Ci, and this phosphorylation appears to be involved in the transcriptional repressor activity of Ci. Also involved in the Hh signaling pathway are the cytoplasmic proteins Cos2 and Su(fu). This rough model is illustrated in Figure 1.

There are, however, a great many pieces missing in this model. First, it is not known how the Hh signal gets from Smo/Ptc cell surface complex to any of the intracellular components of the pathway. Second, it is not known how any of the intracellular components interact. Su(fu), Fu, and Ci interact in in vitro studies, but these interactions have not been shown in vivo. Third, it is not known what phosphorylates Fu and how this phosphorylation of Fu is related to activation of the Hh signaling pathway. Fourth, what cleaves Ci and what precisely regulates this cleavage is unknown. Finally, how phosphorylation of Ci by PKA represses Hh signaling is unclear.

As I shall show in this thesis, we have found that Fused, Cos2, and Ci all interact in a large protein complex. This complex binds to microtubules in the absence of Hh signaling, but is released in response to Hh stimulation. We also show that the C terminus of the Fused protein is required for binding to Cos2. We have further shown that both Fu and Cos2 are phosphorylated on serines, and both are hyperphosphorylated in response to Hh stimulation. Using a baculovirus coinfection system, I have recapitulated the Fused-Cos2 interactions and used this system to show that Fused phosphorylates Cos2. I have mapped the Fu induced phosphorylation site of Cos2 to Serine 572 between the motor domain and the heptad repeat region. I show that mutation of this site to an alanine eliminates several of the phosphotryptic peptides of Cos2 taken from a Fu+Cos2 coinfection. I demonstrate that the

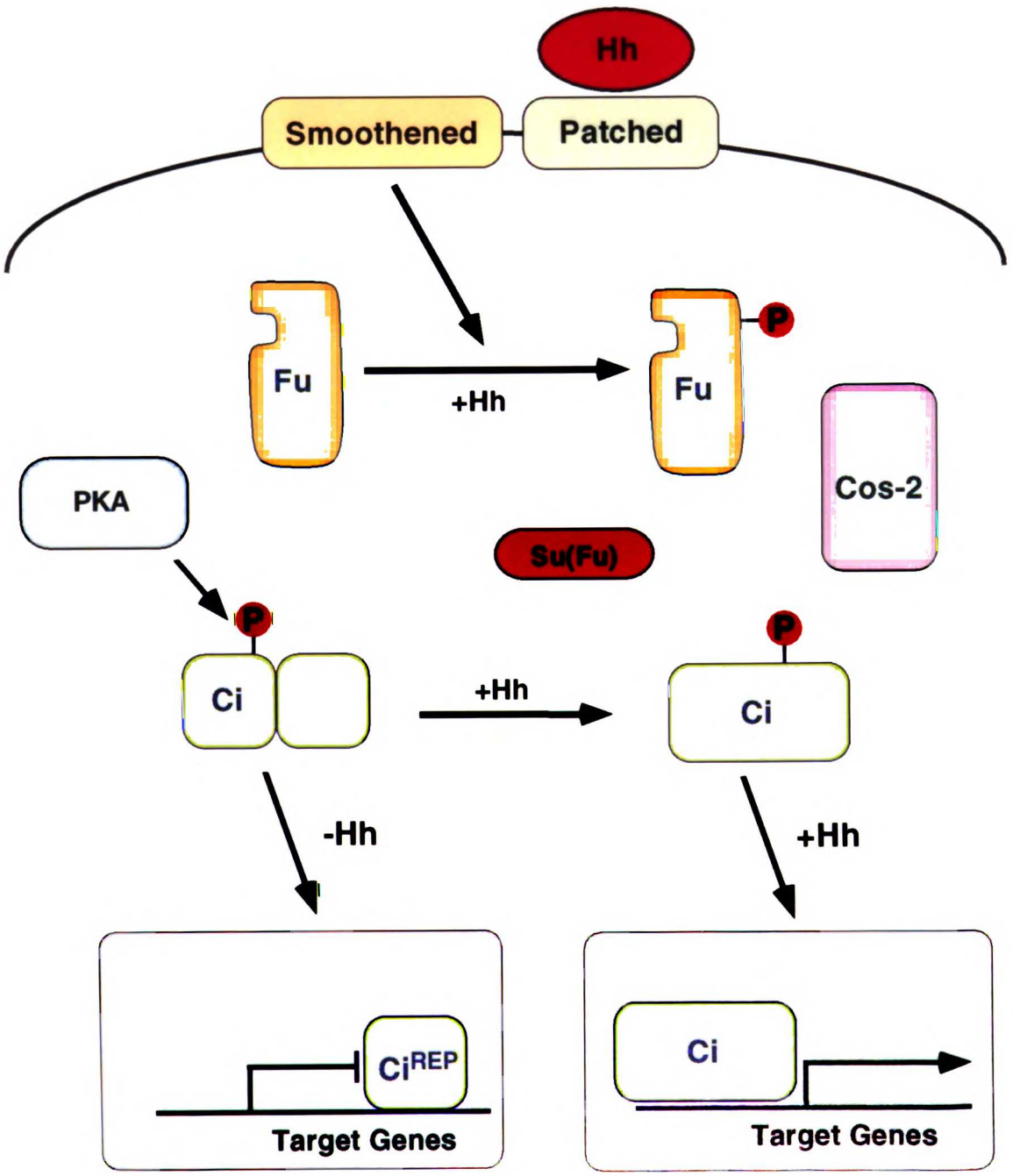
phosphotryptic maps of Cos2 obtained from S2-Hh cells closely matches that of Cos2 obtained from coinfections of Fu and Cos2. I show that the phosphotryptic peptides found in Cos2 from S2 cells matches that found in kinase inactive Fu+Cos2 coinfections. I further identify putative phosphorylation sites in Fu and show that the pattern of Fu phosphorylation found in Fu autophosphorylated in baculovirus matches that of Fu from S2 cells. Finally, I demonstrate that Fu may phosphorylate Ci and contribute to its regulation as a transcriptional activator.

Figure 1. A Model of the Hh signaling pathway.

This model illustrates all known members of the Hh signaling pathway. Their subcellular localizations are shown as are the interactions between them that have so far been identified. Hh binds to Ptc, releasing the Ptc induced inhibition of the activator Smo. This activates the Hh signal which causes Fu to become phosphorylated. This leads, through as yet unidentified means, to a stabilization of Ci. Ci is normally cleaved into a transcriptional repressor form, but Hh signaling prevents this cleavage and causes accumulation of full length Ci, which then induces transcription of target genes.

Figure 1

The Hedgehog Signaling Pathway



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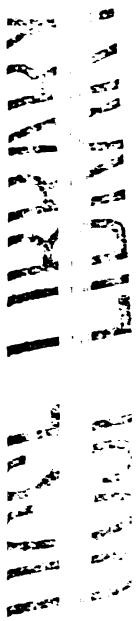
Chapter 2

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



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This work was done in collaboration with David Robbins and Pascal Therond, post-doctoral fellows in the Bishop lab, Ryuji Kobayashi at Cold Spring Harbor Laboratory, and John Sisson in Matt Scott's lab at Stanford University. I made the initial observation that p175 coimmunoprecipitates with Fu, is hyperphosphorylated in response to Hh, and demonstrates a shift in response to Hh similar to that of Fu. I generated all of the Fu antisera used in the experiments in this paper. Dr. Robbins conducted the experiments for Figures 3, 4, and 5. I was an equal participant in producing Figures 1 with Pascal and Dave, and Pascal and I contributed equally to Figure 6. Dr. Therond conducted the experiments in Figure 3A and B, while I did the experiment in Figure 3C. I also helped write and edit the paper. Dr. Kobayashi sequenced p175. John Sisson provided the unpublished Cos2 sequence and Cos2 antiserum.

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

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Summary

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

Introduction

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

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

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

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

§These authors contributed equally to this work.
‖J. C. S. provided the unpublished nucleotide sequence and antiserum required to identify the kinesin-like protein as the product of *cos2*. He did not perform the experiments reported in this manuscript. His own experiments are described in the accompanying manuscript (Sisson et al., 1997 [this issue of *Cell*]).
#Present Address: Institut Jacques Monod, University of Paris VII, Department Dynamique Du Genome et Evolution, 2 Place Jussieu, 75251 Paris Cedex 05, France.

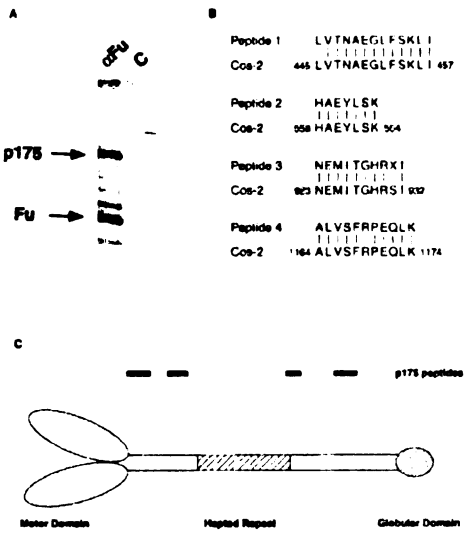

Figure 1. Coprecipitation of FU and p175

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

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

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

Results

The Product of *cos2* Is Associated with FU

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

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

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

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

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

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

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

Multiprotein Complexes Involving FU and COS2 in S2 cells

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

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

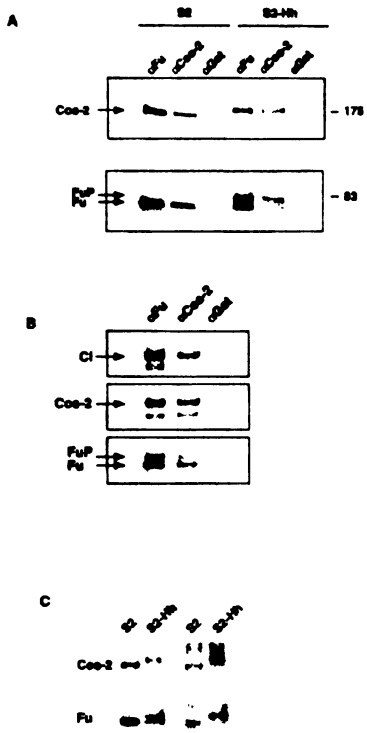

Figure 2. Coprecipitation of FU, COS2, and CI

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

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

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

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

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

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

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

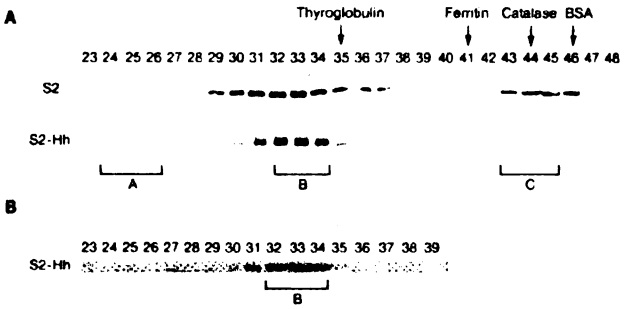

Figure 3. Multiprotein Complexes Involving FU and COS2 in S2 Cells

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

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

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

Binding of FU, COS2, and Ci to Microtubules

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

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

Phosphorylation of COS2 in the Complex with FU

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

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

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

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

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

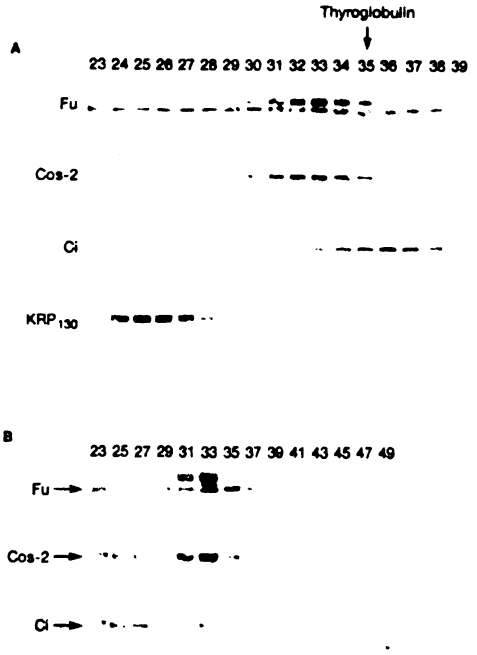

Figure 4. Multiprotein Complexes Involving FU, COS2, and CI in Drosophila Embryos

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

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

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

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

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

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

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

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

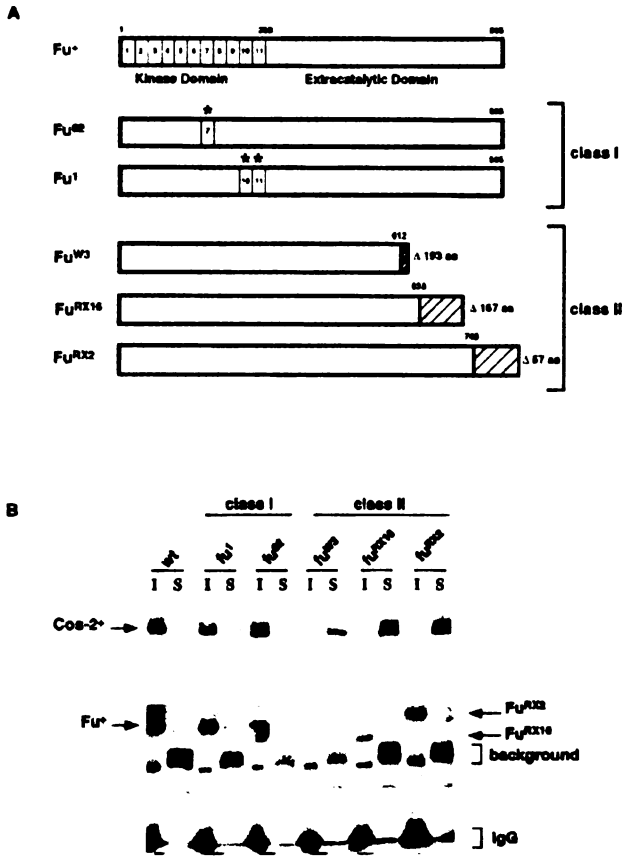

Figure 6. COS2 Binds to the Carboxyterminal Domain of FU

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

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

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

Discussion

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

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

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

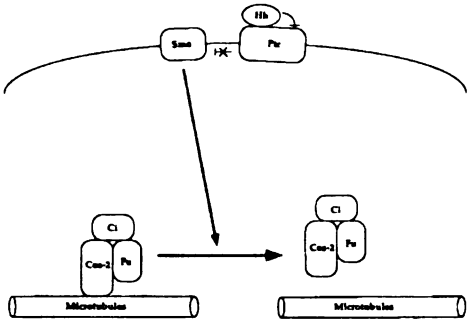

Figure 7. A Protein Machine in the HH Signaling Pathway

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

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

Binding to Microtubules

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

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

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

Phosphorylation of Proteins in the Complexes

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

Functional Significance of the Complexes Involving HH Signaling Proteins

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

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

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

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

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

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

Experimental Procedures

Reagents

Buffer A: 50 mM β-glycerophosphate (pH 7.6), 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM DTT, 10 mM NaF, plus PIC; buffer B: 20 mM Tris (pH 7.5), 20 mM p-nitrophenol phosphate, 1 mM EGTA, 50 mM NaF, 50 μM Na_3VO_4, 5 mM benzamidine; buffer C: 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1 mM EGTA, 50 mM NaF; buffer CX: buffer C supplemented with 1 mM Na_3VO_4, 1 mM Pefabloc (Boehringer Mannheim); buffer D: 50 mM β-glycerophosphate (pH 7.6), 150 mM NaCl, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM DTT, 10 mM NaF; protease inhibitor cocktail (PIC): 0.01 mM benzamidine-HCl, phenantroline (1 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin A (10 μg/ml). All buffers contained 0.1–1.0 × PIC. The 1% NP-40 buffer corresponded to buffer A supplemented with 1% NP-40 and 150 mM NaCl.

Production of Antisera Against FU

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

[^{32}P]Orthophosphate Labeling

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

Purification of p175

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

Preparation of Tissue Extracts for Immunoprecipitation

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

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

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

Immunoprecipitation

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

Analysis by Immunoblotting

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

Gel Filtration Chromatography

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

Microtubule Binding Assay

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

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

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Chapter 3

Costal2 is a Substrate of the Fused Serine/Threonine Kinase.

This chapter presents work done in collaboration with Dr. C. Turck, Adjunct Professor of Medecine, University of California, San Francisco and the Howard Hughes Medical Institute, and Dr. David Robbins, formerly a postdoctoral fellow in the lab, and now an Assistant Professor at the University of Cincinnati School of Medecine. Dr. Turck and I performed the chromatographic separation of radiolabeled peptides and Edman sequencing presented in Figures 2 and 3. Dr. Robbins made the Fu baculovirus. I performed all other experiments and made all the other reagents.

Summary

We have previously shown that hedgehog signal transduction involves a protein complex containing the protein products of the *fused, costal-2,* and *Ci* segment polarity genes, but the functional interactions between the members of this complex are still largely uncharacterized. Here we use a baculovirus coinfection system to demonstrate that the serine/threonine kinase Fu (Fu) stoichiometrically phosphorylates the kinesin-like protein Costal-2 (Cos2) on serine 572. Mutation of serine 572 to alanine eliminates the majority of Cos2 phosphopeptides found in phosphotryptic peptide maps. The phosphopeptide pattern found in Cos2 from Fu/Cos coinfections is almost identical to that found in Cos2 from S2 cells constitutively expressing Hh. This indicates that coinfection of Fu and Cos2 baculoviruses recapitulates the Hh-stimulated state in insect cells. In addition, this phosphorylation correlates with a release of the complex from microtubules since Hh-stimulation results in release of the Fu/Cos2/Ci complex from microtubules. This represents a unique use of a kinase in an intracellular signaling pathway.

Introduction

The secreted products of the hedgehog (Hh) family of cell signaling proteins pattern many tissues in both vertebrates and invertebrates (Concordet et al., 1996; Echelard et al., 1993; Fietz et al., 1994; Krauss et al., 1993; Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Vertebrate Hh homologs are further involved in the generation of certain types of cancer and several genetic syndromes (Belloni et al., 1996; Gailani et al., 1996; Goodrich et al., 1997; Hahn et al., 1996; Johnson et al., 1996; Oro et al., 1997; Raffel et al., 1997;

Reifenberger et al., 1998; Roessler et al., 1996; Wicking et al., 1998; Xie et al., 1998). 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.

Hh is a secreted signaling polypeptide which is thought to be bound to the cell surface via covalently attached cholesterol and palmitic acid moieties (Lee et al., 1992; Pepinsky et al., 1998; Porter et al., 1996; Porter et al., 1996; Tabata et al., 1992). The protein products of the *patched* (*ptc*) and *smoothened* (*smo*) genes encode transmembrane proteins which are the best candidates for Hh receptors (Alcedo et al., 1996; Hooper and Scott, 1989; Nakano et al., 1989; van den Heuvel and Ingham, 1996). Loss of *ptc* causes ectopic expression of Hh target genes, indicating that *ptc* is a negative regulator of the Hh signaling pathway, whereas loss of *smo* causes a loss of Hh target gene expression, indicating it is a positive effector of the Hh pathway (Alcedo et al., 1996; Forbes et al., 1993; Hidalgo and Ingham, 1990; Ingham et al., 1991; van den Heuvel and Ingham, 1996). Studies of the vertebrate homologs of Ptc and Smo, vPtc and vSmo, have demonstrated that vPtc and vSmo bind one another and that vPtc is the receptor for Shh (the closest homolog to Hh of the three vertebrate Hh's). Both receptors, however, seem to be required for transduction of the Hh signal. Physical interactions between Hh, Ptc, and Smo have not been demonstrated in *Drosophila*, but it has been shown genetically that *ptc* seems to be able to titer *hh* function in a dose dependent fashion and that *ptc* is needed to prevent *smo* activity (Chen and Struhl, 1996; Chen and Struhl, 1998).

How the Hh signal is transduced through its receptors to cause transcription is just beginning to be clarified. The distal component of the Hh signaling pathway is Cubitis interruptus (Ci), a zinc finger transcription factor

that is cytoplasmically localized (Alexandre et al., 1996; Dominguez et al., 1996; Eaton and Kornberg, 1990; Motzny and Holmgren, 1995; Orenic et al., 1990). In the absence of Hh stimulation, Ci is cleaved from its full length 155 kD form to a smaller N terminal fragment called Ci75 (Aza-Blanc et al., 1997). This Ci75 lacks the activator portions of the Ci molecule that are found further C terminal in the molecule and is localized to the nucleus where it represses transcription of Hh target genes (Alexandre et al., 1996; Aza-Blanc et al., 1997; Chen et al., 1999; Hepker et al., 1997). Hh stimulation blocks this cleavage and leads to an accumulation of full length Ci (Aza-Blanc et al., 1997) which then acts as a transcriptional activator (Chen et al., 1999). It has recently been demonstrated that what was thought to be a cytoplasmic tethering domain in Ci is, in fact, a nuclear export signal, and that nuclear export of Ci is necessary to maintain the cytoplasmic localization of Ci in the absence of Hh stimulation (Chen et al., 1999).

Four intracellular proteins in addition to Ci play a role in transducing the Hh signal: Fused (Fu), Costal-2 (Cos2), Suppressor of Fused (Su(fu)), and *Drosophila* protein kinase A (dPKA). Fu encodes a novel serine/threonine kinase and Cos2 encodes a kinesin-like protein (Preat et al., 1990; Sisson et al., 1997; Therond et al., 1993), while Su(fu) encodes a protein of unknown function (Pham et al., 1995; Preat, 1992). Su(fu) binds Fused and Ci in vitro, although this has yet to shown in vivo (Monnier et al., 1998). PKA can phosphorylate Ci in vitro and the PKA sites in Ci, and in its vertebrate relatives the Gli's, have been shown to be important in transcriptional activation ability of the Ci/Gli's (Chen et al., 1999; Chen et al., 1998; Dai et al., 1999; Price and Kalderon, 1999). Fu and Cos2 are phosphorylated in response to Hh stimulation (Robbins et al., 1997; Therond et al., 1996). Fu, Cos2, and Ci form a large protein complex that binds to microtubules in the absence of Hh

stimulation but that does not bind when stimulated by Hh (Aza-Blanc et al., 1997; Robbins et al., 1997; Sisson et al., 1997). The kinase activity of Fu is required for Hh signal transduction but the substrate phosphorylated by Fused has not been identified.

To ascertain the substrate of the Fu serine/threonine kinase, we have developed a baculovirus coexpression system which recapitulates the binding of the intracellular components of the Hh signaling pathway. In this coinfection system, Fu kinase activity appears to be constitutive and does not require Hh to be activated. Using this system, we have identified the Cos2 protein as a substrate of the Fu kinase and mapped the phosphorylation site on baculovirus produced Cos2 to serine 572 in a region of Cos2 between the microtubule binding domain and the dimerization motifs. We also show that the phosphorylation pattern of Cos2 from a Fu coinfection is very similar to that of Cos2 from Hh stimulated *Drosophila* S2 cells, suggesting that the phosphorylation events occuring in baculovirus coinfections match those found in response to Hh signaling.

Results

Costal2 is phosphorylated with the same kinetics as Fused and is phosphorylated predominantly on Serine

We have previously shown that Fused and Costal2 are both phosphorylated in the absence of Hh stimulation, and that both become hyperphosphorylated when S2 cells are stimulated by Hh (Robbins et al., 1997). We have further shown that Fu is phosphorylated 30 minutes after Hh stimulation (Therond et al., 1996). As Fu and Cos2 bind tightly to one another, Cos2 is an obvious candidate for a Fu substrate. One might expect that a Fu substrate should

have a similar time course of phosphorylation to Fu and, indeed, Cos2 from S2 cells was phosphorylated 30 minutes after treatment with Hh-N conditioned media, a profile identical to that of Fu (Fig 1A). Phosphoaminoacid analysis of radiolabeled Fu and Cos2 from S2 cells, S2 cells expressing Hh constitutively, and S2 cells treated for one hour with Hh-N conditioned media demonstrated that all are predominantly phosphorylated on serine (Fig 1B).

Costal-2 is hyperphosphorylated and shifted in a Fu-Cos2 baculovirus coexpression system and this phosphorylation is dependent on Fu kinase activity

Using radiolabeled, endogenous Fu and Cos2 from S2 cells, we attempted to map the phosphorylation sites of Cos2 and Fu, but found that the amounts of protein and degree of radiolabeling were insufficient for mapping (data not shown). We therefore looked for an alternate system with to which show that 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 (Bakker et al., 1997; Frolova et al., 1998; Gatti et al., 1999; Glickman et al., 1997; Lin and Hiscott, 1999; Rosenberg et al., 1998; Stempka et al., 1999; Witthuhn et al., 1999). We therefore made baculoviruses expressing Fu or Cos2 and infected them singly or in combination into Sf 21 cells to see if the normal binding between the two was preserved.

When baculoviruses expressing Fu and Cos2 were coinfected into cells, the physical interaction between Fu and Cos2 was preserved as demonstrated by the fact that Cos2 could be coimmunoprecipitated with an anti Fu antisera

(Figure 1C and D) and Fu could be coimmunoprecipitated with an anti-Cos2 antisera (data not shown). In addition, when Sf21 cells were infected with Fu and Cos2, either alone or in combination, radiolabeled, and immunoprecipitated with an anti-Fu antisera, both Fu and Cos2 were phosphorylated (Figure 1C and D), and Cos2 from a Fu coinfection had a slower mobility relative to Cos2 from a Cos2 single infection. This slower mobility was identical 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 to the single Cos2 infection (Figure 1C and D).

To demonstrate that Fu was actually responsible for this reduction in electrophoretic mobility and increase in phosphorylation, we made a baculovirus expressing Fu with a G13V mutation in the kinase domain (FuM) and coexpressed it with Cos2. The G13V mutation removes a residue involved in ATP binding throughout all kinases and should render Fu kinase activity nonfunctional. 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 (Figure 1C). 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 redid the infections and radiolabeling, but this time immunoprecipitated with an anti-Cos2 antisera. Coomassie staining of the gel followed by autoradiogaphy revealed that the amounts of Cos2 being immunoprecipitated were approximately the same, but Cos2 from a FuM coinfection still had a faster electrophoretic mobility and reduced radiolabel incorporation (Figure 1E).

Fu expressed either alone or in combination was radiolabeled and displayed forms of two different electrophoretic mobilities. These two forms

appeared to correspond to unshifted and shifted forms of Fu, the latter of which is associated with Hh stimulation (Robbins et al., 1997; Therond et al., 1996). FuM appeared to incorporate almost no phosphate, although it could still bind to Cos2 as evidenced by its ability to coimmunoprecipitate Cos2 (Figure 1C and D). This indicates that, in baculovirus expression at least, Fu autophosphorylates itself, and, further, that in baculovirus, Fu does not require Hh stimulation for autophosphorylation.

Mapping of Cos-2 Phosphorylation Sites

As Fu/Cos2 coinfection in baculovirus seemed to recreate their physical interaction and the hyperphosphorylation normally found in response to Hh stimulation in vivo, we used this system to map the Fu-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 subjected to digestion with either trypsin or Lys-C proteases. Proteolytic fragments were eluted from the gel slices, separated by HPLC, and the fractions monitored for incorporated radiolabel. Radiolabeled fractions were subjected 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. By comparing the location of the radiolabeled residue in fragments digested with trypsin versus Lys-C, one can ascertain the identity of the residue N terminal to the proteolytic cleavage site since trypsin cuts after Lysine or Arginine whereas Lys-C cuts only after Lysine.

After conducting this analysis on Cos2, we found that the profile of eluted, radiolabeled Cos2 peptides was almost identical when Cos2 was proteolysed with trypsin or Lys-C (Figure 2A). This result indicated there

were most likely, although not necessarily, Lysines at the N and the C terminus of most of the peptides, since an Arginine at a peptide's C terminus or before its N terminal cleavage site would change the size of the proteolytic fragment when digested with Lys-C versus trypsin. We also analyzed tryptic fragments of Cos2 produced from a FuM/Cos2 coinfection to determine which radiolabeled fractions were attributable to Fused kinase activity. Fractions 22, 25, and 27 were only radiolabeled when Fu was active, whereas fraction 19/20 was found whether or not Fused was active (Figure 2B). We therefore focused our analysis on fractions 22, 25, and 27.

When Edman degradation for position was conducted on the radiolabeled fractions 22/23, 25, and 27 from a tryptic or Lys-C digest of Cos2 from a wild type Fu/Cos2 coinfection, we found that the strongest radiolabeled position for fraction numbers 22 and 27 was in position #5 relative to the N terminus of the peptide. This indicated that the peptides in these fractions had a Lysine N terminal to the cleavage site and a serine or threonine in position #5 relative to the N terminus of the peptides. Fractions 22, 25, and 27 from a tryptic digest all gave a radiolabeled position #1, but we believe most of this signal to be the result of a contaminating phosphatase and not a real radiolabeled position as these positions never occurred when Lys-C was used (but see below). Fraction 19/20, whose radiolabeling was independent of Fu activity, also had a radiolabeled residue in position #5. Fractions 23 and 25 usually had a lower level of radiolabeling than fraction 27. Fraction 27 in both the trypsin and Lys-C digests also contained a radiolabeled residue at position #9, although the signal for this position was much more obvious in the Lys-C digest than the trypsin digest and was always considerably less than that for position #5 (Figures 2C and D). (The nature of this site will be discussed below). In searching for the Fu induced

phosphorylation site of Cos2, we therefore focused on theoretical tryptic peptides with a Lysine before the N terminus of the peptide and a serine or threonine in the fifth position relative to 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 to determine the amino acid composition of radiolabeled fractions instead of the radiolabeled position. We radiolabeled Cos2, digested it with trypsin, and again separated the peptides on an HPLC, this time using a shallower elution gradient to provide better separation of the peptides. Using these digestion and elution conditions, we found only two radiolabeled fractions, from only one of which were we able to obtain amino acid sequence information. Not surprisingly, since Cos2 is a large protein, we found several different amino acids in each position, indicating a mix of peptides in the fraction (Figure 3A). By comparing the amino acids in each position with theoretical Cos2 peptide products of cleavage after Lysines, we were able to assign almost all of the amino acids to five possible peptides (Figure 3B). Three of these peptides had three amino acids in common with the residues that we found in our sequencing, and one, containing amino acids 568 to 575 had four amino acids in common. This peptide also contains a Serine at position 572. This Serine did not show up in our Edman degradation, which is expected if this serine is phosphorylated: phosphoaminoacids are very difficult to release using standard Edman sequencing elution conditions and this is why harsher conditions are used to elute phosphoaminoacids when determining radiolabeled residue position. The peptide containing the residues 931 to 937 had two residues in common with our sequencing results, and had a serine in position #5. However, we did not consider this a viable candidate as the N terminus of this peptide is bordered by an Arginine and

hence should not be cut by Lys-C to give a radiolabeled residue in position #5 which we had found with Lys-C digestion. Thus, Serine 572 appeared to be the best candidate site for phosphorylation by Fu in baculovirus.

Mutation of Serine 572 to Alanine eliminates three of the primary Cos2 phosphotryptic peptides

To confirm that Serine 572 was the Fu-induced phosphorylation site of Cos2 in baculovirus, we made a baculovirus expressing a Cos2 with a S572A mutation and compared phosphotryptic peptide maps of radiolabeled Cos2 S572A from a Cos2 S572A/Fu confection with Cos2 from Fu/Cos2 and FuM/Cos2 coinfections. There were 6 major phosphopeptides of differing intensities found in phosphotryptic peptide maps of Cos2 from a Fu/Cos2 coinfection. These phosphopeptides are labeled 1 through 6 and are shown in the actual map in Figure 4A and schematically in Figure 4F. There were also several large blobs of radiolabeled material that only migrated in the chromatographic dimension which we believe to be undigested material. The phosphotryptic peptide map of Cos2 S572A from a Fu/CosS572A coinfection only contained phosphopeptides 1, 3, and 6. Phosphopeptides 2, 4, and 5 were missing from Cos2 S572A, indicating that these phosphopeptides were most likely tryptic peptide isoforms containing serine 572 (Figure 4B). These data strongly suggested that phosphorylation of serine 572 is responsible for phosphorylation of these three phosphopeptides.

As mutation of serine 572 to alanine only eliminated three of the six major phosphopeptides, we were curious whether the other three phosphopeptides were phosphorylated by Fu or by some other kinase. To investigate this possibility, we compared the phosphotryptic peptide maps of Cos2 from a FuM/Cos2 coinfection and a single Cos2 infection with that of

Cos2 from the Fu/Cos2 coinfection (Figures 4A, C, and D). Phosphotryptic peptide maps of Cos2 from the FuM/Cos2 coinfection and the Cos2 single infection only showed phosphopeptide 1 when compared with Cos2 from a Fu/Cos2 coinfection. Several small phosphopeptide spots not seen before can also be seen to the left of phosphopeptide 1 in Figure 4C, but we believe these are background only visible due to the overexposure of this particular film. The fact that only peptide 1 remained when Fu kinase activity was negated in these coinfections indicated that phosphopeptides 2 through 6 of Cos2 were phosphorylated by Fu, whereas phosphopeptide 1 was phosphorylated by an endogenous Sf21 cell kinase.

Verification of Fused induced Phosphorylation of Serine 572 in Baculovirus and in S2 Cells

Because it is possible that the Fu phosphorylation site we identified on Cos2 is an artifact of baculovirus coinfection, we sought to demonstrate that the phosphotryptic peptide map of endogenous Cos2 from radiolabeled S2 cells was similar to that of Cos2 from baculovirus coinfections. Figure 5 shows the comparison of the phosphotryptic maps of Cos2 from S2 cells, S2 cells constitutively expressing Hh (Therond et al., 1996), a Fu/Cos2 baculovirus coinfection, and a FuM/Cos2 coinfection. Cos2 from S2 cells contains only one phosphorylated peptide, which, by position relative to the small amount of undigested material above the origin, appears to correspond to phosphopeptide 1 in the phosphotryptic peptide map of Cos2 from baculovirus coinfections (Compare Figure 5A and 5B). Cos2 from S2 cells constitutively expressing Hh contained five phosphopeptides which appeared to closely correspond to phosphopeptides 1 through 5 found in the phosphotryptic peptide maps of Cos2 from a Fu/Cos2 coinfection (Compare

Figure 5C and 5D). Thus, the major spots found in Fu/Cos2 baculovirus coinfection match those found in S2 cells constitutively expressing Hh, and hence constitutively hyperphosphorylating Fu and Cos2. There are one or two minor spots in the phosphotryptic map that vary between the maps done using S2 cell Cos2 and that using baculovirus expressed Cos2, but overall the phosphotryptic maps are remarkably similar, leading us to conclude that the phosphorylation events occurring on Cos2 obtained from baculovirus expression, either with or without Fu coexpression, are nearly identical to those occurring in S2 cells and, by extension, to those occurring in vivo.

Localization of a Possible Second Fused-Induced Phosphorylation Site in Cos2.

As noted above, fraction 27 contained a radiolabeled position #9 which was also linked to Fu kinase activity, but there were no serines or threonines in a corresponding position in any of the possible peptides found in the Edman sequencing experiment (Figures 2C and D, Figure 3B). Therefore, we tried to determine what Cos2 peptide corresponded to the peptide radiolabeled at position #9. Since the fraction numbers of the Edman sequencing experiment were not comparable to the those used to determine the position of radiolabeled residues because of the shallower elution gradient used for the Edman sequencing, we could not determine how the fractions obtained for radiolabeled position and Edman sequencing correlated. A subsequent sequencing analysis of fractions digested with trypsin and eluted in the same manner as the positioning experiments indicated that the peptides we identified by sequence were eluting in fraction 22 (K. N. and C. T., data not shown), indicating that we would not expect to find a radiolabeled position #9 as we had only seen this position in fraction 27. However, since

we had found a small amount of radiolabel in position #5 in fraction 22 of the original radiolabeled position experiment but a great deal in position #5 in fraction 27, we concluded that the trypsinization of peptide 568-575 was very inefficient in this particular experiment. We hypothesized that the trypsin had instead cut after the Arginine at position 589 or the Lysine at position 590, and that this larger peptide eluted in fraction 27.

If the phosphopeptide in fraction 27 contained Serine 572 and represented a partial proteolytic product, then we reasoned that the position #9 peptide could also represent a partial proteolytic product. After examining the Cos2 sequence for partial proteolytic products with a Lysine at their N terminus, we noticed that Serine 931 would be in position #9 relative to the N terminus of a peptide starting at amino acid 923. This partial digestion product would have a serine in position #9, whereas the complete digestion product, amino acids 931-937, would have a radiolabeled residue in position #1. Some of the radiolabel eluting in position #1 in fractions 22, 25, and 27 of the tryptic digest (Figure 2C), which we originally attributed only to phosphatase activity, could be due to small amounts of this complete digestion product releasing their phosphoserines in the first Edman cycle.

Support for phosphorylation of this residue comes from our previous paper: in the original peptide sequencing of p175, we obtained a Lys-C peptide starting with amino acid 923. We were able to obtain matching sequence for all amino acids between 923 and 932, with the sole exception of the Serine at 931, suggesting that this residue was somehow modified and so could not be identified by Edman sequencing (Robbins et al., 1997). Confirmation of the phosphorylation of this site came when we made a baculovirus with Serines in positions 927, 931, and 935, and a threonine in position 934 all mutated to alanine. We coinfected this quadruple mutant with Fu into Sf21 cells and

analyzed the phosphotryptic peptide maps of this mutant Cos2. We found that phosphopeptide 3 was missing in these coinfections (Figure 4E). This result, combined with the original sequencing data, suggests that this phosphopeptide contains a phosphorylated serine 931. However, it is possible that threonine 934 or serine 935 could also contribute to radiolabeling of phosphopeptide 3. We are presently mutating these sites individually to address this possibility.

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 large molecular weight complex, but the role of Fu in the complex was indeterminate (Robbins et al., 1997). We have shown here that Fu phosphorylates the kinesin-like protein Cos2 in a baculovirus coexpression system and that the pattern of phosphorylation of Cos2 from baculovirus and S2-Hh cells is almost identical. We have mapped the Fu-induced phosphorylation site of Cos2 to Serine 572 in a region of the protein between the microtubule binding domain and the heptad repeats. Serine 572 is closer to the heptad repeats than the microtubule binding domain and is not within the the "neck" region of kinesins which has been shown to be involved in kinesin movement (Vale and Fletterick, 1997)

Several observations indicate that the Fu-induced phosphorylation of Cos2 that we observe in baculovirus is a physiologically relevant Hh signaling event. First, the electrophoretic mobility shift of Cos2 seen in cells and embryos 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 physical interaction between these two proteins is preserved in coinfections. Most convincing, however, is the fact that both the positions and relative intensities of the phosphopeptides found in phosphotryptic peptide maps of Cos2 isolated from S2 cells and from baculovirus coinfections correspond very closely. This indicates that the Fu-induced phosphorylation of Cos2 observed in baculovirus correlates with the Hh induced phosphorylation of Cos2 found in cells. This assertion is further supported by the observation that the phosphotryptic peptide map of Cos2 from a FuM/Cos2 coinfection 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 while the phosphorylation state of Cos2 from a FuM coinfection mimics that found in S2 cells in the absence of Hh signaling.

It should be noted that, in our previous study, we asserted that Fu was probably not responsible for Cos2 hyperphosphorylation. Immunoblots of extracts made from homozygous Fused mutant larvae initially indicated that Cos2 was still shifted in these extracts even when the mutant Fused did not bind to Cos2 or had a lesion in the kinase domain (Robbins et al., 1997). This immunoblot was not definitive, however, since the Cos2 blotted from one Fu mutant did not show a shifted band and appeared to have less protein than the Cos2 blotted from two other mutants, both of which appeared to show a shifted band. This hinted that perhaps the shifting seen for some of these mutants was a loading effect and not a true change in mobility. Also, we could not see separation of the two Cos2 bands, which we could clearly see in our initial immunoblots showing physical association. In any case, we

believe that phosphotryptic peptide maps are a far more sensitive test of phosphorylation status than one dimensional gels.

Effects of Fu Phosphorylation of Cos2 on Cos2 Activity

The only other kinesin like molecule in which a phosphorylation site has been mapped is the HsEg5 kinesin related protein from humans which is involved in cell division (Blangy et al., 1995). In that instance, HsEg5 was shown to be phosphorylated by $p34^{cdc2}$ on a site in the C terminal tail domain of the protein, and this phosphorylation was shown to regulate binding of HsEg5 to dynactin and to the microtubule spindle in dividing cells. We have demonstrated here that Fu primarily phosphorylates Cos2 in a region between the microtubule binding domain and the heptad repeats, a region not previously known to be phosphorylated in kinesin-like molecules. Because Cos2 is a divergent member of the kinesin family, we have not been able to determine if this phosphorylation site is conserved.

Previously we demonstrated that the Hh induced hyper-phosphorylation of Cos2, which we now attribute to Fu kinase activity, corresponded to a reduction in the binding of the Fu/Cos2/Ci complex to microtubules (Robbins et al., 1997; Sisson et al., 1997). Thus, the Fu-induced phosphorylation of Cos2 most likely regulates the binding of Cos2 to microtubules. This regulation could be achieved directly by regulation of the binding activity of Cos2 or indirectly by a modulation of the interactions of two members of a Cos2 dimer. It is also possible that the Fu induced phosphorylation does not regulate binding of Cos2 to microtubules, but rather regulates Cos2 movement. In this case, Cos2 would normally be immobile and bound to microtubules. Hh signaling would then trigger Cos2 to move and the lack of binding of Cos2 to microtubules in our assay would reflect the

Cos2 molecules attempting movement. But again, it is not known if Cos2 can function as a motor.

A Second Fu induced Phosphorylation site on Cos2.

In the description of the identification of candidate peptides by Edman sequencing, we point out that one of the candidate peptides is Cos2 amino acids 931-937 (Figure 3B), and demonstrate that it appears to cause a loss of phosphopeptide #3 in Cos2 phosphotryptic peptide maps but does not affect the major phosphopeptide 1 and 2 (Figure 4E). At the present time, we have not discovered the purpose of this second Fu induced phosphorylation of Cos2, but it is interesting to note that it falls into roughly the same area of the molecule as the phosphorylation site of HsEg5 which is important in binding to the dynein interacting protein, dynactin. Given the recent evidence that Fu kinase activity promotes Ci processing (Alves et al., 1998; Ohlmeyer and Kalderon, 1998), it is conceivable that phosphorylation of this site could regulate Ci binding by Cos2. Loss of Fused kinase activity or loss of the phosphorylation site could reduce Ci binding to Cos2 which could, in turn, reduce Ci processing. It has already been demonstrated that loss of Cos2 increases Ci155 levels in the wing disc (Sisson et al., 1997). It is also possible that Cos2 does not bind to the tail of Ci but, in fact, binds to some other protein involved in the pathway, such as Ptc, or to some as yet unidentified protein involved in vesicular transport.

Fu and Cos2 Interactions are Accurately Recapitulated in Baculovirus.

Most analysis of protein postranslational modification occurs in cell lines because of the uniformity of responses to different stimuli and, in the case of phosphorylation studies, because of the relative ease of radiolabeling.

Unfortunately, *Drosophila* cell lines, while providing the uniformity of response, are more difficult to radiolabel than mammalian cell lines, requiring extended incubation times, increased levels of radiolabel, and the use of incompletely defined media often containing free phosphate (K.N., unpublished data). Many previous studies have demonstrated that the physical and catalytic interactions between proteins are maintained upon baculovirus coexpression, and we show here that this is true for members of the Hh signaling pathway. This is illustrated by the presence of a basal phosphorylation in Cos2 taken from baculovirus that is found regardless of Fu activity and correlates with a non-Hh induced phosphorylation in Cos2 from S2 cells (phosphopeptide 1, Figure 4).

We have demonstrated that the unique segment polarity kinase Fu phosphorylates the kinesin-like protein, Cos2, and that this phosphorylation is very likely required to mediate the Hh signal, both in flies and in insect derived cell lines. The discovery that Fused phosphorylates Cos2 is an interesting finding for several reasons. First, although kinesins have been shown to be phosphorylated (Blangy et al., 1997; Blangy et al., 1995; Dorner et al., 1998; Lee and Hollenbeck, 1995; Lindesmith et al., 1997; Marlowe et al., 1998; Okada et al., 1995), there are few examples wherein the actual kinase has been identified (Blangy et al., 1995; Okada et al., 1995). Second, this is the first example that we are aware of where the kinase and the substrate kinesin have been shown to bind together. This intimate relationship of kinesin and kinesin-kinase probably functions to ensure that phosphorylation of Cos2 occurs in a stoichiometric fashion instead of a catalytic manner, as in the example of HsEg5 and $p34^{CDC2}$ (Blangy et al., 1995). Third, the HsEg5 kinesin phosphorylation site, the only one previously mapped, was found in the

"tail" domain of HsEg5, whereas we demonstrate the phosphorylation relevant to signaling occurs in the coiled regions near the motor domain. Finally, previous studies of kinesin phosphorylation have focused on the role that the phosphorylation plays in regulating either kinesin movement or in the cell cycle, whereas we demonstrate a new role for kinesin phosphorylation in transducing extracellular signals.

Experimental Procedures

Constructs, Mutagenesis, and Cloning

A full length Cos2 cDNA was a kind gift of J. Sisson, K. Ho, and M. Scott (Stanford University). pKN5 was created as a shuttle vector by excising the Kpn I/Sac II fragment from pBluescript KS(-) (Stratagene) and inserting a new multiple cloning site with a central stop codon in frame with all restriction sites (details available upon request). Bam HI/Not I cleaved Cos2 was cloned into the Bam HI/Not I 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 on the full length Fu cDNA, D6, or, in the case of Cos2, on pieces of Cos2 encompassing the site to be mutated cloned into pKN5 or pBluescript KS(-) (Stratagene). FuM was made by mutating Glycine 13 to Valine. The Cos2 mutants were made by mutating the appropriate Serine(s) and/or Threonine(s) to Alanine. Pfu polymerase (Stratagene) was used and for both Fu and Cos2, minimal portions of the mutated plasmid were excised with the appropriate enzymes and ligated into similarly cut D6 or pKNCos2, respectively, and the junctions confirmed by sequencing (details available upon request).

To create the UAST-Cos mutant vectors, UAST (Brand and Perrimon, 1993), kindly provided by K. Hill (UCSF), was cleaved with Bgl II and Sac II and ligated to mutant Cos2 similarly cleaved. To make metallotheinin inducible vectors for stable cell transfection, an Eco RI fragment containing the Fu cDNA was ligated into an Eco RI cut pMTV5HisA (Invitrogen) to create pMTFu and, for Cos2, a Kpn I/ Sma I cleaved pKN5Cos2 or Cos2 mutant was ligated into a Kpn I/ Eco RV cleaved pMTV5HisA to create pMTCos2.

Immunoprecipitation

Cells were lysed by addition of 0.5 ml per 10 cm plate (0.25 ml for 3.5 cm plates) of NP40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 % Nonidet P-40, 10 mM NaF, 1 mM EGTA, 0.01 mM benzamidine-HCL, 1 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml Pepstatin A) and rocked occasionally at room temperature for 15 minutes. The lysate was collected and cleared by centrifugation at 10,000 X g for 10 minutes at 4°C. Protein A sepharose beads which had been preincubated with Fu antisera or anti Cos2 antisera (Robbins et al., 1997; Sisson et al., 1997) for 30 minutes were added to the resultant supernatant and immune complexes were collected by incubation of this mixture for 2-4 hours at 4°C. The immunoprecipitates were then washed three times for 30 minutes each with NP40 lysis buffer + 0.25 M NaCl and separated on 8% SDS-PAGE gels.

Baculovirus Production and Infection

Baculoviruses were produced using the Bac to Bac system, essentially as described by the manufacturer (Life Technologies). For the Fu and FuM baculoviruses, a EcoRI fragment containing the full length wild type or

mutant Fu cDNA was inserted into the EcoRI site of pFastBac1. For the Cos2 and Cos2 mutant baculoviruses, wild type or mutant Cos2 was excised from the pKN5 shuttle vector using BamHI and SacII and inserted into the BamHI and SacII sites of pFastBac1. The Cos2-6His was constructed by inserting the BamHI /Not I fragment of Cos2 into the BamHI/Not I sites of pFastBac HtB. After the initial infection of Sf21 cells, viruses were amplified by infecting Sf21 cells at an MOI of 0.1 and harvesting virally infested medium 4 days after infection. Infections were conducted by spinning down $12.5X10^6$ cells per 10 cm plate, adding virally infested medium to an MOI of 3.5-4.0 for each virus, resuspending the cells, and incubating the cells at 28°C for 1 hour, shaking every 10 minutes. The infected cells and medium were then plated out on 10 or 15 cm plates and Grace's medium sufficient to give 12 ml per 10 cm/ 25 ml per 15 cm plate was added. All baculoviruses were confirmed to be expressing the appropriate protein by immunoblotting.

Cell Culture and ^{32}P Orthophosphate Labeling

S2 cells, S2-Hh-N, and S2-Hh cells were cultured in supplemented Schneider cells medium as previously described (Therond et al., 1996). cl-8 cells were cultured as previously described, with the exception that the fly extract was heat treated for 90 minutes at 55°C (Cumberledge and Krasnow, 1994). Sf21 cells were cultured in spinner flasks in Grace's Insect Cells Medium (Life Technologies) supplemented with 10% fetal bovine serum and 1X Penicillin/Streptomycin. S2 cell labeling was as previously described (Robbins et al., 1997), with the exception that cells were lysed and immunoprecipitated as above. Each tryptic map represents the Fu and/or Cos2 immunoprecipitated from 12 10 cm plates. Sf21 cells were labeled by infecting 12.5 X 10^6 cells/ 10 cm tissue culture plate as above, then 24 hours

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 and 1X Pen/Strep, and ^{32}P orthophosphate to 0.4 mCi/ml (NEN). Plates were then incubated 14 hrs at room temperature, followed by a 5 ml wash of phosphate free Grace's medium. Lysis and immuno-precipitation were then conducted as above. Immunoprecipitates were run out on 8% SDS-PAGE gels and autoradiographed.

Phosphoaminoacid and Phosphotryptic Peptide Analysis

Radiolabeled Cos2 and Fu proteins were excised from SDS-PAGE gels and cut into small pieces. These fragments were placed in tubes and washed twice for 30 minutes each with 100µl of 50 mM ammonium bicarbonate, pH=8.0 /50% acetonitrile. The gel fragments were dried overnight in a fume hood before the fragments were reduced by rehydration and incubation for 1 hr at 55°C in 50 mM ammonium bicarbonate supplemented with 10 mM DTT. The DTT solution was removed and the fragments acylated by incubation for 45 minutes with 50 mM ammonium bicarbonate with 55 mM iodoacetamide. The fragments were washed 2 X 10 minutes with 50 mM ammonium bicarbonate, and then 2 X 30 minutes with 50 mM ammonium bicarbonate/50% acetonitrile and dried overnight in a fume hood. The samples were then digested by adding 200 µl of 50 mM ammonium bicarbonate + 5 µg/ml modified trypsin (Promega) and incubating for 24-48 hrs at room temperature. Peptides were then eluted with two washes of 300 µl each 60% acetonitrile + 0.1 % TFA in dH_2O. 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 dH_2O 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 in Boyle et al (Boyle et al., 1991), 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 (Hellman et al., 1995). The resultant peptides were then separated using reverse phase HPLC on a microbore C8 column (Vydac, Hesperia, CA) and an aliquot of each collected fraction was scintillation counted. It should be noted that, however, that, while radiolabeled fraction profiles were relatively consistent, a radiolabeled fraction could change elution fraction by ±1 and the number of counts per fraction could also vary between different experiments.

Individual radiolabeled fractions were subjected to covalent Edman degradation on a Sequelon AA membrane (Perseptive Biosystems, Cambridge, MA) with a Perkin-Elmer Model 492 Protein Sequencer (Perkin-Elmer, Foster City, CA). The ATZ-amino acids were extracted from the membranes with neat TFA 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 ATZ-amino acids were eluted using standard conditions instead of neat TFA and sequenced on a Perkin-Elmer Model 492 Protein Sequencer. Amino acids found at each position were then compared to the

known Cos2 sequence and candidate peptides identified based on a similarity of at least two sequential amino acids.

Transient Transfections

cl-8 cells were resuspended and plated onto 3.5 or 10 cm plates then allowed to grow until they reached 60-80% confluence, then washed once with complete M3 medium before transfection. For transient transfections, 10 μg of the appropriate UAS plasmid was combined with 4 μg of Ub-Gal4 for 3.5 cm plates or 50 μg of the appropriate UAS plasmid was combined with 20 μg of Ub-Gal4 for 10 cm plates, and transfected using a standard calcium phosphate transfection protocol (Ashburner, 1989). The precipitates were left on the cells for 12-24 hours before the cells were washed twice with 2-5 ml of complete M3 medium. The cells were then incubated for a further 10-14 hours before being lysed in NP40 lysis buffer and analyzed by SDS-PAGE.

Figure 1. Time course and phosphoaminoacid analysis of Cos2 from S2 cells and Fused dependent phosphorylation of Cos2.

(A) Time course of phosphorylation of Fused and Costal-2 from S2 cells. S2 cells were treated with Hh-N conditioned media for the indicated times. Both Cos2 and Fu begin shifting between 15 and 30 minutes after exposure to conditioned media. Cos2 from S2 cells constitutively expressing Hh or S2 cells constitutively expressing Hh-N appear to have an even slower electrophoretic mobility than that from cells treated with conditioned media.
(B) Cos2 from S2 cells and S2 cells constitutively expressing Hh is predominantly phosphorylated on serine.
(C) Coinfection of baculoviruses expressing Cos2 and FuM, a Fu with a G13V mutation which abrogates kinase activity, results in reduced levels of phosphorylation of Cos2 and eliminates the slower migrating form of Cos2 in these coinfections.
(D) Fu and Cos2 interact physically when coinfected into baculovirus and Cos2 coinfected with Fu migrates slower than Cos2 infected alone.
(E) Immunoprecipitation by a Cos2 antisera of Cos2 from different coinfections yields similar amounts of Cos2 by Coomassie staining. Cos2 from a Fu coinfection is more strongly radiolabeled than that from a FuM/Cos2 coinfection.

Figure 1

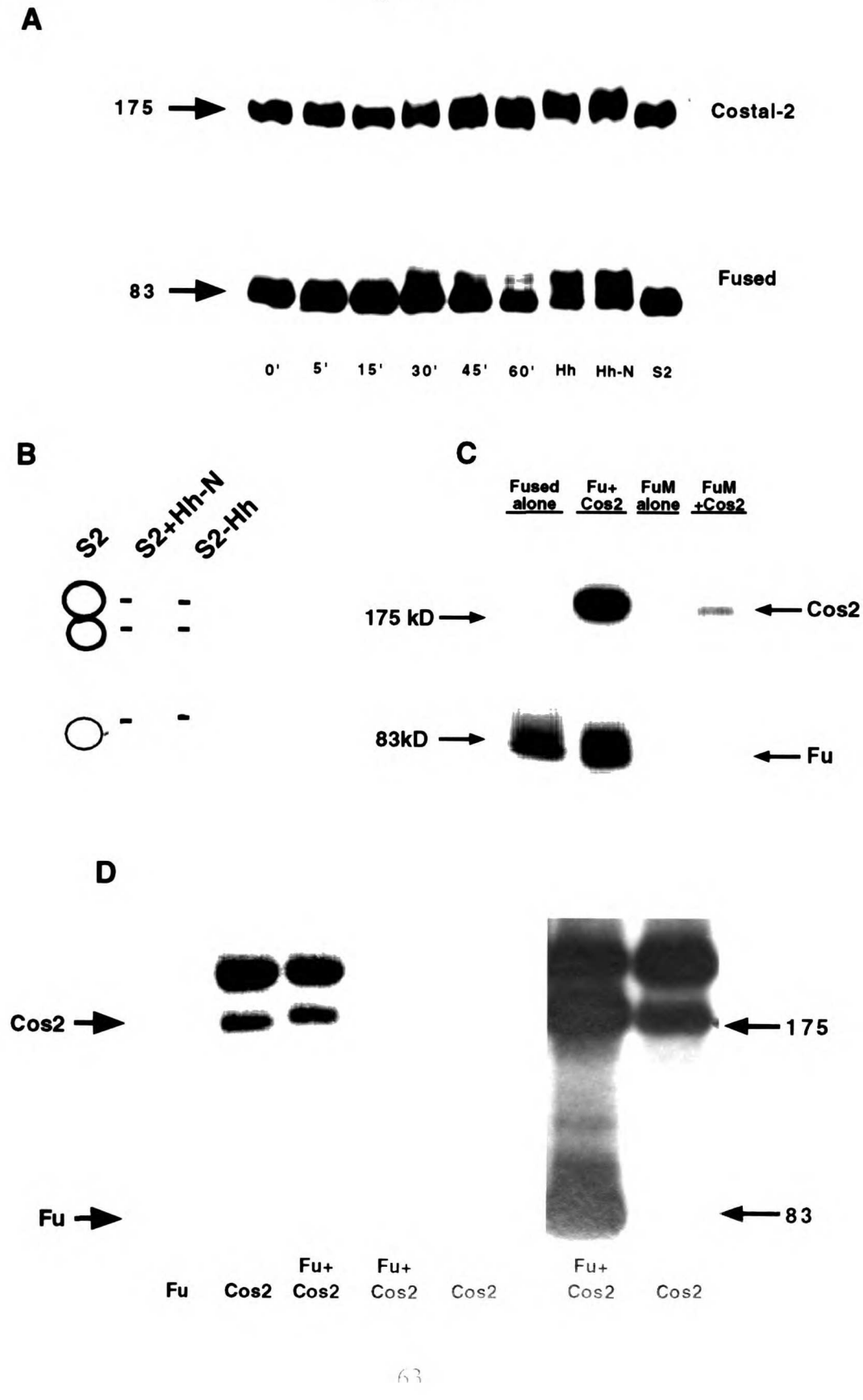

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Figure 1

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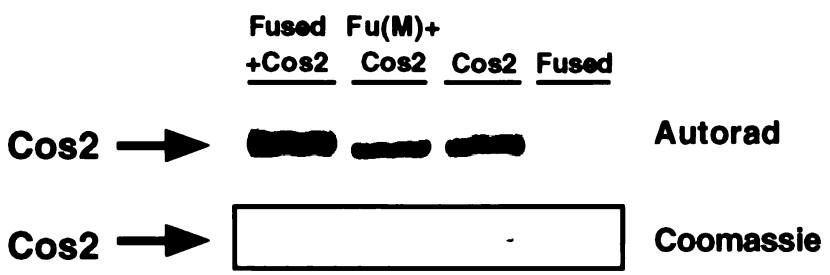


Figure 2. Positioning of radiolabeled phosphoaminoacids relative to the N termini of Cos2 phosphopeptides.

(A) Profile of Radiolabeled Cos2 peptides from trypsin versus Lys-C digests. The pattern of radiolabeled fractions is nearly identical in early fractions for both proteases.

(B) Profile of radiolabeled Cos2 peptides from coinfections of FuM/Cos2 and Fu/Cos2. Three of the earlier eluting fractions, fractions #22, 25, and 27, are found only when Fu kinase activity is present.

(C) Location of the radiolabeled residue in the three earlier eluting fractions of a trypsin digest of a Fu/Cos2 coinfection. Fractions 22 gave a radiolabeled position at position #5. Fraction 25 did not give any radiolabeled positions, while fraction 27 gave radiolabeled positions #5 and #9.

(D) Location of the radiolabeled residue in the three earlier eluting fractions of a Lys-C digest of a Fu/Cos2 coinfection. Fraction 23 gave a radiolabeled position #4. Fraction 25 again gave no radiolabeled position, while fraction 27 gave hot positions #5 and #9, as found in the trypsin digest. The radiolabeled position #6 we believe to be later eluting phosphoaminoacids from position #5.

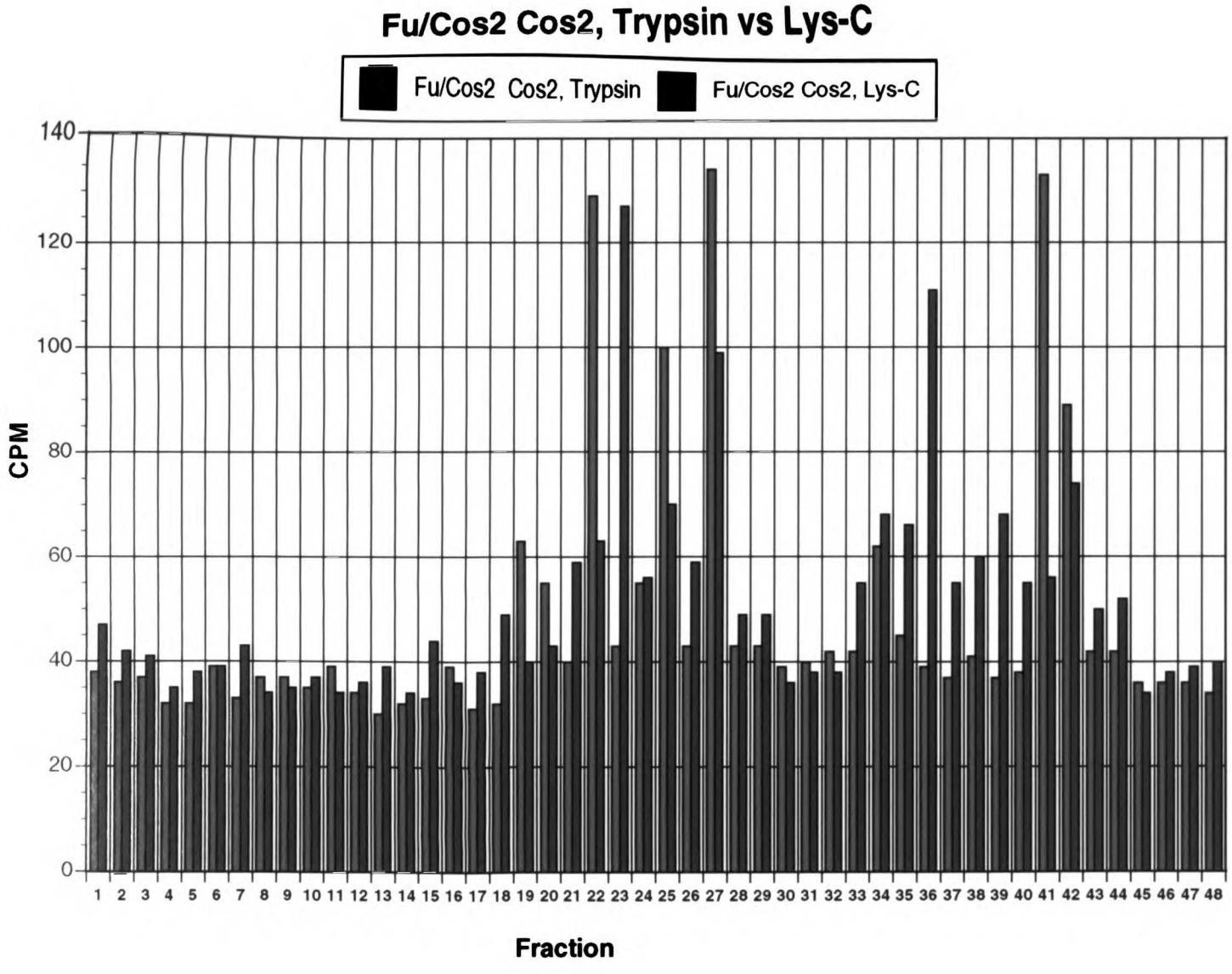

Fu/Cos2 Cos2, Trypsin vs Lys-C
Fu/Cos2 Cos2, Trypsin
Fu/Cos2 Cos2, Lys-C
CPM
0
20
40
60
80
100
120
140
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
Fraction

Figure 2A

Figure 2B

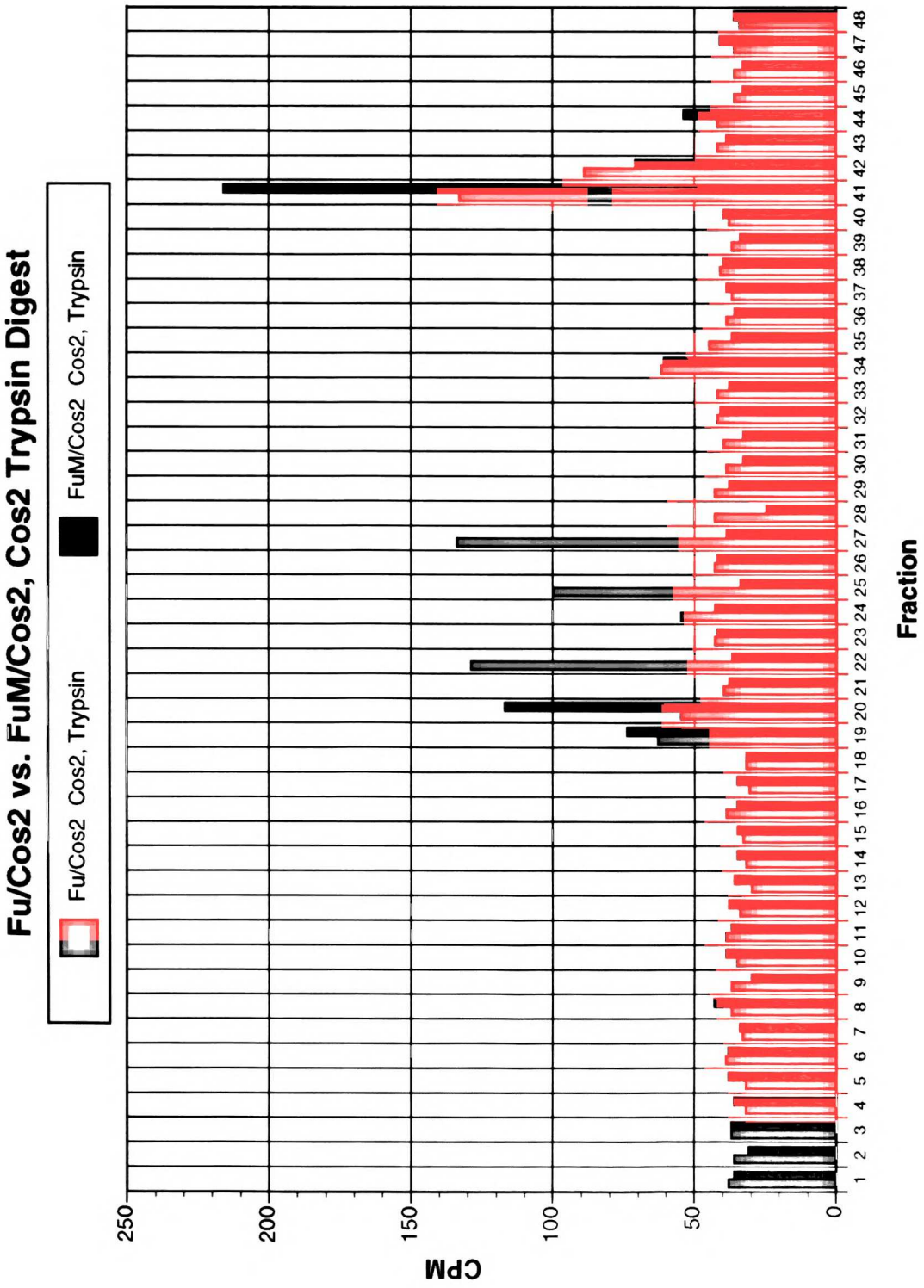

Fu/Cos2 vs. FuM/Cos2, Cos2 Trypsin Digest
Fu/Cos2 Cos2, Trypsin
FuM/Cos2 Cos2, Trypsin
CPM
0
50
100
150
200
250
Fraction
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48

Figure 2C

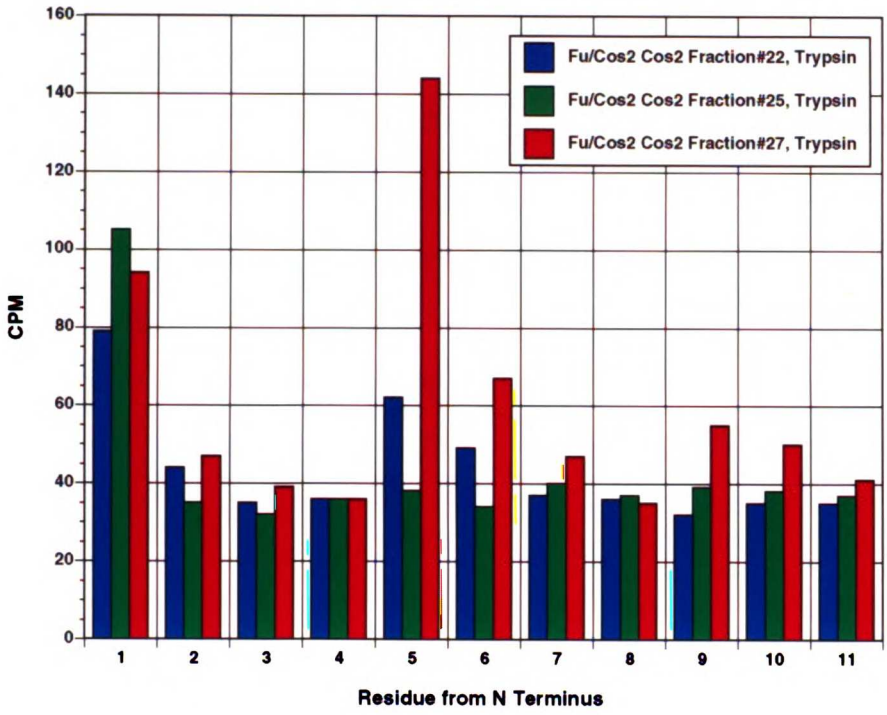


Figure 2D

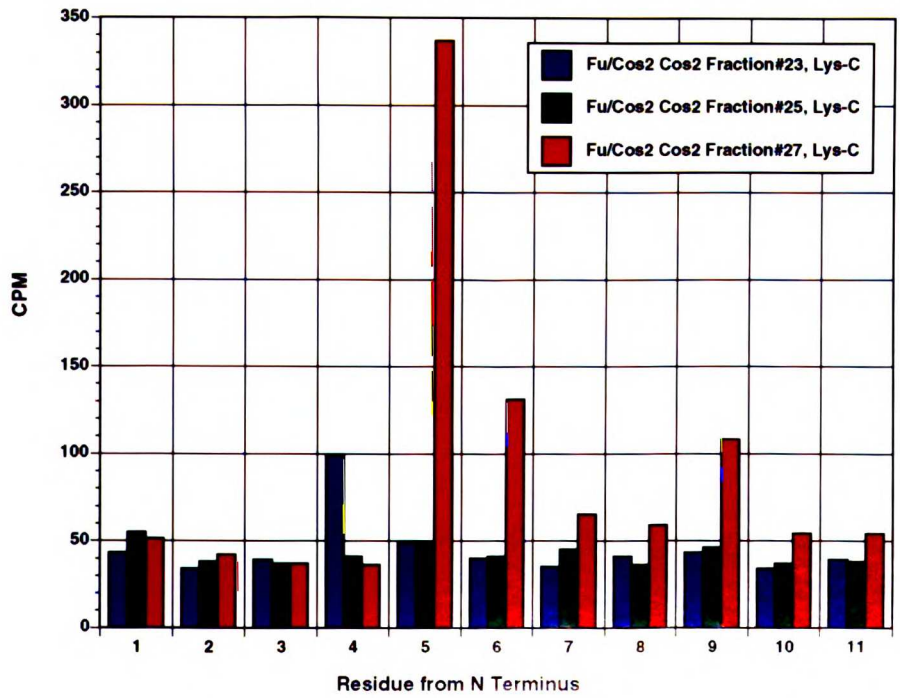


Figure 3. Amino acid sequencing of radiolabeled Cos2 fraction.

(A) The various amino acids found at each position of the peptides from fraction 25 relative to the N terminus of the peptide. Due to the nature of the sequencing method, the amino acids in the first position can not be determined. There is a mix of amino acids at each position relative to the N terminus, indicating a mix of peptides in the fraction.

(B) Sequences of the theoretical tryptic peptides of Cos2 that have at least two, consecutive, matching amino acids to those found in each position in (A).

Figure 3

A Amino Acid Positions from Edman Sequencing

2	3	4	5	6
Val	Asp	Ser	Lys	Gln
Iso	Tyr	Gln	Gly	
Leu	Met	Leu		

B Amino Acid Positions of Corresponding Peptides

-1	1	2	3	4	5	6	7	8
Lys	Val 896	Leu	Thr	Gln	Lys			
Lys	Ala 568	Val	Met	Gln	Ser	Gln	Asp	Arg
Lys	Leu 456	Iso	Asp	Ser	Lys			
Lys	Val 971	Iso	Asp	Leu	Arg			
Arg	Ser 931	Iso	Asp	Thr	Ser	Asp	Arg	

Figure 4. Verification of Serine 572 as a Fu induced phosphorylation site of Cos2.

(A) Phosphotryptic peptide (PTP) map of wild type Fu and Cos2 coinfected. There are two large blobs of radiolabeled material which only migrate in the chromatography direction (which we believe to be undigested material) and some faint spots in the upper reaches of the map to which we have not assigned numbers. The principal phosphotryptic peptides which are found are numbered 1 through 6.

(B) Phosphotryptic peptide map of Cos2 from a Fu/Cos2 S572A coinfection. Phosphotryptic peptides #2, 4, and 5 are all missing as compared to a Fu/Cos2 coinfection (A), indicating that these are probably tryptic isoforms of phosphorylated Cos2 peptide 568-575.

(C) PTP map of Cos2 from a FuM/Cos2 coinfection. All of the phosphopeptides found in a Fu/Cos2 coinfection are missing except phosphotryptic peptide #1 when Fused kinase activity is removed.

(D) PTP map of Cos2 from a single Cos2 infection alone. As in FuM/Cos2 coinfection, only phosphotryptic peptide #1 is found; all other phosphotryptic peptides are missing.

(E) PTP map of Cos2 from a Fu/Cos 927-935 AAAA coinfection. This Cos2 construct contains three Serines and one Threonine that have been mutated to Alanine, including the two Serines at positions # 931 and 935 that could be phosphorylated as a part of peptide 931-937. All phosphotryptic peptides except peptide #3 are present, indicating phosphotryptic peptide #3 correlates to Cos2 peptide 931-937.

(F) Schematic representation of a Fu/Cos2 PTP map, with directions of thin layer electrophoresis and chromatography indicated.

Figure 4

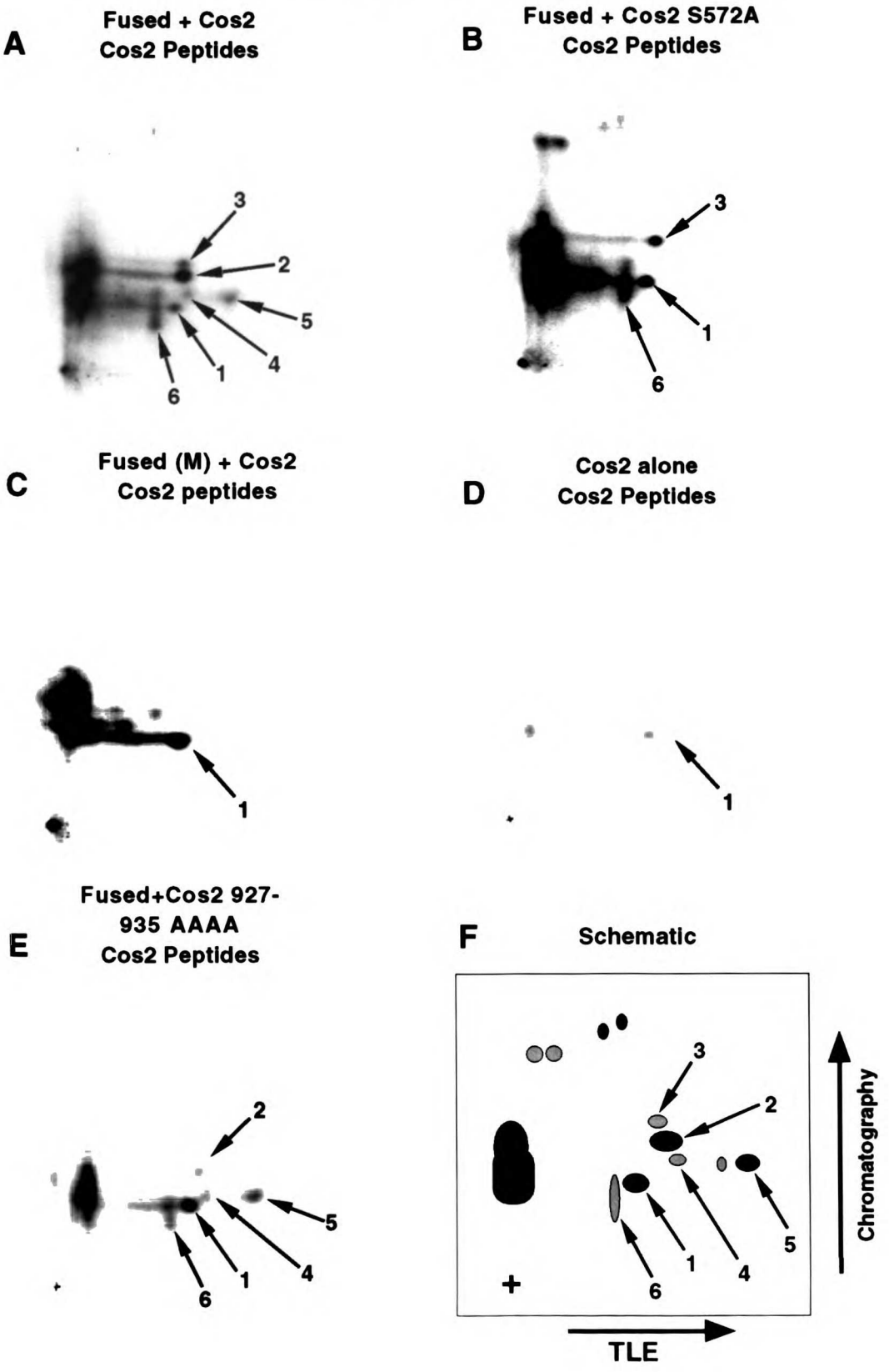

A
Fused + Cos2
Cos2 Peptides
3
2
5
4
1
6
B
Fused + Cos2 S572A
Cos2 Peptides
3
1
6
C
Fused (M) + Cos2
Cos2 peptides
1
D
Cos2 alone
Cos2 Peptides
1
E
Fused+Cos2 927-
935 AAAA
Cos2 Peptides
2
5
4
1
6
F
Schematic
3
2
1
4
5
6
+
Chromatography
TLE

Figure 5. Comparison of PTP maps of Cos2 from S2 cells and baculovirus.

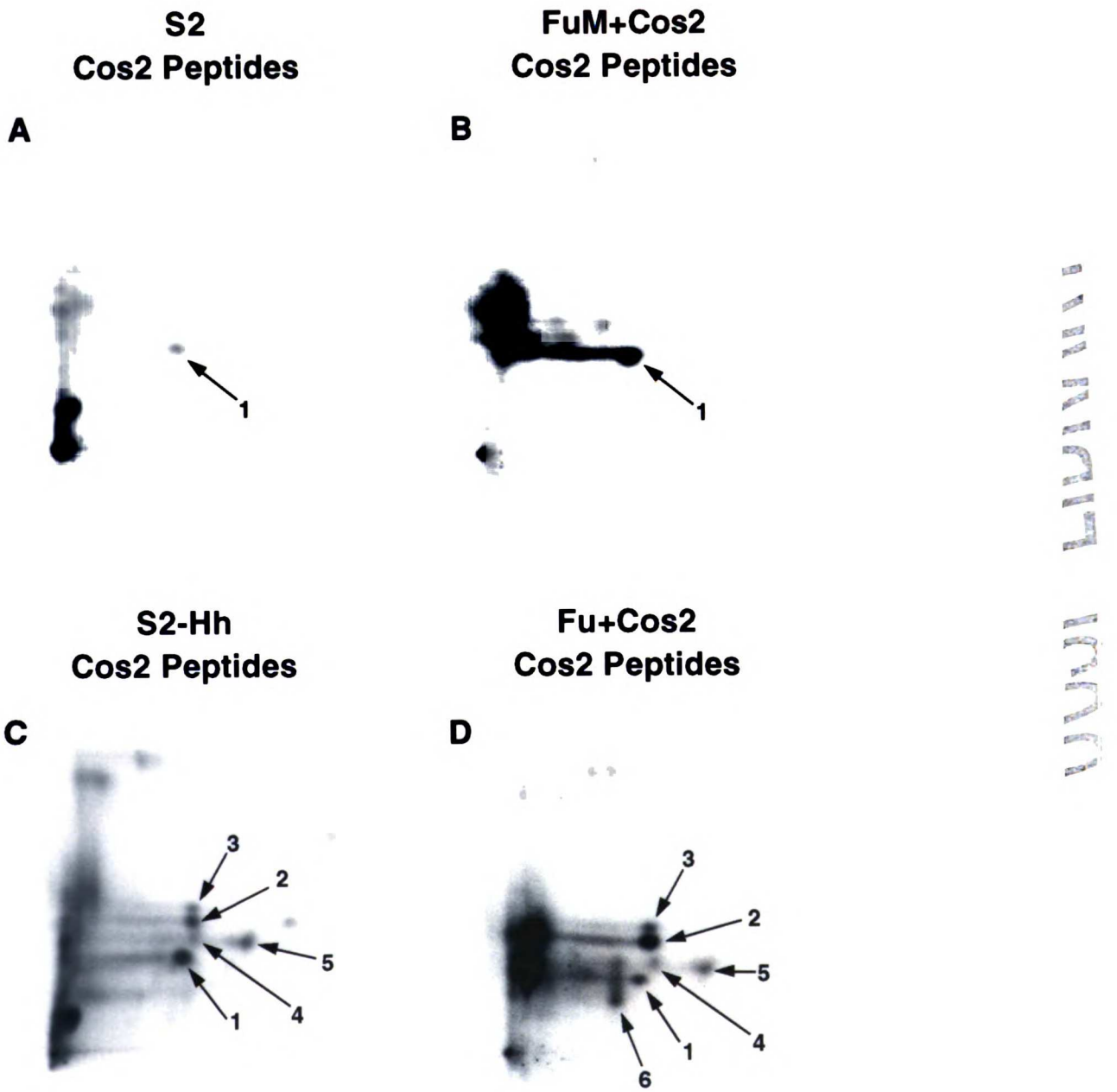
(A) PTP map of Cos2 from wild type S2 cells. This map varies from that of Cos2 obtained from a FuM/Cos2 coinfection in having large blobs of radiolabel on and above the origin, and in having distinct spots above the origin where there are normally blobs found in PTP of baculovirus produced Cos2. But it is similar to the map of Cos2 from FuM/Cos2 in having only one major phosphotryptic peptide--peptide #1.

(B) PTP map of Cos2 obtained from a FuM/Cos2 coinfection for comparison to the map of Cos2 from S2 cells.

(C) PTP map of Cos2 from S2 cells constitutively expressing Hh. Cos2 from S2-Hh cells contains all the phosphotryptic peptides found in PTP maps of Cos2 taken from a Fu/Cos2 coinfection except peptide #6. As in (A), the blobs of radiolabel found in and near the origin and the reduction of blobs above the origin distinguish this map from the baculovirus produced Cos2 map.

(D) PTP map of Cos2 from a Fu/Cos2 coinfection.

Figure 5


S2
Cos2 Peptides
A
1
FuM+Cos2
Cos2 Peptides
B
1
S2-Hh
Cos2 Peptides
C
3
2
5
1
4
Fu+Cos2
Cos2 Peptides
D
3
2
5
4
1
6

Chapter 4

Regulation of the Fused Serine/Threonine Kinase by Phosphorylation and the Role of Fused in Ci Processing.

Abstract

The serine/threonine kinase encoded by the *fused* segment polarity gene is phosphorylated in response to hedgehog signaling. Exactly what causes the phosphorylation of Fused (Fu), where the phosphorylation sites are located, and the function of these phosphorylation events, however, remain unknown. We show, using a baculovirus coexpression system, that Fu autophosphorylates on Serine 313 and possibly also on Threonine 80. Mutation of Threonine 80 to Alanine eliminates the Fu induced phosphorylation of Costal-2 in baculovirus. As phosphorylation of Fu is not changed in this mutant, this suggests that Threonine 80 is required for phosphorylation of Costal-2, but does not eliminate kinase activity and hence is likely involved in kinase specificity. The importance of phosphorylation of Serine 313 is not yet known.

We further show that Fu kinase activity is only required at the anterior/posterior compartment boundary, and expression of a kinase deleted form increases the levels of full length Ci. Furthermore, Fu kinase activity correlates with Ci155 phosphorylation in baculovirus coexpression experiments. While Fu kinase activity is required for generation of one of the electrophoretic isoforms of Ci, another isoform of Ci appears to be maintained by a kinase activity found in Sf21 cells. These results suggest that processing of Ci requires phosphorylation by Fu and by another kinase.

Introduction

The Hedgehog (Hh) family of secreted signaling factors is one of the most diverse morphogen families found in animals in terms of the number

of tissue patterning events affected by its members (Goodrich and Scott, 1998; Hammerschmidt et al., 1997). Hedgehog was originally identified in *Drosophila melanogaster*as a member of a family of genes called the segment polarity genes (Nusslein-Volhard and Wieschaus, 1980). These genes are involved in maintaining the borders between the segments and in specifying the identity and borders of the anterior and posterior compartments that make up each segment (Lawrence and Struhl, 1996). While the biochemistry of Hh secretion has been examined in detail, the mechanism by which Hh transduces its signal from the extracellular environment to the nucleus is still largely unsolved. Other members of the segment polarity gene family comprise the downstream transducers of Hh signals. The segment polarity genes *patched* (*ptc*) and *smoothened* (*smo*) have been shown to encode transmembrane proteins involved in Hh signal reception (Fuse et al., 1999; Marigo et al., 1996; Stone et al., 1996). Another segment polarity gene, *fused*, has been shown to be epistatic to these genes in the *Drosophila* embryonic epidermis and imaginal discs (Forbes et al., 1993).

fused has been cloned and encodes a member of the serine/threonine kinase superfamily (Preat et al., 1990; Therond et al., 1993). Fu is found in both hypo- and hyperphosphorylated forms (Therond et al, 1996, Chapter 2). In the *Drosophila* S2 cell line, Fu is hyperphosphorylated in response to stimulation with Hh, indicating the dependence of this phosphorylation on Hh-stimulation (Therond et al., 1996). We have previously shown that Fused binds to the product of another segment polarity gene, *costal2*, and that this binding is dependent on the C terminus of Fu and not on the kinase domain (Chapter 2). This Fu-Costal2 complex is of high molecular weight and binds one population of another segment polarity gene product, Ci (Chapter 2: Sisson et al, 1997).

A Hh signal ultimately impinges on *Ci*, which encodes a Zn finger transcription factor (Motzny and Holmgren, 1995; Orenic et al., 1990). Ci is only found in the anterior compartments of segments and, at a distance from the anterior/posterior compartment boundary, Ci is normally cleaved into N and C terminal fragments (Aza-Blanc et al., 1997; Hepker et al., 1997). The N terminal fragment is referred to as Ci75, and it localizes to the nucleus where it is thought to act as a repressor, preventing transcription of Ci target genes such as *ptc* and *dpp* (Alexandre et al., 1996; Aza-Blanc et al., 1997; Dominguez et al., 1996; Hepker et al., 1997). Near the compartment border, Hh signal from the posterior compartment prevents this cleavage and full length Ci accumulates. This full length Ci then acts as a transcriptional activator, stimulating expression of the Ci target genes *ptc* and *dpp* in the border region of the wing imaginal disc (Alexandre et al., 1996; Aza-Blanc et al., 1997; Chen et al., 1999; Hepker et al., 1997; Methot and Basler, 1999).

In Chapter 3, we demonstrated that Fu phosphorylates Cos2 and this phosphorylation correlates with a reduction in binding of the Fu/Cos2 complex to microtubules. However, important questions still remain: what are the phosphorylation sites of Fu and what kinase phosphorylates Fu? Since Fu does not bear any particular homologies to other kinases, there are no good candidates for a Fu-activating kinase. In chapter 3, we also demonstrated that Fu autophosphorylates itself in baculovirus. As Fu autophosphorylation could be either the normal method of Fu activation in vivo or could reflect sites normally phosphorylated by another kinase, I sought to identify these sites. Here we identify Serine 313 as an autophosphorylation site of Fu. We also demonstrate that Threonine 80, while not an autophosphorylation site, appears to be important for Fu phosphorylation of Cos2.

In addition, mutant Fu constructs were tested both in the baculovirus system and in transgenic flies to assay their effects on accumulation and processing of Ci. Expression of a Fu kinase-domain deleted protein at the anterior/posterior compartment border of *Drosophila* wing imaginal discs mimicked a *fused* phenotype, demonstrating that the kinase activity of Fused is required in vivo as well as in baculovirus. Expression of the kinase truncated Fu caused an increase in the levels of full length Ci, indicating that Fu is required for proper processing of Ci. Finally, we demonstrate that Fu phosphorylates Ci in baculovirus coexpression and that Ci is also constitutively phosphorylated by an endogenous Sf21 cell kinase. These two phosphorylations of Ci may account for the heterogeneity seen in electrophoretic migration of Ci from embryonic lysates, and, more importantly, appear to play a role in regulation of Ci processing and activation.

Results

Fu is phosphorylated on serine and this phosphorylation can be recapitulated by expression of Fu in baculovirus

Fu is hyperphosphorylated in response to Hh stimulation, and this hyperphosphorylated form of Fused demonstrates a reduced electrophoretic mobility on SDS-PAGE gels (Therond et al., 1996). As a prelude to identifying the Fu phosphorylation sites, we first determined the nature of the phosphorylated residue(s) of Fu. Phosphoaminoacid analysis of Fu from radiolabeled S2 cells constitutively expressing Hh indicated that Fu was predominantly phosphorylated on Serine (Figure 1A).

We next sought to determine the phosphorylation sites of Fu. S2 cells proved to be impractical for this purpose due to the relatively low levels of Fu and Cos2 proteins and the low levels of radiolabel incorporation into these proteins (data not shown). We therefore attempted to map the phosphorylation sites using a baculovirus expression system. As shown in Chapter 3, and again in Figure 1B, Fu expressed in baculovirus was autophosphorylated, both in the absence and presence of coexpressed Cos2. This phosphorylation was not due to different amounts of Fu protein, as was seen by comparing the amounts of Fu by Coomassie staining with levels of autophosphorylation (data not shown). Because there only appeared to be two phosphorylated Fu isoforms in Fu/Cos2 coinfections, a number identical to that seen in S2 cells, we conducted our analysis using Fu from Fu/Cos2 coinfections.

Fu is autophosphorylated at positions #5 and #7 in Baculovirus.

To begin mapping the autophosphorylation sites of Fu, radiolabeled Fu was digested with trypsin and Lys-C proteases to determine the position of the radiolabeled residue in the proteolytic fragment and the nature of the basic residue abutting the N terminus of the fragment. Fu digested with trypsin produced significant radiolabel incorporation into column fractions 19, 20, 22, and 29 (Figure 2A). Since radiolabeled fractions 19 and 20 are found in elutions of both Fused and Cos2 peptides, and since they occur at the very beginning of the acetonitrile elution gradient, we believe that they represent free phosphate eluting off the column rather than actual peptides.

Lys-C digestion of radiolabeled Fu resulted in a significantly different profile of radiolabeled peaks. We were not able to obtain radiolabelled

position information from fraction 21, and so we did not analyze it further. We now found that fractions 33 and 47+48 incorporated radiolabel, while fractions 22 and 29 did not (Figure 2B). Edman degradation, followed by stringent elution conditions to obtain radiolabeled position, revealed that radiolabeled fraction 22 from the trypsin digest and fraction 33 from the Lys-C digest both contained a radiolabeled residue at position #7 (and possibly also at position #8 for the Lys-C digest) (Figure 2C and D). In fractions 47+48 of the Lys-C digest and fraction 29 of the trypsin digest, we also found a radiolabeled residue at position #5 in a peptide of that fraction (Figure 2C and D). If the trypsin and Lys-C fractions with the same radiolabeled position corresponded to the same peptides, then the residue at the N-terminal boundary of these peptides was most likely Lysine since the position of the radiolabeled residue did not change when digested with trypsin versus Lys-C.

At least one radiolabeled fraction from the trypsin digest did not appear in the Lys-C digest while two new radiolabeled fractions appeared in the Lys-C digest that were not found in the trypsin digest. This suggested that at least one of the peptides had an Arginine at either its N or C terminus. As trypsin digest fraction 22 and Lys-C digest fraction 33 both had a radiolabeled residue in position #7, it seemed likely that the same peptide was responsible for the labeling in both these fractions. And since the radiolabeled position remained position #7 in both proteolytic digests, it was likely that this Arginine was at the C terminus of the peptide. Because we detected a radiolabeled position #5 for trypsin digest fraction 29 and Lys-C fraction 47+48, we believe that these two fractions could also represent the same peptide. Without sequence information from these fractions, however, we were not able to validate these hypotheses.

To clarify the nature of these peptides, we therefore digested radiolabeled Fu with trypsin and conducted Edman degradation for amino acid sequence on the radiolabeled fractions. We used a shallower acetonitrile elution gradient to obtain better separation of the peptides. Consequently, the fraction numbers were not comparable between the radiolabeled position experiment and this amino acid sequence experiment. Two radiolabeled fractions were obtained from this experiment, but only one provided usable sequence. Fraction 25 contained a mixture of amino acids at each position relative to the N terminus (Figure 3A), corresponding directly to the tryptic peptides starting at amino acid #307 and amino acid #557 (Figure 3B). The peptide containing amino acids # 557-560 does not have any phosphorylatable residues in it and so was discounted, whereas peptide 307-314 has two threonines and a serine in positions #5-7 relative to the N terminus. Since the serine in position #5 was detected in the Edman sequencing, and we never found a radiolabeled residue at position #6, we conclude that Serine 313 is the phosphorylated residue in this peptide. Due to the fact that the fraction containing this peptide gave the strongest radiolabeling signal in our radiolabeled fraction profile, we also conclude that Serine 313 is the major target of autophosphorylation in Fu. We are presently testing the effect of Fu S313A expression on Fu and Cos2 phosphorylation in the baculovirus coexpression system.

Determination of the peptide phosphorylated on position #5

Since we could not identify the fraction that contained the radiolabeled position #5 using Edman degradation for sequence, we determined the possible candidate peptides that had a threonine or a serine in position #5 and

mutated them to Alanine. These mutants were then tested by baculovirus coinfections to see what their effect was on Fused and Cos2 phosphorylation using SDS-PAGE gels and phosphotryptic peptide (PTP) mapping. We also did the same for candidate peptides having a serine or threonine in position #7. A list of the candidate tryptic peptides with phosphorylatable residues at positions #5 or #7 is shown in Figure 4A and 4B, respectively. We presumed that, since both the position #5 and position #7 fractions had changed their migration positions in response to digestion with trypsin versus Lys-C and still retained the same radiolabeled position relative to the N terminus, both radiolabeled peptide in the fractions likely had an arginine at their C-terminus and Lysine at their N-terminus. This then allowed us to eliminate all peptides without a Arginine at their C terminus. For position #5, this left only the tryptic peptide 612-624. We then mutated Serine 616 to an Alanine and made baculoviruses expressing this mutated Fused protein. These viruses we then coinfected with Costal-2, radiolabeled, and analyzed by the aforementioned methods.

We found that, when Fu S616A was coinfected with Cos2, the phosphotryptic maps of Fused and Costal-2 remained unchanged compared to wildtype Fused/Costal-2 coinfection, indicating that we had not identified the appropriate position #5 phosphorylation site (Figure 4C). We are presently mutating all candidate peptides containing a Lysine at the N terminal border and serine or threonine in position #5, irrespective of the nature of the C terminal residue.

Determination of the Peptide Phosphorylated at position #7.

The serines in position #7 of the peptides beginning with residues 74 and 564 of Fu were mutated to Alanine because these peptides had a Lysine at

their N-terminus and an Arginine at their C-terminus. Fu S570A had no effect on either Fu or Cos2 phosphorylation by the PTP assay (data not shown). Fu T80A appeared to eliminate most of the shifted form of Fused in a one dimensional SDS-PAGE gel (Figure 5A), indicating that phosphorylation of this threonine could be responsible for shifted Fu. However, the PTP map of Fu T80A was the same as that of wild-type Fu (compare Figures 5B and C), indicating that Threonine 80 was not a major phosphorylation site. PTP maps of Cos2 from a Fu T80A/Cos2 coinfection showed that all but the basal phosphorylation of Cos2 was eliminated when compared to PTP maps of Cos2 from a Fu/Cos2 coinfection (Figure 5D and E). Since Fu T80A autophosphorylation appeared normal, this indicated that Threonine 80 does not affect Fu kinase ability, but rather the ability of Fu to phosphorylate its substrates. It is also possible that Fu T80A impairs Fu kinase ability only enough to prevent Cos2 phosphorylation but not Fu autophosphorylation. We are currently designing experiments to address these hypotheses.

Phosphorylation Patterns of Fu are very similar in Baculovirus expression and S2 cells.

While the baculovirus expression system has an obvious utility in identifying phosphorylation sites, there always exists the concern that it does not faithfully recapitulate the phosphorylation patterns of endogenous Fu or Cos2 in cells or flies. To address this concern, we compared the PTP maps of Fu immunoprecipitated from radiolabeled S2 cells constitutively expressing Hh with that from Sf21 cells infected with Fu baculovirus and radiolabeled. As baculovirus-expressed Fu from a Fu/Cos2 coinfection seems to mimic the

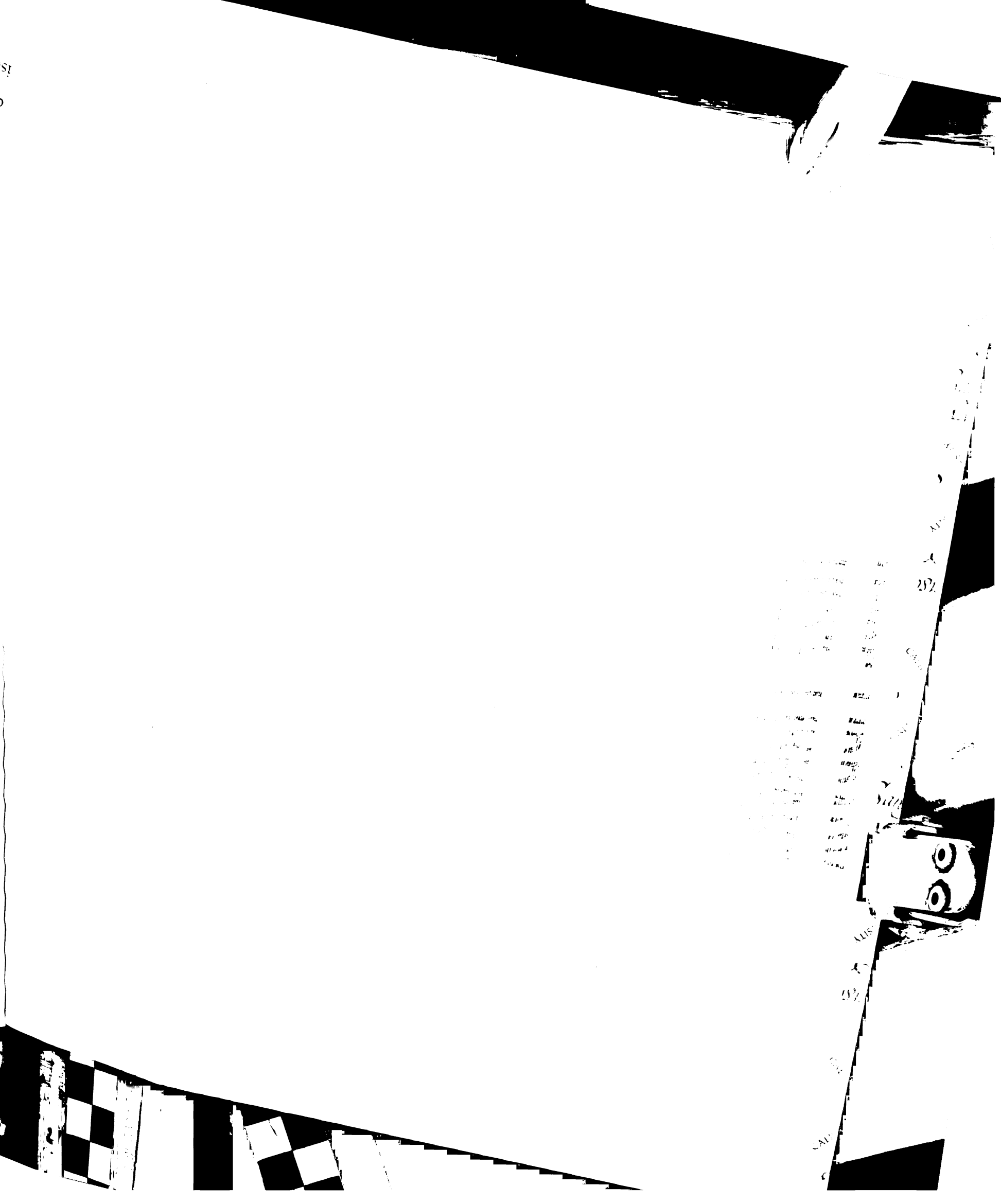
Hh-stimulated state, we compared baculovirus Fu with endogenous Fu from S2-Hh cells. While the patterns were not identical, the PTP maps of the baculovirus-produced Fu and the endogenous Fu from S2-Hh cells were remarkably similar. As can be seen in Figure 6A/B and C, phosphopeptides 1'-5' were present in both phosphotryptic peptide maps and only two of the major phosphotryptic peptides, 6' and 7', found in baculovirus produced Fu could not be seen in the map of S2-Hh Fu. Figure 6D shows a PTP map of endogenous Fu taken from unstimulated S2 cells. In addition to a considerable reduction in overall radiolabeling, the PTP map showed that only phosphopeptides 1' and 5' were present in Fu taken from unstimulated S2 cells. We did not attempt to compare Fused from S2 cells with FuM from baculovirus infections because of the almost complete lack of FuM radiolabeling. Because the phosphotryptic peptide patterns were so similar between Fu from S2-Hh cells and Fu expressed in baculovirus, we are confident that phosphorylation site data obtained from baculovirus produced Fu will be similar to that of endogenous Fu from *Drosophila*.

Expression of a kinase deletion construct of Fused mimics the Fused phenotype when expressed along the wing compartment border.

In addition to identifying Fu phosphorylation sites, we wanted to ascertain whether or not *fu* mutants would behave as dominant negatives when expressed in a wild type background. This information would tell us whether we needed to test our mutant *fu* constructs in a severely hypomorphic (or null) *fu* background or whether we could simply test these constucts in a wild type background. To resolve this issue, transgenic flies were created expressing either wild-type Fu, or a truncated form of Fu in

which the entire kinase domain had been deleted, under control of UAS promoters. Expression of these transgenes was tested by crossing them to different Gal-4 driver lines and examining progeny wings for phenotype (Brand and Perrimon, 1993). When wild-type Fu was expressed at the wing compartment border, using the ptc-Gal4 (Hinz et al., 1994) or dpp-Gal4 (Staehling-Hampton et al., 1994) driver lines at either room temperature, 25°C, or 29°C, there was no effect on any part of wing morphology as compared to wild type (Figure 7B and E). Expression of wild-type Fu also had no effect on wing morphology when expressed using a Hh-Gal4 line, which only expresses in the posterior compartment, or when expressed using the 3Sb-Gal4 line (*brinker*-Gal4) which expresses only in the far anterior and posterior compartments (data not shown). When the truncated Fu (FuD) was expressed using these Hh-Gal4 and 3Sb-Gal4 lines, there was also no effect on wing morphology (data not shown). However, when FuD was expressed at the anterior-posterior compartment border using either the ptc-Gal4 or the dpp-Gal4, a severe *fu* mutant phenotype was observed (Figure 7C,D, F, and G). These data were consistent with previous observations, using gynandromorphs and large mitotic clones, that *fu* function was only required in the anterior compartment at the anterior-posterior compartment border (Fausto-Sterling, 1978; Wurst, 1979).

The effects of FuD on Ci were also examined. In the absence of Hh-stimulation, Ci is cleaved into two fragments. The N terminal portion appears to be stable and is termed Ci75 for its molecular weight (Aza-Blanc et al 97). When Hh signaling is activated, this cleavage is suppressed and full-length Ci, called Ci155, accumulates. This Ci155 accumulation can be detected on immunoblots of extracts or by immunofluoresence of imaginal discs. In wing imaginal discs, Hh signaling from the posterior compartment induces



an accumulation of Ci155 at the compartment border, but not in the far anterior reaches of the disc, and so we anticipated that interfering with the Hh signaling pathway would cause a reduction in the amount of Ci155 accumulation at the border. We stained wing imaginal discs from wild type Fu or FuD transgenic flies crossed to a dpp-Gal4 line with antisera to Fu and to the N terminus of Ci and found that overexpression of wild type Fu had no effect on the normal Ci accumulation at the border (Figure 8). But, surprisingly, when FuD was overexpressed, the region of Ci155 accumulation expanded anteriorly and became more intense. Yet, as shown in Figure 7, this accumulation of Ci155 correlated with a loss of function *fu* phenotype. This indicated that accumulation of Ci155 was not sufficient for activation of Ci and that some other event is required in additon to accumulation to create the fully active form of Ci155. This is contrary to what was expected, but in agreement with recent reports that describe the same phenomena (Alves et al., 1998; Ohlmeyer and Kalderon, 1998).

Fu Phosphorylates Ci in Baculovirus.

That Fu appears to be required for the generation of both Ci75 and the active form of Ci155 led us to investigate whether Ci might be a substrate of the Fu kinase. The baculovirus expression system was again chosen to test this hypothesis. Baculovirus expressing Ci were coinfected with Fu, FuM, and/or Cos2 and extracts immunoblotted for Ci to ascertain whether Fu kinase activity had any effect on the migration of Ci in a SDS-PAGE gel. Figure 9A shows that four isoforms of Ci could be detected in these coinfection experiments. Ci and Cos2 expressed together (Ci/Cos2) yielded Ci isoforms 1 and 3, while Fu expressed with Ci (Fu/Ci) yielded Ci isoform 2,

which appeared to have an electrophoretic mobility intermediate to that of the two Ci isoforms generated from the Ci/Cos2 coinfection. Coinfection of FuM, Cos2, and Ci virus (FuM/Cos2/Ci) generated two forms of Ci that had the same electrophoretic mobilities as those found in the Cos2/Ci coinfection (isoforms 1 and 3). These data suggested that two phenomena were occurring. First, Ci was phosphorylated by some endogenous kinase in Sf21 cells, and, second, Fu was also phosphorylating Ci.

Coinfection of Fu, Cos2, and Ci (Fu/Cos2/Ci) generated a smeary band of Ci. This band could be accounted for either by a single band that runs electrophoretically different than the aforementioned three isoforms, or by a set of three very closely apposed bands, one of which corresponds to the Fu/Ci isoform, one of which corresponds to the upper Ci isoform from the Ci/Cos2 coinfection, and one of which is an isoform novel to this triple infection, isoform 4 (Figure 9A). Fu/Cos2/Ci coinfections in which the Fu dose was doubled had no apparent effect on the amount of this isoform(s). Similarly, doubling the infectious titer of FuM did not alter the Ci immunoblot pattern from that seen with the initial FuM viral titer (Figure 9B). We also coinfected Fu, Cos2, and Ci using two different mutant Cos2 viruses, one of which carried a S572A mutation and the other a S595A mutation, to examine whether these point mutants affected Ci cleavage. These Cos2 point mutants did not affect Ci cleavage, but they did convert all of the Ci to the slowest electrophoretic isoform (Figure 9A). However, these two viruses had a considerably higher titer than the normal Cos2 virus (Figure 9A, lower panel), and we believe that this conversion was probably not due to the point mutations present, but rather to the increased amounts of Cos2 relative to Ci. We also tried adding either Ptc or Smo to the Fu/Cos2/Ci coinfection and examined the Ci isoforms produced. We found that coinfection of

Fu/Cos2/Ci and Smo generated only isoform 2. Coinfection of Fu/Cos2/Ci and Ptc appeared to generate two forms, although the immunoblot is smeary and it is not clear if they correspond to isoforms 2 and 4 or to isoforms 1 and 3 (Figure 9B).

We also examined the effect these different coinfections had on the abundance of Ci75. Ci75 was found in baculovirus coinfections, but there was considerably less Ci75 relative to Ci155 than is normally found in embryonic and cell lysates by immunoblotting (Aza-Blanc et al., 1997). Even so, the amount of Ci75 is increased in Fu/Cos2/Ci coinfections relative to infections lacking Fu kinase activity or Cos2 (Figure 9B), indicating that Fu kinase activity is required for generation of Ci75. The three bands below Ci75 could be background bands, except that they change abundance depending on the presence of Cos2 and Ptc/Ci coinfections seem to increase the amounts of bands C and D (Figure 9B, lower panel).

Discussion

Constitutive activation of Fused by baculovirus expression

Simple infection of Sf21 cells with Fu baculovirus yields a hyper-phosphorylated form of Fu which closely resembles the hyperphosphorylated form of Fu found in S2-Hh cells. This would indicate that Sf21 cells either contain an activator of Fu or lack an inhibitor of Fu normally found in S2 cells. However, Sf21 cells infected with Fu baculovirus and then treated with Hh conditioned medium or coinfected with another baculovirus expressing Hh-N do not show any change in the ratio of electrophoretic isoforms of Fused (K. N., unpublished data). Moreover, FuM overexpressed by baculovirus infection does not itself become shifted, as would be expected if

the Hh signaling pathway activated a kinase that phosphorylated Fu. This strongly suggests that Fu expression alone is responsible for the generation of the shifted form of Fused. We cannot, however, rule out the possibility that Sf21 cells do constitutively express Hh and that FuM expressed by baculovirus blocks Hh signaling by acting as a dominant negative in a manner that prevents Fu hyperphosphorylation.

Fused expressed in Sf21 cells seems to constitutively present both the hypo- and hyper-phosphorylated forms. The distribution of protein quantity between the two electrophoretic forms of Fused taken from baculovirus infections seems to mimic that found in S2 cells stimulated by Hh in that the ratio of shifted to unshifted Fu isoforms, as determined by immunoblot, is never higher than 1:1. However, the distribution of radiolabel between the two isoforms of Fu obtained from baculovirus is different from that of the Fu isoforms obtained from S2 cells stimulated with Hh. In S2 cells stimulated by Hh, almost all of the radiolabel is found in the shifted form of Fu and almost none is found in the unshifted form, whereas in baculovirus produced Fu, at most, 50% of the radiolabel is found in the shifted form and the remainder is found in the unshifted form (Robbins et al., 1997). It is possible that there is a phosphatase not found in Sf21 cells but expressed in S2 cells and *Drosophila* tissues which dephosphorylates the lower Fu isoform in response to Hh signaling. Indeed, phosphatases have been implicated in signaling by Hh and Shh, although it is thought they act principally on Ci or other transcription factors (Chen et al., 1999; Krishnan et al., 1997). Another possibility is that phosphopeptides 6' and 7', which were only found in the PTP maps of Fu from baculovirus infections and not in PTP maps of S2 or S2-Hh cell Fu, could represent ectopic autophosphorylation sites which do not cause Fu to shift.

Fu autophosphorylation in baculovirus: could inhibition of Fu autophosphorylation be used to maintain the Hh off state?

The fact that Fu autophosphorylates itself upon infection into Sf21 cells to generate phosphoisoforms identical to those found in cells raises the question as to whether autophosphorylation could be a relevant signaling mechanism in vivo. The only other kinase shown to function in the Hh signaling pathway, PKA, does not affect Fu phosphorylation (Therond et al., 1996; our unpublished results) and there are presently no other good candidates for a kinase phosphorylating Fu. Moreover, there are several well established examples of kinases trans-autophosphorylating to activate signaling pathways. The most notable examples are the transmembrane receptor tyrosine kinases that autophosphorylate once close association of receptor dimers is achieved by ligand binding (Schlessinger and Ullrich, 1992; Weiss and Schlessinger, 1998). If Fu autophosphorylation is relevant mechanism to Hh signaling in vivo, then this raises the question as to what prevents autophosphorylation of Fu in the absence of Hh stimulation. A simple model would be that Ptc, being a negative regulator of the pathway, could physically interact with Fu bound to Cos2 and inhibit its kinase activity. Hh binding to Ptc would then remove this inhibition, possibly through the intervention of Smo. However, we are not aware of any evidence that Fu and Ptc (or Smo) can physically interact. The interaction could be weak, or could be mediated through other proteins, but certainly this hypothesis deserves further investigation.

Another possible mechanism of Fu action is that a mix of autophosphorylation and exogenous phosphorylation activities could be required for complete activation of Fu. There is precedent for such an

activation mechanism. PKC, for example, requires an activating phosphorylation by an exogenous kinase followed by two autophosphorylation events before it is competent to phosphorylate its own substrates (Newton, 1995). In baculovirus infections, overexpression of Fu could lead to ectopic autophosphorylation of Fu on the site normally phosphorylated by an exogenous kinase, followed by natural autophosphorylation events and subsequent activation of Fu' ability to phosphorylate Cos2.

Causes of the Distribution of Phosphorylated Fu Isoforms.

As noted above, in baculovirus overexpression and S2 cells no more than 50% of Fu protein is actually incorporated into the hyperphosphorylated isoform, even in the presence of soluble phosphatase inhibitors (Figure 1, Therond et al, 1996, Robbins et al, 1997, our unpublished data). This argues that there is some intrinsic mechanism which prevents more than a certain percentage of Fused from being hyperphosphorylated at any one time. It is possible that there is an endogenous phosphatase in all insect cell lines so far tested that is refractory to the phosphatase inhibitors we used, but we believe that it is more likely that this phenomena is a direct reflection of how Fu is normally regulated. Cos2 probably forms a dimer since it contains the heptad repeats known to confer dimerization ability on kinesin-like molecules (Vale, 1990; Vale and Fletterick, 1997). Fu could bind to Cos2 in a one to one ratio with Cos2 meaning that two Fu molecules would bind per Cos2 dimer. Phosphorylation of one of this pair of Fu molecules could lead to an activation of its ability to phosphorylate the other Fu molecule, but that particular Fu molecule would not shift. The other Fu molecule attached to the Cos2 dimer would then be phosphorylated by the unshifted but activated

Fu. The phosphorylation site of the second Fu molecule would result in its presenting a shifted form of Fu in gels. This shifted form of Fu could then phosphorylate both members of the Cos2 dimer, causing their hyperphosphorylation. In baculovirus, the phosphorylated but unshifted form of Fu would persist, whereas it would normally be dephosphorylated in S2 cells and embryos (Figure 10).

Fu Phosphorylates Ci in Baculovirus.

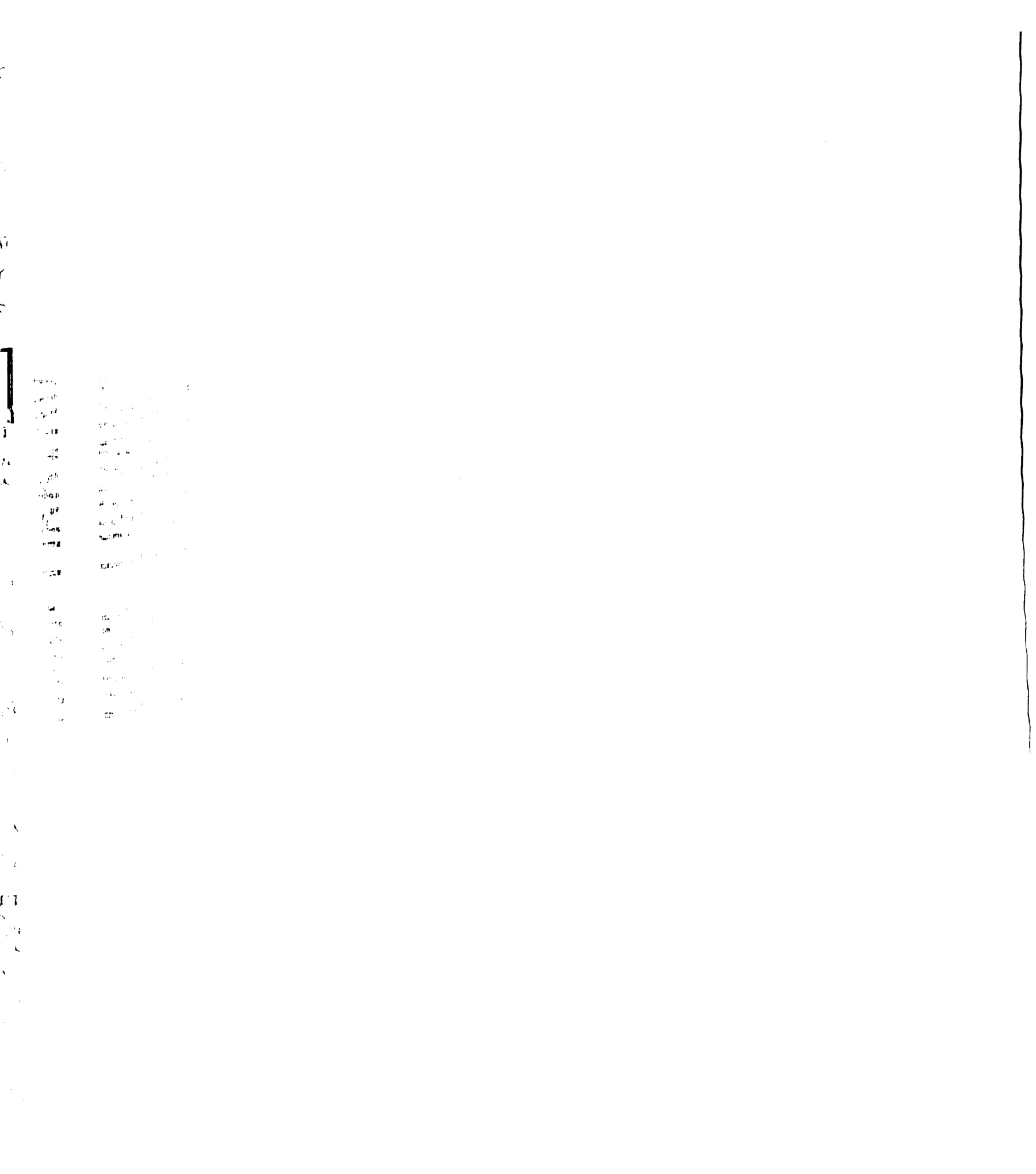
We have shown previously that Fu can phosphorylate Cos2 and we originally surmised that the only effect that Fu would have on Ci would be an indirect one through Cos2. That inhibiting Fu activity actually increased Ci155 levels led us to hypothesize that Fu might play a more direct role in the activation of Ci. It has been shown that loss of *fu* results in a reduction in Ci75 amounts and an accumulation Ci155 farther in the anterior compartment than is normal (Alves et al., 1998; Ohlmeyer and Kalderon, 1998). Phosphorylation of Ci155 has further been shown to be regulated by Hh signaling and simple inhibition of processing of Ci155 is not sufficient to create activated Ci (Chen et al., 1999; Ohlmeyer and Kalderon, 1998).

We have shown here that Fu appears to directly phosphorylate Ci155 in baculovirus-infected cells and that this phosphorylation accounts for at least one of the four isoforms of baculovirus-produced Ci that we see in immunoblots. The fastest migrating isoform of Ci (isoform 1) may or may not be phosphorylated, but we believe the other three isoforms are the result of phosphorylation events. In particular, we believe PKA may be responsible for at least one of the Ci isoforms because PKA has been shown to phosphorylate Ci in vitro (Price and Kalderon, 1999), and because several of the PKA sites in Ci and its vertebrate homologs, the Gli's, have been shown

to be important for transcriptional activation by Ci/Gli's (Chen et al., 1998; Dai et al., 1999). Isoforms of Ci phosphorylated only by Fu or only by PKA could account for two Ci isoforms. A third isoform could then be accounted for by Ci that had been phosphorylated by both Fu and PKA.

It is not yet clear which of these baculovirus-produced isoforms of Ci155 exists in cells and/or embryos and what function each might serve. At least two isoforms of Ci155 can be seen in immunoblots of embryonic extracts and extracts from cl8 cells, a *Drosophila* imaginal disc cell line (Chen et al., 1999; Ohlmeyer and Kalderon, 1998). Loss of function studies of PKA in the wing imaginal disc indicate that PKA is normally active throughout the anterior compartment (Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995). It is therefore a reasonable assumption that Ci is phosphorylated by PKA throughout the anterior compartment and that Ci155 solely phosphorylated by PKA accounts for the most abundant, lower isoform of Ci155 seen in immunoblots of Ci155 from embryos and cl8 cells (Chen et al., 1999; Ohlmeyer and Kalderon, 1998). This isoform could correspond to either isoform 1 or 3 of Ci155 seen in the Cos2/Ci coinfection. Since it has been shown that Fu kinase activity is only required at the anterior/posterior compartment border, Fu may only phosphorylate Ci at the border. An isoform of Ci155 phosphorylated by PKA and Fu in cells would then account for the smaller amounts of slower migrating Ci seen in embryonic and cl8 immunoblots. This slower migrating Ci155 isoform from cells could correspond to isoforms 2 or 4 of Ci155 seen in the Fu/Ci and Fu/Cos2/Ci coinfections (Figure 9A and B).

Previous work has demonstrated that PKA activity negatively regulates Ci activator function and Fu activity positively regulates Ci function. PKA seems to affect the formation of Ci75, the repressor form of Ci, and so its role

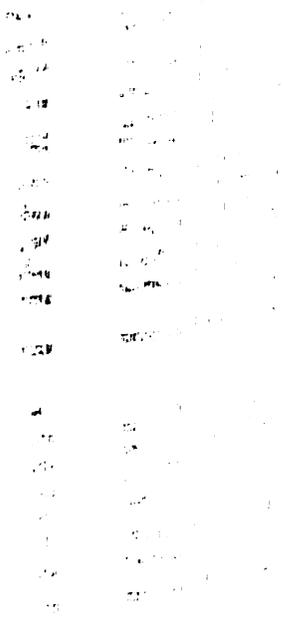


in Ci function may be to prime Ci for processing by the proteasome (Chen et al., 1998; Dai et al., 1999; Price and Kalderon, 1999). Loss of Fu activity prevents this processing, but does not allow Ci to become an activator. Fu phosphorylation of Ci therefore seems to play a dual role in Ci function. Fu phosphorylation of Ci seems to be required for recognition of Ci by its processing enzyme at the compartment border, but it also is required for activation of Ci. The latter effect could be an indirect effect of the requirement of Fu phosphorylation of Cos2 and the subsequent release of the complex from microtubules, or Ci may require phosphorylation by Fu for transcriptional activity as well as processing.

An interesting question arises in considering the effects produced by different coinfections on the amounts of Ci75; if Fu kinase activity affects the amounts of Ci75, then why do we not see a change in overall Ci levels in anterior compartment areas distant from the border when FuD is expressed there? It is possible that this increase in overall Ci does occur, but we cannot detect the difference in the immunfluoresence and it does not cause a large enough increase to produce a phenotype. Since the antisera used to detect Ci in Figure 8, 2A1, detects both Ci155 and Ci75, the amount of Ci could only stay the same in these regions if the amounts of Ci155 increased to compensate for the loss Ci75. We are planning to examine the relative staining of Ci155 alone at the border versus the far anterior compartment using an antisera that only recognizes the C-terminus of Ci to address this question.

A Model of Fu and Cos2 Function.

The information we have presented in these three chapters allow us to create a more detailed model of how Fu and Cos2 interact, with each other and with Ci, to transduce the Hh signal (Figure 10). Cos2 is thought to



dimerize, as do most kinesin-like molecules, and one Fu molecule is bound to each Cos2 molecule (D. R. and K. N., unpublished results). In the absence of Hh signal, Cos2 is dimerized and attached to microtubules. Two Fu molecules are bound to each Cos2 dimer, and at least one Ci molecule is bound to the complex through an physical interaction with either Fu or Cos2 (both possible interactions are shown here). Neither Fu molecule is an active kinase in the absence of Hh signal. When Hh signaling occurs, one of the Fu molecules is activated and proceeds to phosphorylate the other. This leaves one Fu molecule phosphorylated and one unphosphorylated. The phosphorylated Fu molecule then proceeds to phosphorylate both Cos2 molecules on Serine 572. Simultaneously, one or both of these Fu proteins then also phosphorylates Ci. The phosphorylation of the Cos2 dimer on both Serine 572's then induces a confirmational change in Cos2 which causes the whole complex to release from microtubules. From here the whole complex could then undergo proteolysis or processing to generate Ci75, or Ci could release from the Fu/Cos2 complex and then be processed into Ci75 separately. The remaining Fu/Cos2 complex could then either be proteolysed or dephosphorylated. Dephosphorylation could allow Ci to be bound again and the whole Fu/Cos2/Ci complex could then rebind to microtubules and start the cycle over again.

Experimental Procedures

Constructs and Cloning

FuM was made as previously described (Chapter 3). FuD was made by amplifying a portion of the *fu* D6 cDNA (Therond et al., 1993) corresponding to codons 270 to 806 by PCR using Pfu polymerase (Stratagene) and the oligos 5'-ACTATCGATCCTTTCACAAATCCCGAAGCCAAGG-3' and 5'-

ATCACTAGTAGATCTCTAGGTGACGAAAAAAGTGAAGTGACTGATC TCG-3' which result in a truncated *fu* coding sequence which contains an in frame Cla I site at the 5' end of the coding region and an in-frame stop codon followed by Bgl II and SpeI sites at the 3' end of the coding region. Identity of this truncated *fu* was confirmed by sequencing. A 6 X His tagging vector was constructed by annealing the oligos 5'-TCGAGGAATTCATGCATCAC CATCATCATCACCATAT-3' and 5'-CGATATGGTGATGATGATGGTGATG CATGAATTCC-3' and ligating this fragment into a Xho I/ Cla I cut pBluescript KS (-) (Stratagene). This tagging vector was then digested with Cla I and Spe I and was ligated to a similarly digested truncated *fu* fragment. This gave pKN1-FuD.

Mutagenesis of single residues of Fu was accomplished using PCR based site directed mutagenesis on the full length Fu cDNA, D6. Pfu polymerase (Stratagene) was used and minimal portions of the mutated plasmid were excised with the appropriate enzymes and ligated into similarly cut D6 and the junctions confirmed by sequencing (details available upon request).

To create the UAST-Fu, UAST (Brand and Perrimon, 1993), kindly provided by K. Hill (UCSF), was cleaved with Eco RI and ligated to a D6 Fu cDNA similarly cleaved. UAST-FuD was generated by cleaving UAST with Xho I and Xba I and ligating in a Xho I/Spe I cleaved FuD fragment. To make metallotheinin inducible vectors for stable cell transfection, an Eco RI fragment containing the Fu cDNA was ligated into an Eco RI cut pMTV5HisA (Invitrogen) to create pMTFu.

Immunoprecipitation

Cells were lysed by addition of 0.5 ml per 10 cm plate (0.25 ml for 3.5 cm plates) of NP40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 % Nonidet P-

40, 10 mM NaF, 1 mM EGTA, 0.01 mM benzamidine-HCL, 1 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml Pepstatin A) and rocked occasionally at room temperature for 15 minutes. The lysate was collected and cleared by centrifugation at 10,000 X g for 10 minutes at 4°C. Protein A sepharose beads which had been preincubated with 2 μg of polyconal anti-Fu antisera per 10 cm plate (Robbins et al., 1997) for 30 minutes were added to the resultant supernatant and immune complexes were collected by incubation of this mixture for 2-4 hours at 4°C. The immunoprecipitates were then washed three times for 30 minutes each with NP40 lysis buffer + 0.25 M NaCl and separated on 8% SDS-PAGE gels.

Baculovirus Production and Infection

Baculoviruses were produced using the Bac to Bac system, essentially as described by the manufacturer (Life Technologies). For the Fu, FuM, and other Fu mutant baculoviruses, a EcoRI fragment containing the full length wild type or mutant Fu cDNA was inserted into the EcoRI site of pFastBac1. For the Cos2 baculoviruses, wild type Cos2 was excised from the pKN5 shuttle vector using BamHI and SacII and inserted into the BamHI and SacII sites of pFastBac1. After the initial infection of Sf21 cells, viruses were amplified by infecting Sf21 cells at an MOI of 0.1 and harvesting virally infested medium 4 days after infection. Infections were conducted by spinning down $12.5X10^6$ cells per 10 cm plate, adding virally infested medium to an MOI of 3.5-4.0 for each virus, resuspending the cells, and incubating the cells at 28°C for 1 hour, shaking every 10 minutes. The infected cells and medium were then plated out on 10 or 15 cm plates and Grace's medium sufficient to give 12 ml per 10 cm/ 25 ml per 15 cm plate was added. All baculoviruses were confirmed to be expressing the appropriate protein by immunoblotting.

Cell Culture and ^{32}P Orthophosphate Labeling

S2 cells, S2-Hh-N, and S2-Hh cells were cultured in supplemented Schneider cells medium as previously described (Therond et al., 1996) cl-8 cells were cultured as previously described, with the exception that the fly extract was heat treated for 90 minutes at 55 °C (Cumberledge and Krasnow, 1994). Sf21 cells were cultured in spinner flasks in Grace's Insect Cells Medium (Life Technologies) supplemented with 10% fetal bovine serum and 1X Penicillin/Streptomycin. S2 cell labeling was as previously described (Robbins et al., 1997), with the exception that cells were lysed and immunoprecipitated as above. Each tryptic map represents the Fu and/or Cos2 immunoprecipitated from twelve 10 cm plates. Sf21 cells were labeled by infecting 12.5 X 10^6 cells/ 10 cm tissue culture plate as above, then 24 hours 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 and 1X Pen/Strep, and ^{32}P orthophosphate to 0.4 mCi/ml (NEN). Plates were then incubated 14 hrs at room temperature, followed by a 5 ml wash of phosphate free Grace's medium. Lysis and immuno-precipitation were then conducted as above. Immunoprecipitates were run out on 8% SDS-PAGE gels and autoradiographed.

Phosphoaminoacid and Phosphotryptic Peptide Analysis

Radiolabeled Cos2 and Fu proteins were excised from SDS-PAGE gels and cut into small pieces. These fragments were placed in tubes and washed twice for 30 minutes each with 100µl of 50 mM ammonium bicarbonate/50% acetonitrile. The gel fragments were dried overnight in a fume hood before

the fragments were reduced by rehydration and incubation for 1 hr at 55°C in 50 mM ammonium bicarbonate supplemented with 10 mM DTT. The DTT solution was removed and the fragments acylated by incubation for 45 minutes with 50 mM ammonium bicarbonate with 55 mM iodoacetamide. The fragments were washed 2 X 10 minutes with 50 mM ammonium bicarbonate, and then 2 X 30 minutes with 50 mM ammonium bicarbonate/50% acetonitrile and dried overnight in a fume hood. The samples were then digested by adding 200 µl of 50 mM ammonium bicarbonate + 5 µg/ml modified trypsin (Promega) and incubating for 24-48 hrs at room temperature. Peptides were then eluted with two washes of 300 µl each 60% acetonitrile + 0.1 % TFA in dH_2O. 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 dH_2O 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 in Boyle et al., (Boyle et al., 1991), 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 (Hellman et al., 1995). The resultant peptides were then separated using 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 a Sequelon AA membrane (Perseptive Biosystems, Cambridge, MA) with a Perkin-Elmer Model 492 Protein Sequencer (Perkin-Elmer, Foster City, CA). The ATZ-amino acids were extracted from the membranes with neat TFA 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 ATZ-amino acids were eluted using standard conditions instead of neat TFA and sequenced on a Perkin-Elmer Model 492 Protein Sequencer. Amino acids found at each position were then compared to the known Cos2 sequence and candidate peptides identified based on a similarity of at least two sequential amino acids.

Stable and Transient Transfections

cl-8 cells were resuspended and plated onto 3.5 or 10 cm plates then allowed to grow until they reached 60-80% confluence, then washed once with complete M3 medium before transfection. For transient transfections, 10 μg of the appropriate UAST plasmid was combined with 5 μg of Ub-Gal4 for 3.5 cm plates, 50 μg of UAST plasmid with 10 μg of Ub-Gal4 for 10 cm plates, and transfected using a standard calcium phosphate transfection protocol (Ashburner, 1989). The precipitates were left on the cells for 12-24 hours before the cells were washed twice with 2-5 ml of complete M3 medium. The cells were then incubated for a further 10-14 hours before being lysed and analyzed by SDS-PAGE. For stable transfections, 50 μg of the appropriate MT plasmid was mixed with 5 μg of pCoHygro and stable cells transfection and selection were conducted using the manufacturers protocol (Invitrogen).

Immunoblotting

Immunoblotting was done as previously described (Therond et al, 96) except that NP40 was not included in the wash solutions. Polyclonal rabbit anti-Ci antisera was used at a 1:5000 or 1:40,000 dilution and was kindly provided by P. Aza-Blanc and T. Kornberg (UCSF). Polyclonal rat anti-Cos2 antisera was a kind gift of J. Sisson, K. Ho, and M. Scott (Stanford University) and was used at a 1:5,000 dilution. Donkey anti-rabbit IgG and anti-rat IgG (Amersham) were used at a 1:5000 dilution.

Immunofluorescence

Wing imaginal discs were dissected out of wandering third instar larvae in PBS. The discs were fixed in PBS-TX (PBS + 0.2% TX100) + 4% formaldehyde (Ted Pella) for 10 minutes and then washed three times with PBT (PBS + 0.1% Tween-20). The discs were then incubated with primary antibody in PBT + 5% donkey serum (Jackson Labs) for 1 hour, and the rest of the staining conducted as in (Aza-Blanc et al., 1997). Anti-Fused antisera (Robbins et al., 1997) was used at 1:20,000 and 2A1 anti-Ci was used at 1:5,000 (Motzny and Holmgren). Cy5 conjugated anti-rabbit (Jackson Labs) and FITC conjugated anti-rat (Jackson Labs) were both used at 1:5,000. Imaging was conducted on a Leica TCS NT confocal microscope (Leica America).

***Drosophila* Strains and Generation of Transgenic Flies**

Drosophila strains were maintained as previously described (Therond et al., 1996). Transgenic flies were made using the aforementioned constructs according to Rubin and Spradling (1982) using *yw* flies as hosts and maintained at 18°C. dpp-Gal4 (Staehling-Hampton et al., 1994), ptc-Gal4 (Hinz

et al., 1994), and en-Gal4 were kind gifts of the Kornberg laboratory. Crosses to detect adult wing phenotype were conducted at 29°C.

For wing preparations, whole flies were placed in isopropanol overnight and the wings were dissected and placed into 100% ethanol. The wings were then washed twice in ethanol, mounted in a 6:5 lactic acid:ethanol solution, a imaged using a Leica microscope with a CCD camera.

Figure 1. Fu is phosphorylated on serine and hyperphosphorylated in baculovirus infections.

(A) Phosphoaminoacid analysis of Fu from S2-Hh cells. Although the signals are faint, serine is clearly the predominant phosphorylated amino acid in Fu.

(B) Autoradiograms of Fu and FuM, a kinase inactive form of Fu created by mutating G13V, expressed by infection in baculovirus. The degree of radiolabeling of FuM is almost as little as the control Cos2 alone infection. Fu expressed with Cos2 shows two isoforms, while Fu infected alone shows at least three isoforms.

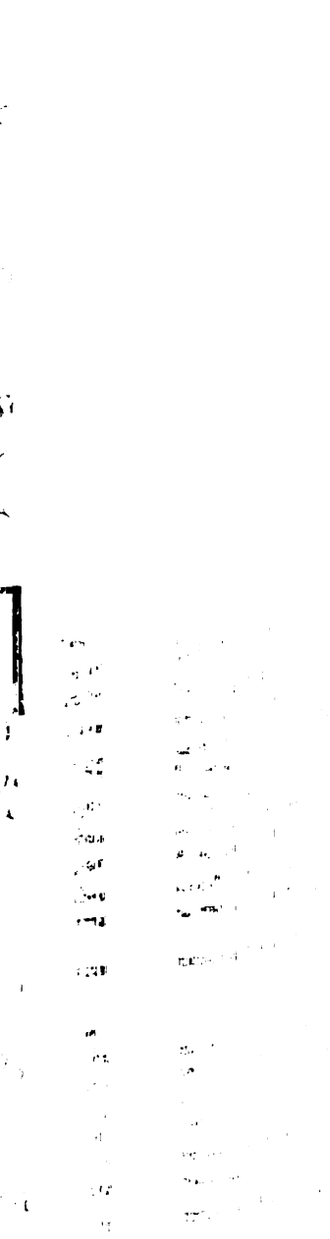


Figure 1

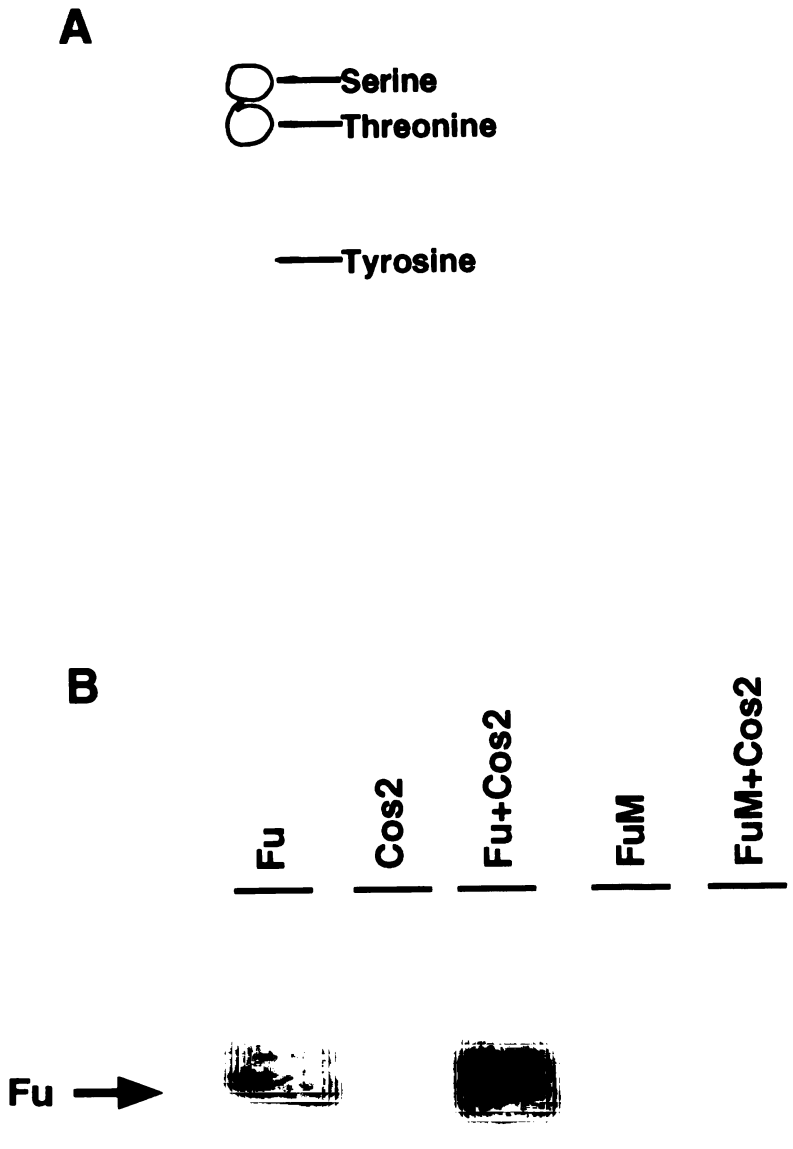
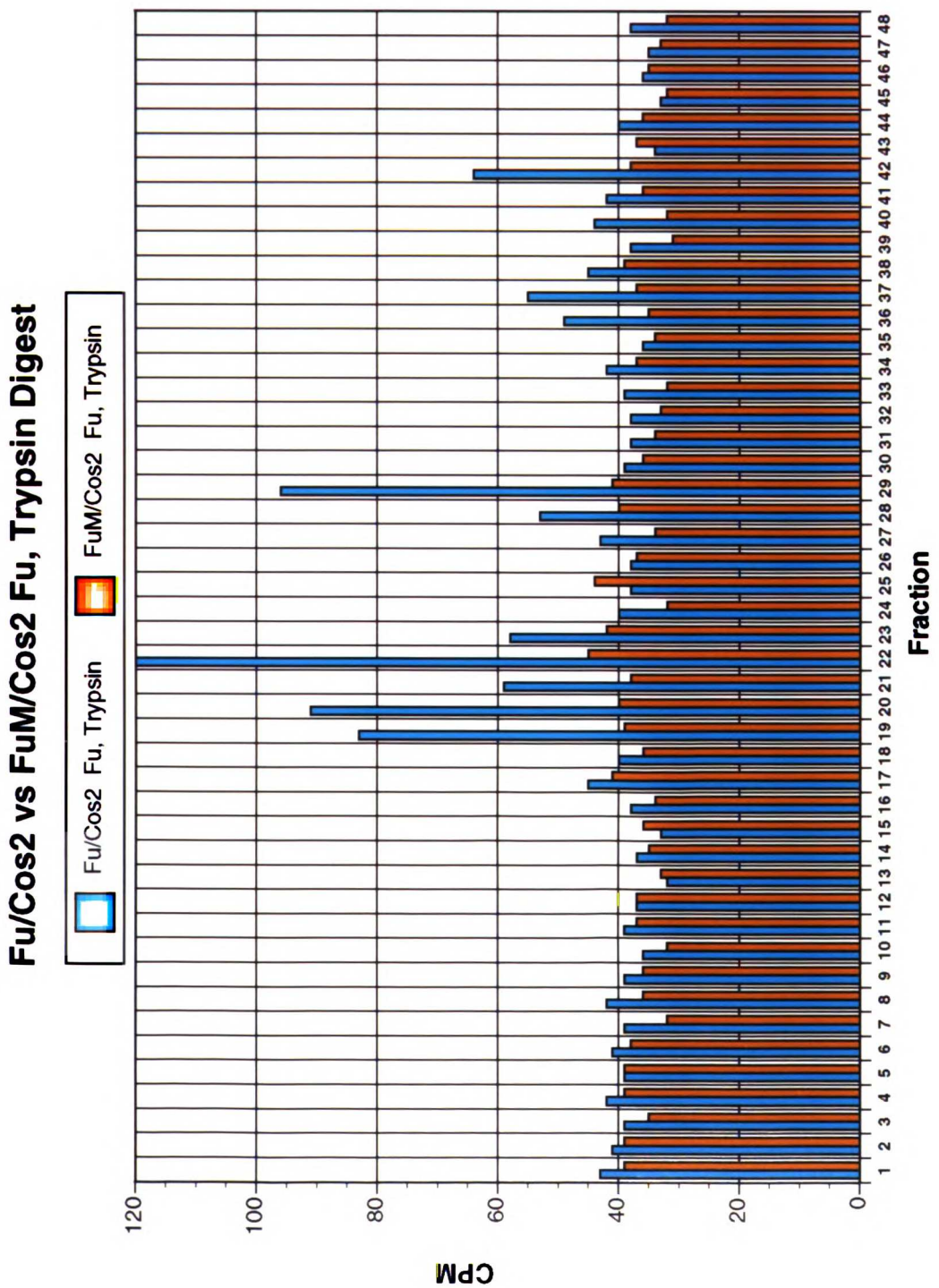


Figure 2A


Fu/Cos2 vs FuM/Cos2 Fu, Trypsin Digest
Fu/Cos2 Fu, Trypsin
FuM/Cos2 Fu, Trypsin
CPM
0
20
40
60
80
100
120
Fraction
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48

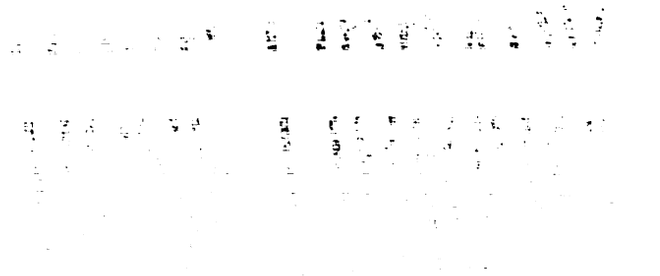


Figure 2B

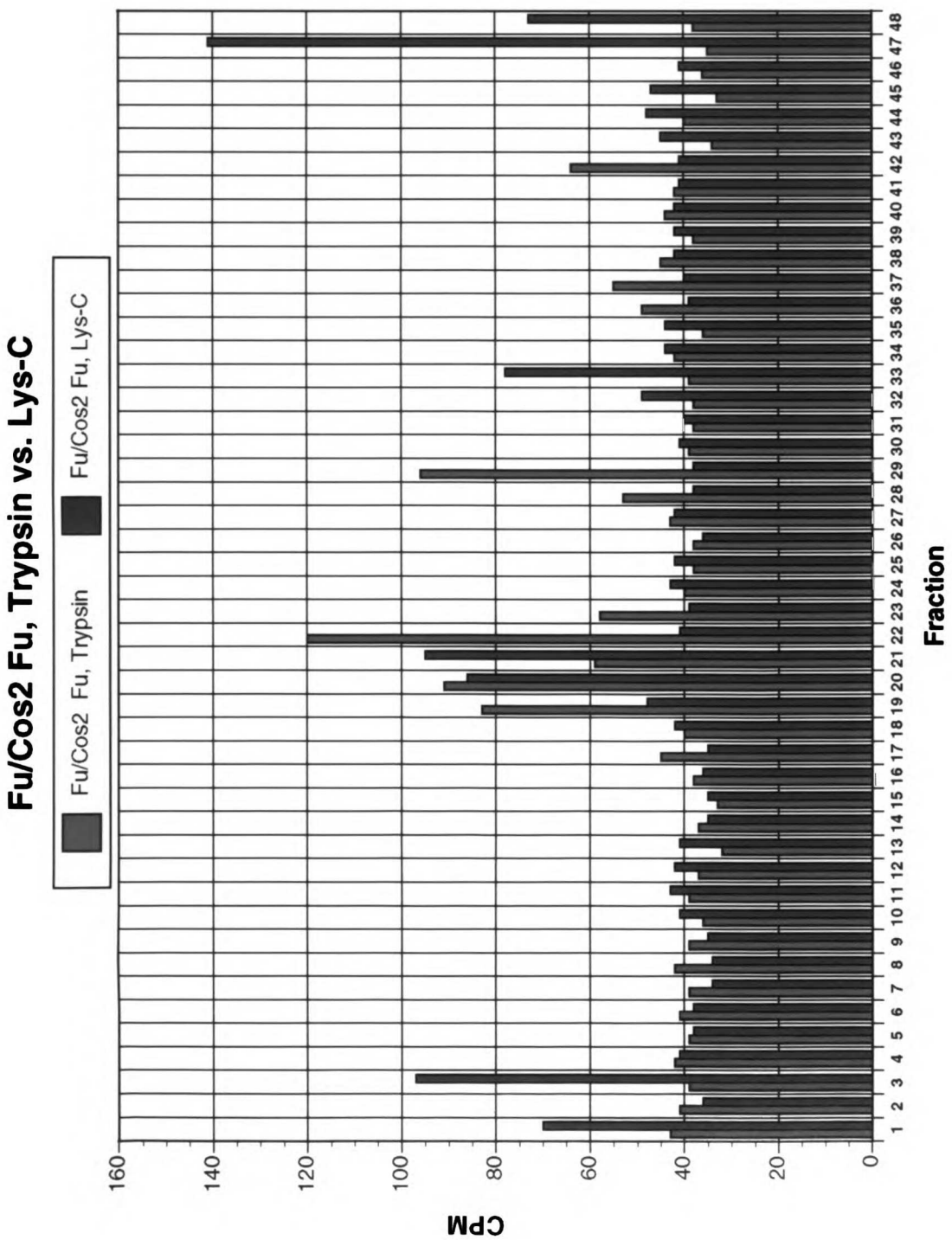

Fu/Cos2 Fu, Trypsin vs. Lys-C
Fu/Cos2 Fu, Trypsin
Fu/Cos2 Fu, Lys-C
CPM
0
20
40
60
80
100
120
140
160
Fraction
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48

Figure 2C

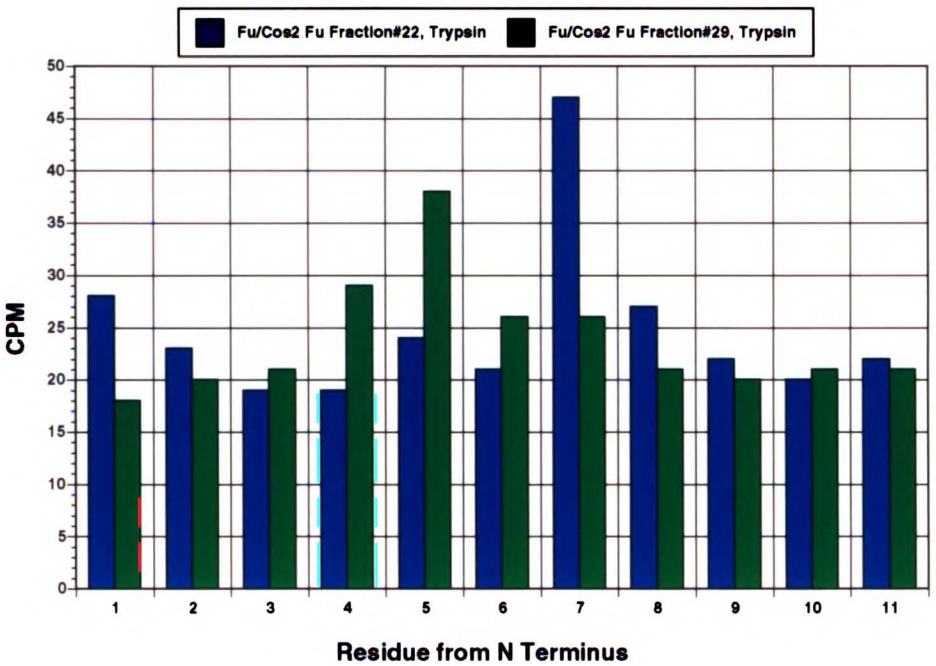


Figure 2D

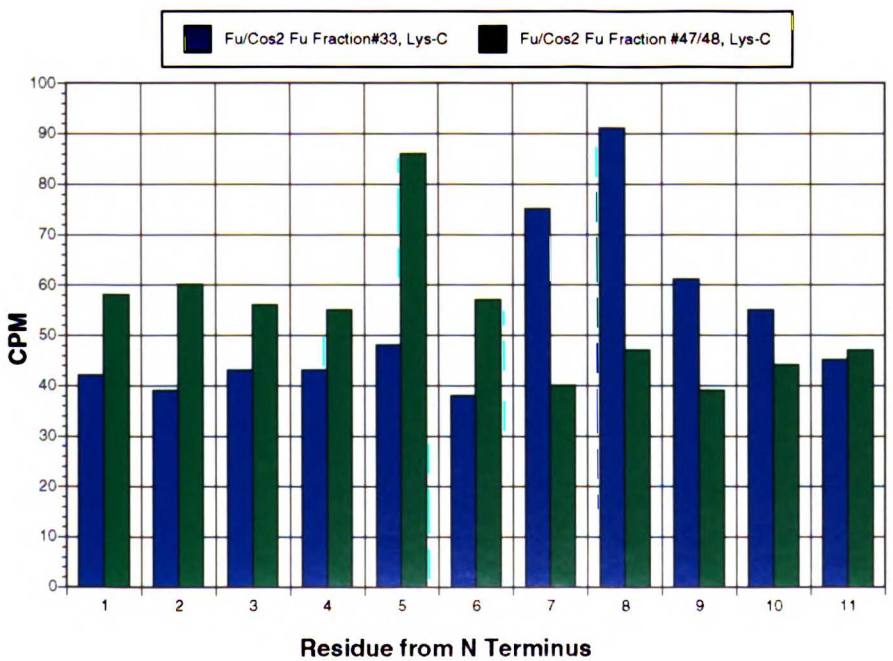


Figure 3. Amino acid sequencing of a radiolabeled Fu fraction.

(A) The various amino acids found at each position of the peptides from fraction 25 relative to the N terminus of the peptide. Due to the nature of the sequencing method, the amino acids in the first position can not be determined. There is, as expected, a mix of amino acids at each position relative to the N terminus, indicating a mix of peptides in the fraction.
(B) Sequences of the two theoretical tryptic peptides of Fu that have at least two, consecutive, matching amino acids to those found in each position in (A). Two tryptic peptides of Fu, 557-560 and 307-314, account for all the amino acid residues found in (A).

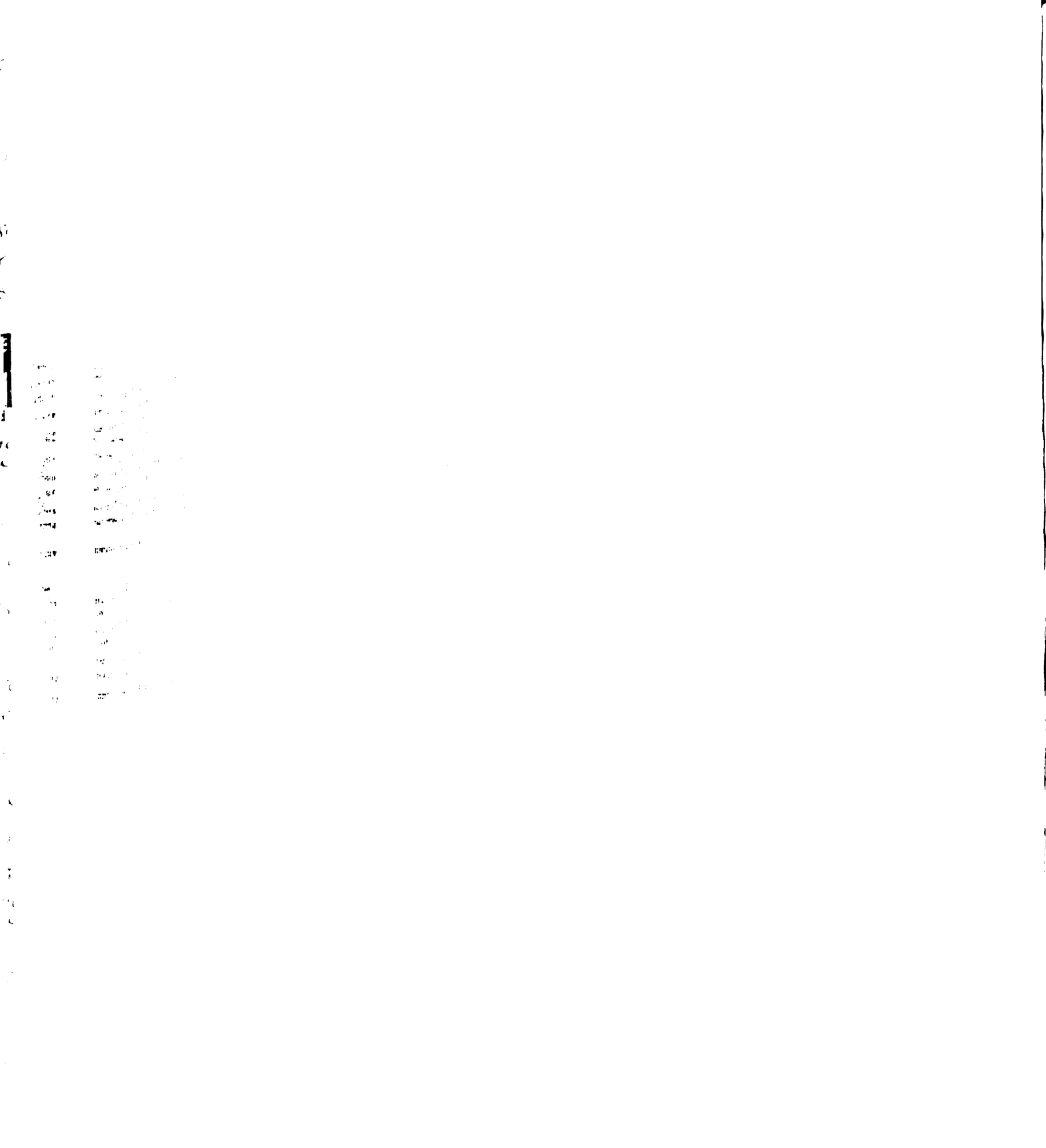


Figure 3

A **Amino Acid Positions from Edman Sequencing**

2	3	4	5
Iso	Asn	Arg	Thr
Glu	Pro	Leu	

B **Amino Acid Positions of Corresponding Tryptic Peptides**

-1	1	2	3	4	5	6	7	8
Lys	Ala 557	Iso	Pro	Arg				
Arg	Gln 307	Glu	Asn	Leu	Thr	Thr	Ser	Arg

Figure 4. Theoretical Tryptic Peptides of Fu containing Serines or Threonines at Positions #5 or #7.

(A) Theoretical tryptic peptides of Fu with a Serine or Threonine at position #5.

(B) Theoretical tryptic peptides of Fu with a Serine or Threonine at position #7.

(C) PTP map of Cos2 from Fu S616A/Cos2 and Fu/Co2 coinfections. Fu S616A has no effect on Cos2 PTP maps in coinfections.

Figure 4

A

	-1	1	2	3	4	5	6	7	8	9	10
3	R	Y	A	V	S	S	L	V	G	Q	G...K
270	K	E	S	P	F	T	N	P	E	A	K
307	R	Q	E	N	L	T	T	S	R		
481	K	H	S	M	H	S	T	N	E	E	K
612	K	Q	L	S	D	S	A	A	S	A	P...R
734	R	L	N	F	V	S	L	L	Q	S	R

B

	-1	1	2	3	4	5	6	7	8	9	10
74	K	T	D	L	F	V	V	T	E	F	A...R
105	R	V	T	G	H	L	V	S	A	L	Y...R
307	R	Q	E	N	L	T	T	S	R		
459	K	P	P	A	E	A	I	S	Y	A	N...K
564	K	S	V	A	Q	L	L	S	L	P	F...R
772	R	F	A	L	Q	Q	L	S	E	H	H...R

Figure 4C

Fu S616A+Cos
Cos Peptides

Fu+Cos2
Cos2 Peptides

Figure 5. Threonine 80 in the Fu kinase domain appears to regulate Cos2 phosphorylation but not autophosphorylation.

(A) Autoradiography of a one dimensional SDS-PAGE gel on which ^{32}P radiolabeled immunoprecipitates of wild type and various Fu mutants coinfected with Cos2 have been separated. Fu T80A has a strongly radiolabeled lower isoform of Fu but has little to no upper isoform of Fu. Cos2 phosphorylation in a Fu T80A coinfection is still robust, however.
(B) PTP map of Fu from a Fu/Cos2 coinfection. Phosphopeptides 1' through 7' are labeled.
(C) PTP map of Fu T80A from coinfection with Cos2. The map looks identical to a wild-type Fu PTP map.
(D) PTP map of Cos2 from a Fu/Cos2 coinfection. Phosphopeptides 1 trough 6 are labeled.
(E) PTP map of Cos2 from a Fu T80A/Cos2 coinfection. Only peptide 1 is apparent.

Figure 5

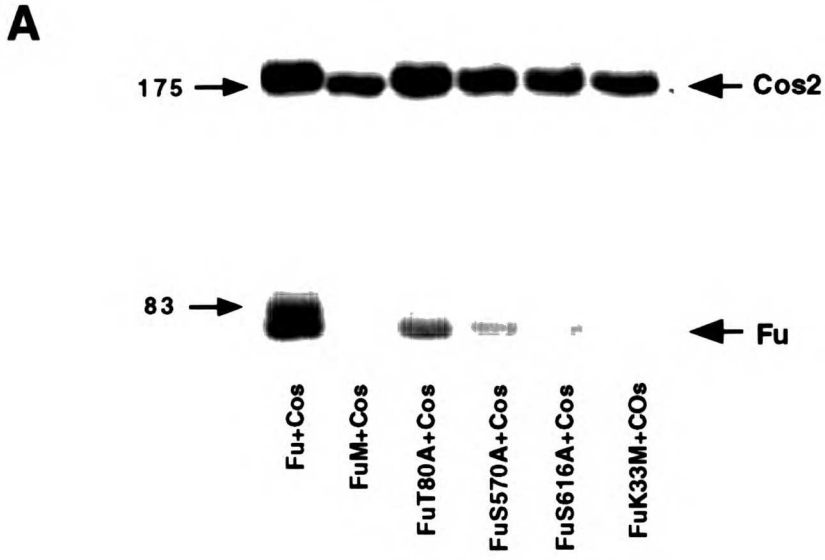
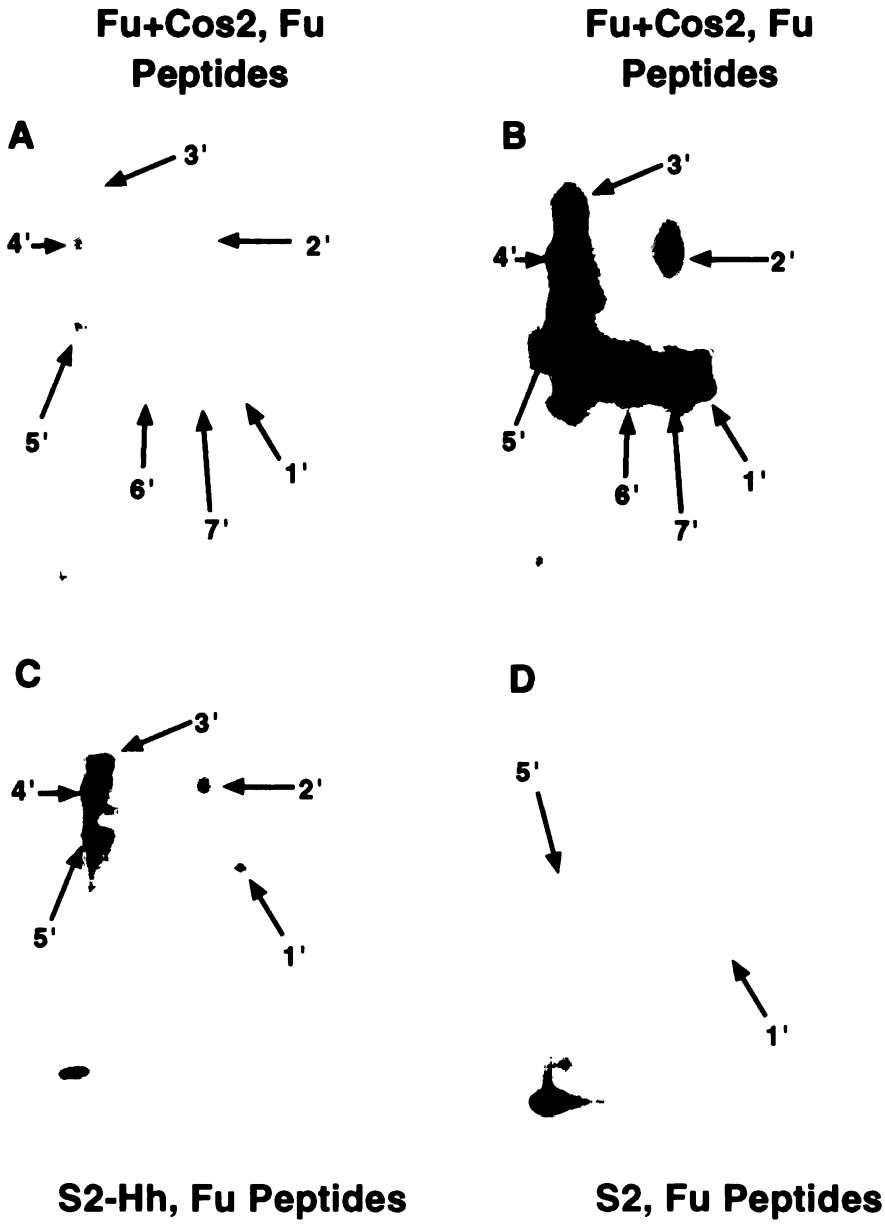

A
175
Cos2
83
Fu
Fu+Cos
FuM+Cos
FuT80A+Cos
FuS570A+Cos
FuS616A+Cos
FuK33M+COs
B
Fu+Cos2
Fu Peptides
C
Fu T80A+Cos2
Fu Peptides
3'
4'
2'
5'
6'
7'
1'
D
Fu+Cos2
Cos2 Peptides
E
Fu T80A+Cos2
Cos2 Peptides
3
2
5
4
1
6

Figure 6. Comparison of PTP Maps of Fu from S2 cells and Baculovirus Coinfections.

(A) PTP map of Fu from a Fu/Cos2 coinfection. There are numerous ^{32}P radiolabeled peptides found which are numbered 1' to 7' (to prevent confusion with the Cos2 peptide numbers).
(B) A darker exposure of (A).
(C) PTP map of endogenous Fu from S2 cells constitutively expressing Hh. All phosphotryptic peptides except peptide #7 are found in the S2-Hh Fu PTP map.
(D) PTP map of endogenous Fu from S2 cells. In addition to the great overall reduction in radiolabeling, it appears that only peptides # 1' and 5' are present in Fu from non-Hh stimulated S2 cells.

Figure 6


Fu+Cos2, Fu Peptides
Fu+Cos2, Fu Peptides
A
B
3'
4'
2'
5'
6'
7'
1'
C
D
S2-Hh, Fu Peptides
S2, Fu Peptides

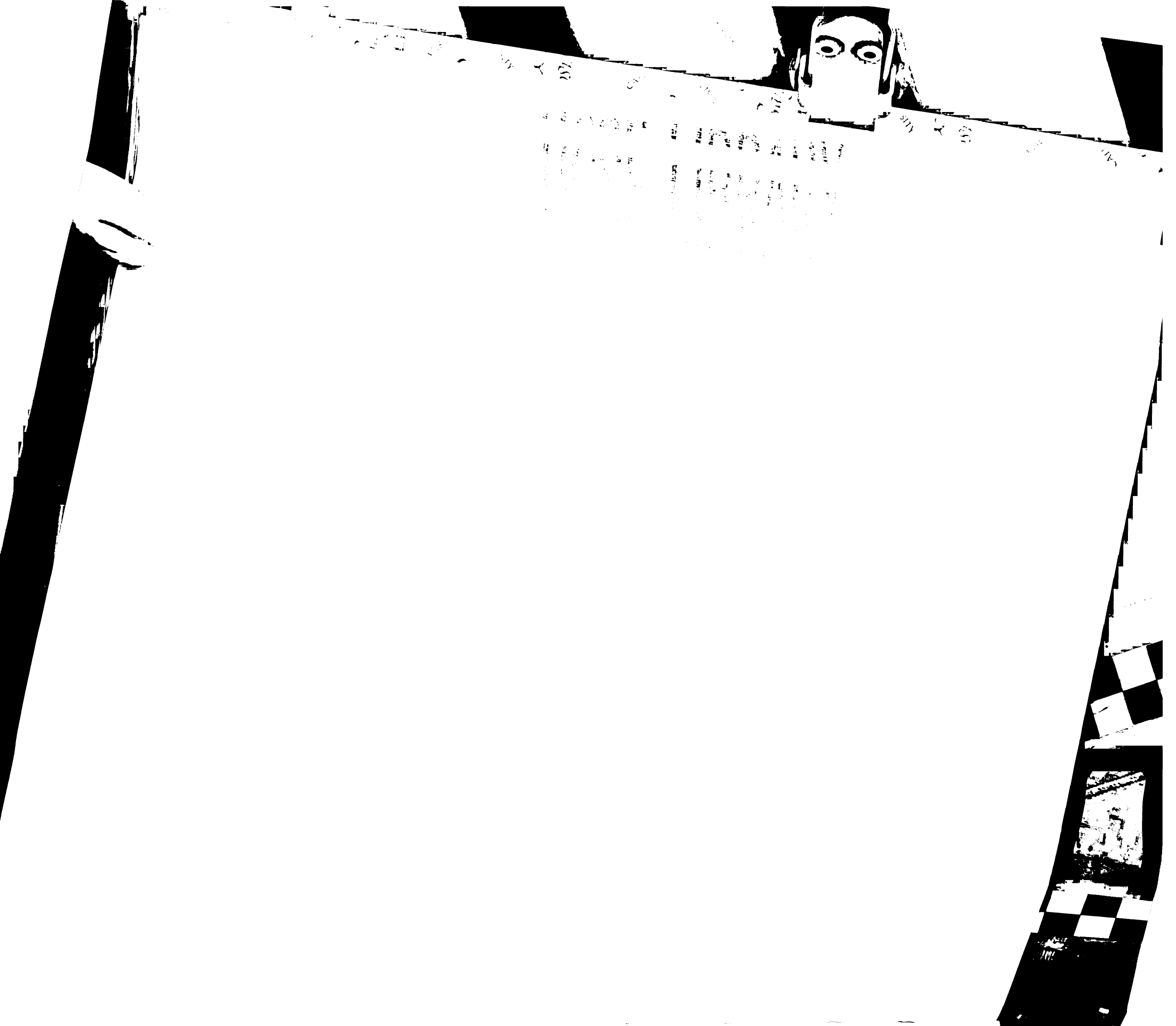


Figure 7. Expression of FuD, but not wild-type Fu, in the anterior wing imaginal disc recapitulates a *fu* phenotype.

(A) Schematic diagram of the wild type and truncated forms of Fused used to make transgenic flies.
(B) A wild-type *Drosophila* wing. There is continuous separation between longitudinal wing vein 3 and 4.
(C) A wing from a *fu62* homozygous adult fly. Proximal fusions between wing veins 3 and 4, as well as narrowing of the distance between these veins distally, can be seen.
(D) Wing from a *fuRX16* homozygous adult fly. More severe distal fusions between wing vein 3 and 4 can be seen.
(E) Wing from a UAST-Fu X Dpp-Gal4 fly raised at 29°C. The wing is normal and has no fusion of longitudinal wing veins 3 and 4.
(F) Wing from a UAST-FuD X Ptc-Gal4 raised at 29°C. The wing has proximal fusions of longitudinal wing veins 3 and 4.
(G) Wing from a UAST-FuD X Dpp-Gal4 raised at 29°C. The wing has more severe proximal fusions of longitudinal wing veins 3 and 4 than that in (F). In addition, there are distal ectopic veins.

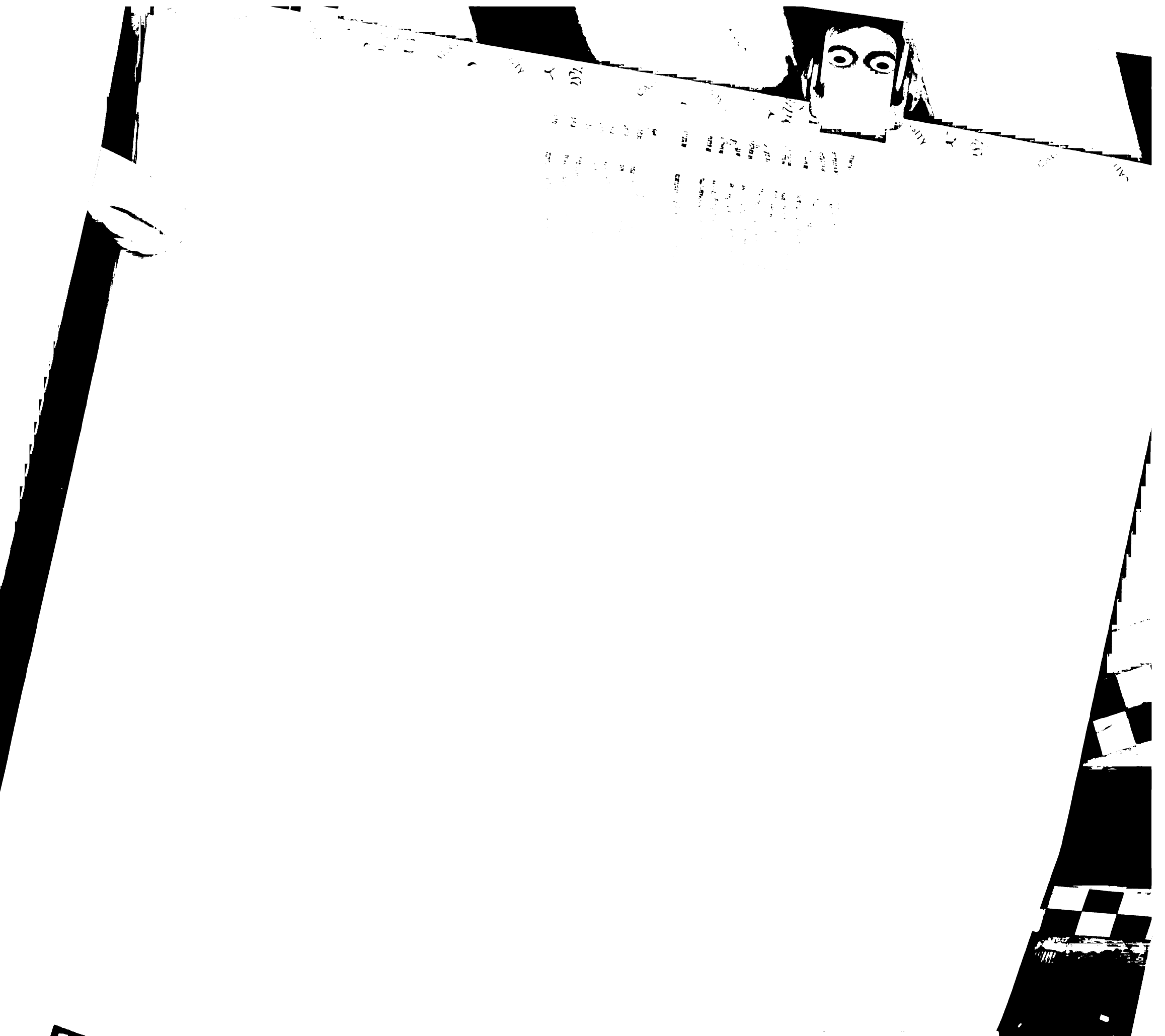


Figure 7

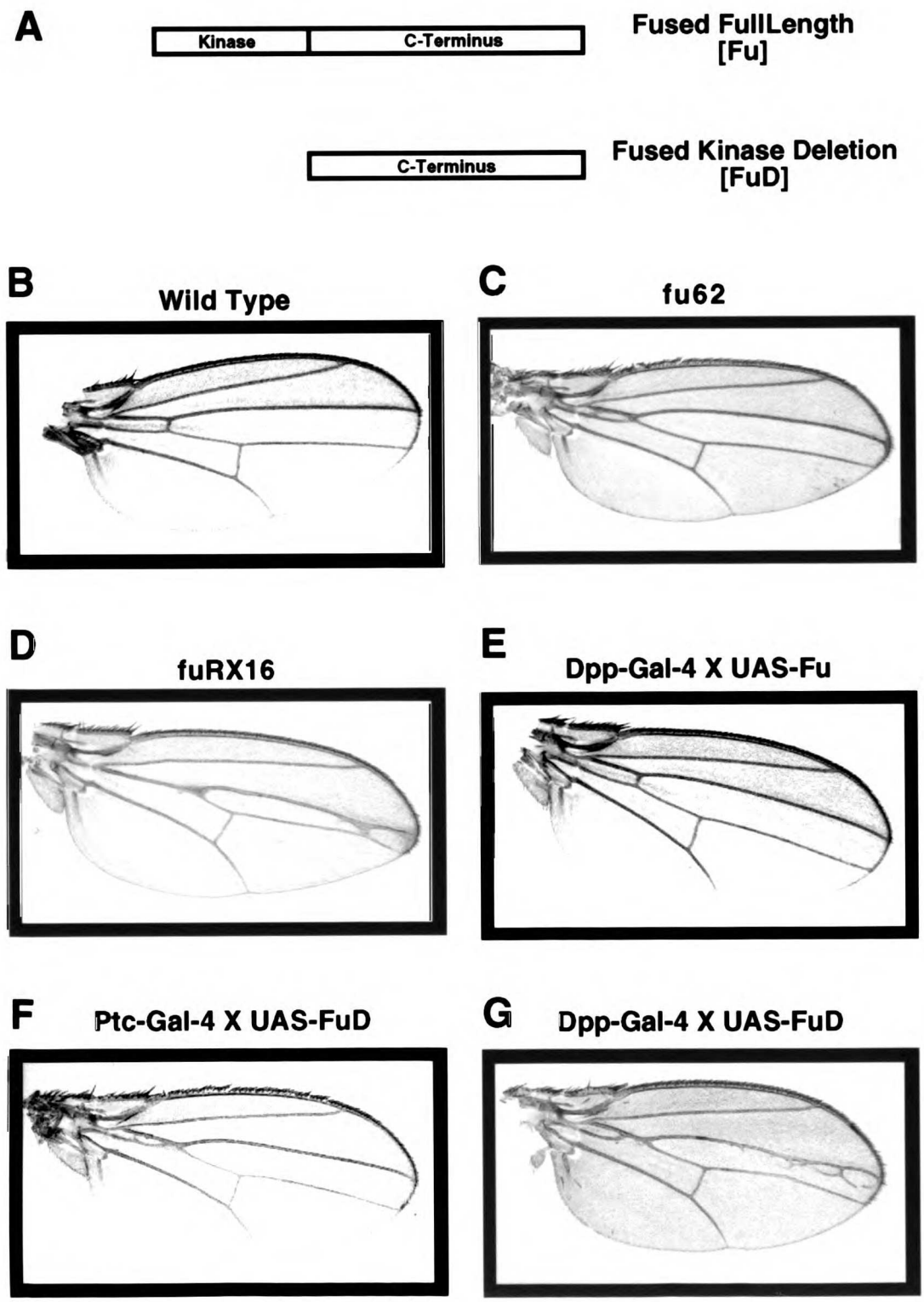

A
Kinase
C-Terminus
Fused FullLength
[Fu]
C-Terminus
Fused Kinase Deletion
[FuD]
B
Wild Type
C
fu62
D
fuRX16
E
Dpp-Gal-4 X UAS-Fu
F
Ptc-Gal-4 X UAS-FuD
G
Dpp-Gal-4 X UAS-FuD

Figure 8. Increased accumulation of Ci155 in areas of the wing where FuD is expressed.

(A, (C), (E), (G), and (I), immunofluorescence for Fu with an antisera that recognizes both Fu and FuD. (B), (D), (F), (H), and (J), immunofluorescence with the AbN antisera to detect full length Ci accumulation. (A) and (B) show Fu and Ci fluorescence of wild type wing imaginal discs. The accumulation of full length Ci at the anterior-posterior compartment border can clearly be seen, as can the slightly increased levels of Fu in the anterior compartment. (C) and (D) show a close up view of the anterior-posterior compartment border in these same discs. (E) shows the increased staining of Fu at the compartment border when UAST-Fu is driven by dpp-Gal4. (F) shows that the normal increase in Ci at the border is not affected when wild-type Fu is overexpressed in this way. (G) demonstrates the wide band of FuD overexpression when UAST-FuD is driven by dpp-Gal4. (H) illustrates that the band of full length Ci accumulation has widened to include all areas where FuD is overexpressed. (I) and (J); magnifications of (G) and (H).

Figure 8

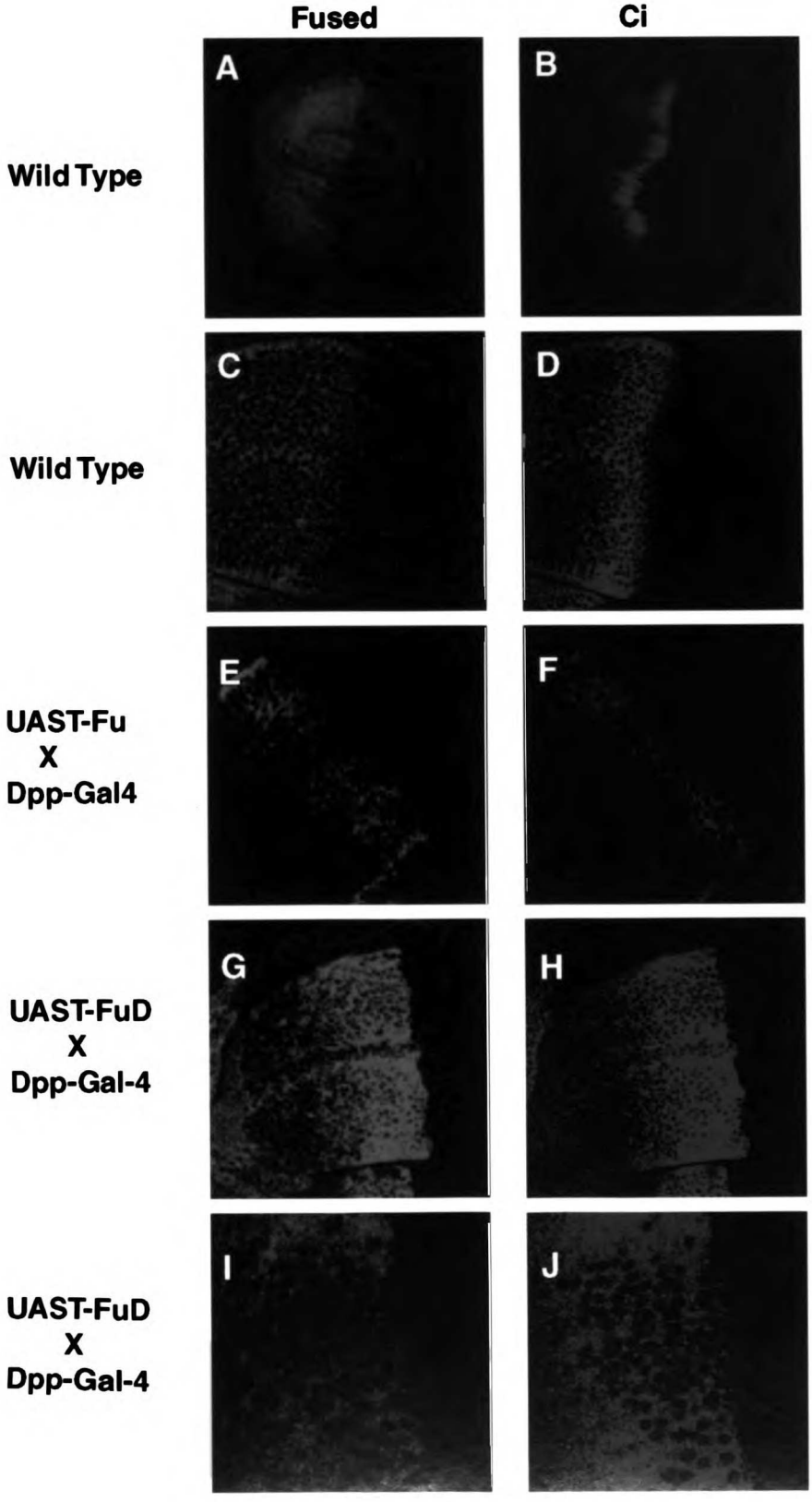

Fused
Ci
A
B
Wild Type
C
D
Wild Type
E
F
UAST-Fu
X
Dpp-Gal4
G
H
UAST-FuD
X
Dpp-Gal-4
I
J
UAST-FuD
X
Dpp-Gal-4

Figure 9. Fu phosphorylates and shifts Ci155 in baculovirus coinfections.

(A) Combinations of coinfections of Fu, Cos2, and Ci. The various coinfection combinations shown generated four isoforms of Ci155 as determined by immunoblot. Two isoforms of Ci155, #1 and #3, are found Cos2 and Ci are coinfected, and these same two forms are found when Cos2, Ci and FuM are coinfected. Coinfection of Fu and Ci gave a new isoform, #2. Fu, Cos2, and Ci coinfection gave a fourth isoform of Ci155, #4. Mutant Cos2 viruses appear to force all Ci155 into the upper, fourth isoform, probably due to higher titer of these viruses.

(B) Further combinations of coinfections showing similar results to those in (A), although only three isoforms can be distinguished in this immunoblot. Doubling the titer of Fu or FuM does not change the Ci155 isoforms found in that particular coinfection. Ptc and Smo both do not appear to have significant effects on Ci155 isoforms when coinfected with Fu, Cos2, and Ci. The lower panel shows the effects these coinfections have on formation of Ci75. Loss of Fu kinase activity correlates with a reduction in the amount of Ci75. The lower panel was overexposed since the abundance of these lower molecular weight Ci isoforms is considerably less than that of Ci155 in these coinfections.

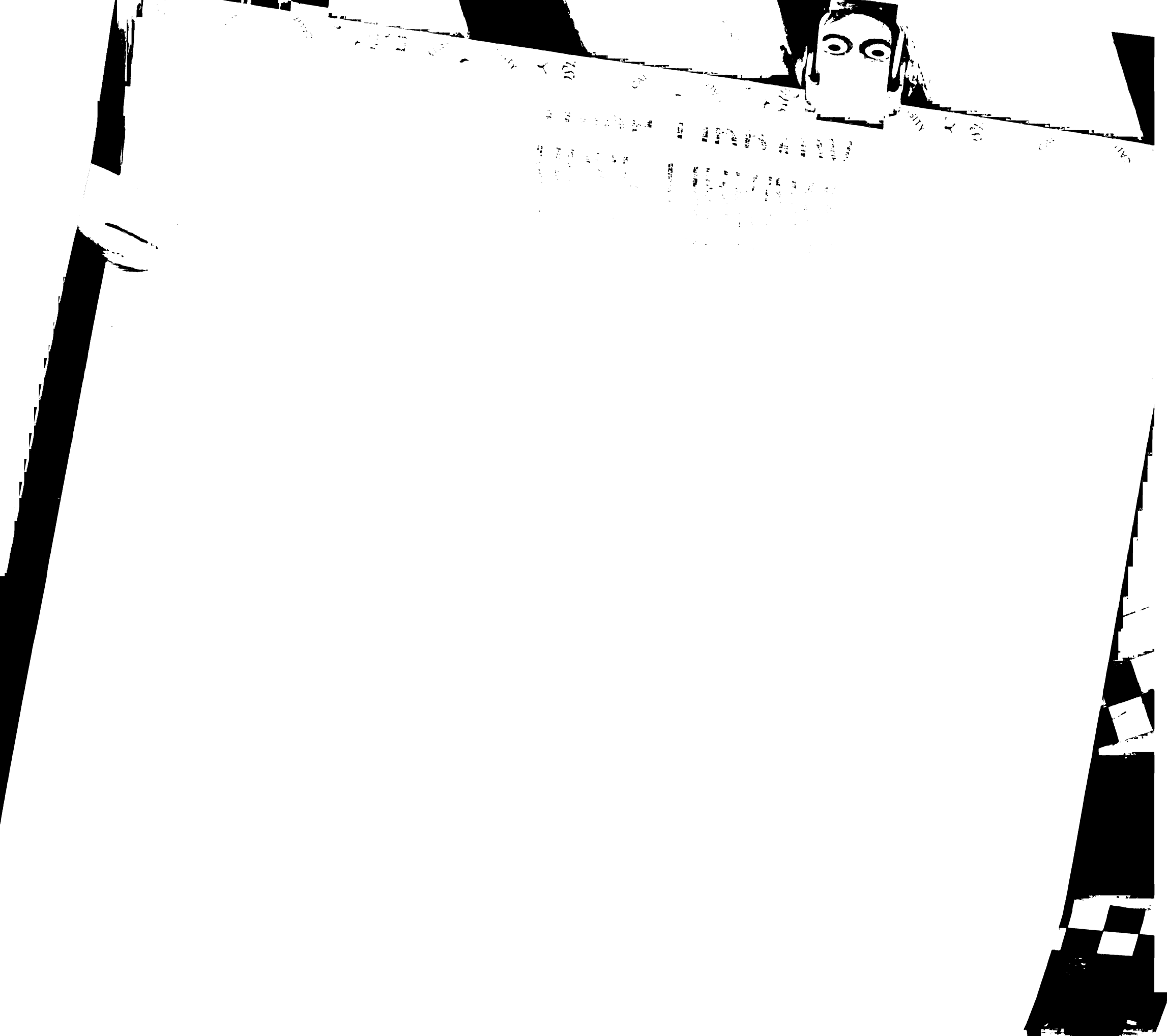
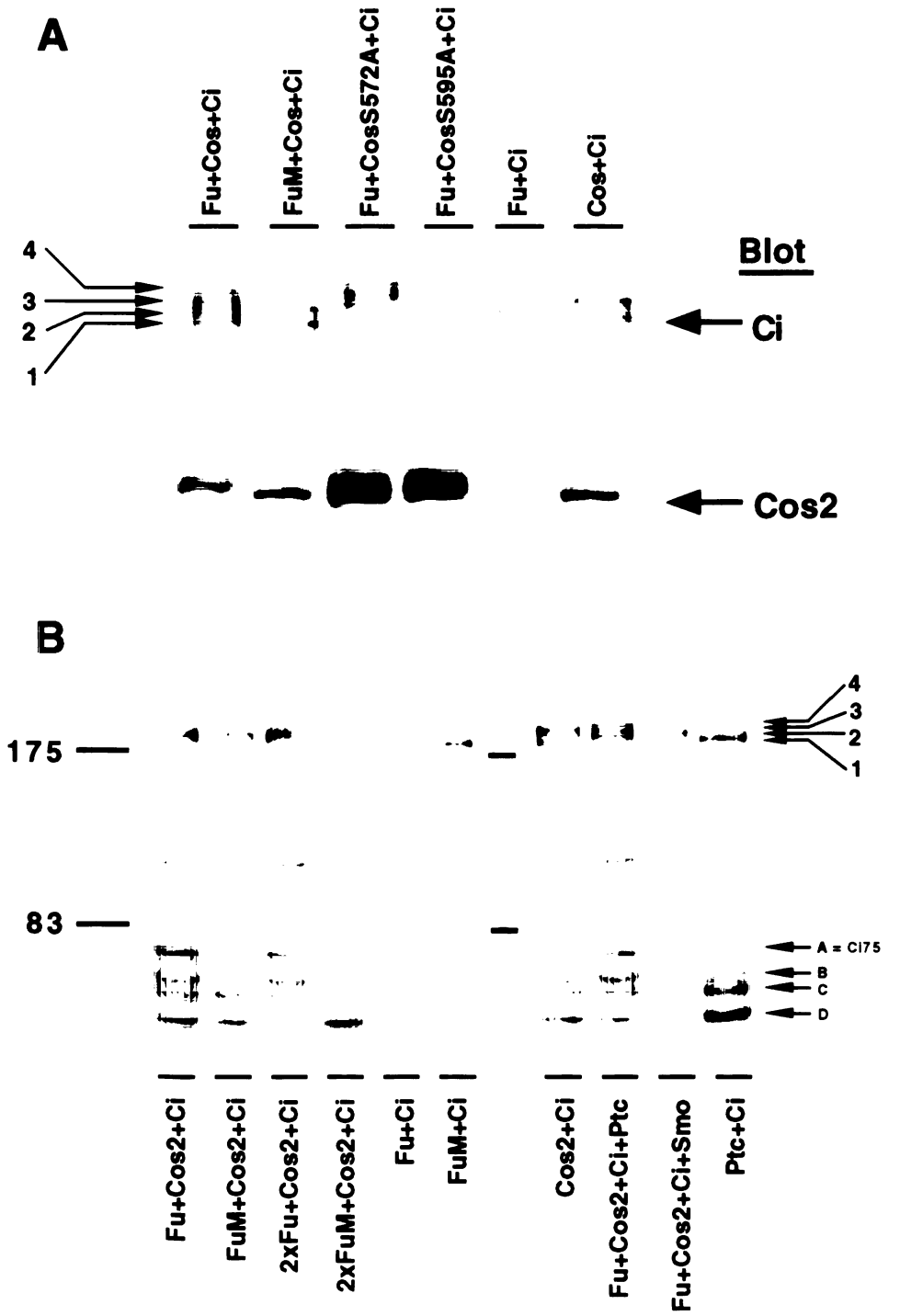
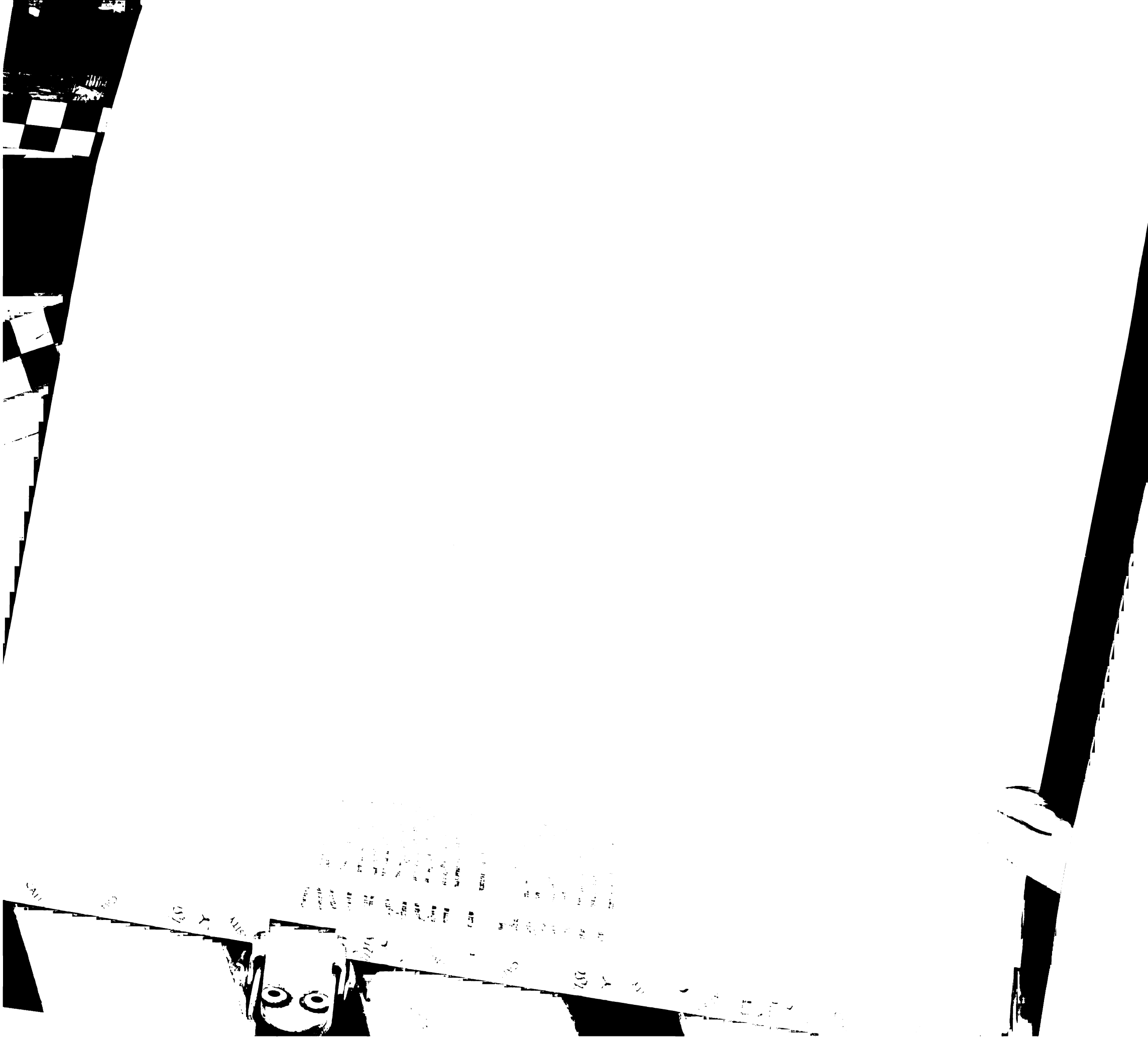


Figure 9


A
Fu+Cos+Ci
FuM+Cos+Ci
Fu+CosS572A+Ci
Fu+CosS595A+Ci
Fu+Ci
Cos+Ci
Blot
4
3
2
1
Ci
Cos2
B
175
83
4
3
2
1
A = Ci75
B
C
D
Fu+Cos2+Ci
FuM+Cos2+Ci
2xFu+Cos2+Ci
2xFuM+Cos2+Ci
Fu+Ci
FuM+Ci
Cos2+Ci
Fu+Cos2+Ci+Ptc
Fu+Cos2+Ci+Smo
Ptc+Ci



Proteolysis
??
Ci
Release
Ci
Cleavage
Ci75
Protease
Ci
Fu
Fu
Cos2
Cos2
Release/
Movement
Microtubules
Microtubules
Hh
Signal
Ci
Binding
Phosphatase
??

Figure 10

Chapter 5

Future Directions

As with most scientific research, this work raises as many questions as it answers and points to many new avenues of research in the Hh signaling field. In this final chapter, I will highlight some of the specific questions that still remain to be answered as well as pointing out the directions this work might take in the future.

Identification and verification of the phosphorylation sites of Fu still needs to be finished. Once the sites of autophosphorylation of Fu are identified, it will need to be confirmed which are genuine autophosphorylation sites and which are normally phosphorylated by an exogenous kinase. If there is an exogenous kinase that phosphorylates Fu, then isolation of this kinase will certainly be a priority in future studies of the signaling pathway. In the long term, examination of the mechanism of autophosphorylation will illuminate how this unusual signaling mechanism functions in such a divergent Serine/Threonine kinase as Fu.

Clarifying the role that Fu plays in phosphorylating Ci is another important area still to be investigated. Determination of the Fu generated phosphorylation site on Ci will allow an examination of how this site(s) affects Ci cleavage and/or transcriptional activity. A more thorough study of the binding interactions of Fu, Ci, and Cos2 will help verify if Fu can bind and phosphorylate Ci in the absence of Cos2, as we have seen. Cos2 might normally be required for Fu to phosphorylate Ci and the phosphorylation of Ci by Fu we see in baculovirus in the absence of Cos2 could be an artifact of overexpression in the baculovirus system. The interaction of the phosphorylation events catalysed by PKA and Fu is also a subject that is open to investigation.

With respect to mechanism(s) of action of Fu and Cos2 in Hh signaling, there are a great many questions still to be answered. An obvious question, but one of great import, is: does Cos2 function like a classical kinesin and actually move along microtubules? We and others have shown that Cos2 does bind microtubules, but we have not examined whether the nucleotide binding motifs found in the microtubule motor domain of Cos2 have ATPase activity or are required for Hh signaling (Chapter 2, Sisson et al, 1997). Nor have we investigated whether the kinesin domains found in Cos2 which have been shown to be specific to motor activity in other kinesins, such as the DLL and L12 domains, are required for Hh signaling (Vale and Fletterick, 1997). Cos2 is a divergent member of the kinesin family and has no particular homologies to other kinesin subfamilies, and so it is difficult to ascertain by sequence comparison whether its primary function is one of binding or of movement (Sisson et al., 1997). Answers to these questions will have to await the results of ATPase assays and in vitro microtubule movement tests.

Our studies have shown that Hh signaling is associated with a substantial reduction in binding of the Fu/Cos2/Ci complex to microtubules in an in vitro microtubule rebinding assay, and we have shown here that Fu phosphorylation of Cos2 correlates with Hh signaling. This strongly implicates Fu as an effector of Cos2 release from microtubules. But is this inhibition of binding a consequence of Cos2 trying to execute some sort of movement, or is it simply that the Fu induced phosphorylation is a release signal causing a loss of microtubule binding? Again, in vitro movement assays and development of an in vitro binding/release assay will be necessary to resolve these questions.

If Cos2 does function as a motor, then where does it move to and from and for what purpose? Immunofluorescent staining of both cells and

embryonic and imaginal tissues have not demonstrated any specific localization or accumulation of Fu or Cos2. Immunofluorescent staining of embryos has demonstrated that Cos2 is not found in the nuclei and appears to be enriched in cleavage furrows, but otherwise seems to have a non-specific cytoplasmic staining (Sisson et al., 1997). Similarly, in imaginal discs, Cos2 seems to have a non-specific cytoplasmic staining. Cos2 is also found overlapping immunofluorescently stained microtubules, but it does not overlap all microtubules and no specific patterns of overlap are discernable (Sisson et al., 1997). Fused has a similiar non-specific cytoplasmic staining (Alves et al., 1998). Fu and Cos2 may not require any sort of specific subcellular localization, but rather, movement along any microtubule may suffice for signaling. It is also possible that Fu and Cos2 move to a microtubule bound protein complex or vesicle that is common and widely distributed, and so specific localization would never be seen.

One possible protein complex that the Fu/Cos2/Ci complex could move to is the proteasome. The processing of Ci is thought to be carried out by the proteasome, a very large, multisubunit protein machine involved in regulated proteolysis in the cell (Chen et al., 1999; Jiang and Struhl, 1998). Perhaps Cos2 and Fu are needed to carry Ci in complex along microtubule tracks to the proteasome for processing. In this case, the proteasome may be attached to microtubules and the Fu/Cos2 complex could actually have a direct physical interaction with the proteasome. Alternatively, Fu/Cos2 could deliver Ci to a region near the proteasome where Ci could then be shuttled to the proteasome by other factors. Since Ci function appears to be regulated by nuclear import and export, it is also conceivable that Cos2 could move along microtubules in the process of delivering Ci into the proximity of the nucleus. Recent studies have suggested that Su(fu) is part of the Fu/Cos2/Ci

complex and that it may be a nuclear import factor (Monnier et al., 1998; Pearse et al., 1999). The Fu/Cos2 complex could thus bring a Ci/Su(fu) complex to the nuclear pores where they could then enter the nucleus via Su(fu) action. The one caveat to this theory is that Su(Fu) alleles thought to be nulls exhibit a very mild phenotype, whereas one would expect a profound segment polarity phenotype if the Ci155 transcriptional activator was prevented from entering the nucleus (Preat et al., 1993). It is possible that the Su(fu) alleles now known are not complete nulls, or that there are other molecules that are functionally redundant to Su(fu).

There is also some evidence to implicate Fu/Cos2 in vesicular transport. The principal reason to suspect that Fu/Cos2 could be involved with vesicular transport is that both Hh and Ptc are found in particles, or "multivesiculate bodies", in those cells abutting a population of Hh producing cells (Capdevila et al., 1994; Tabata et al., 1992). These multivesiculate bodies seem to be enriched at the basal cell membrane (Tabata, 94, Capdevila, 94, Taylor 93) (Capdevila et al., 1994; Tabata and Kornberg, 1994; Taylor et al., 1993). Moreover, the discovery that Ptc bears a sterol sensing domain and a region of homology to the NPC1 protein, and the fact that most sterol sensing domains are involved in endocytosis or metabolism of lipid containing particles, further suggests that Ptc could be involved in vesicular transport (Carstea et al., 1997). Cos2, being downstream of Ptc genetically, would then be an obvious candidate for a motor to move a Ptc bearing vesicle or particle. No interaction between Fu/Cos2 and Ptc has ever been demonstrated, however, and the connection between Fu/Cos2 and Ptc remains a missing piece in the Hh signaling puzzle.

Complexes containing Cos2 may not be capable of any movement at all and may instead only achieve signaling through their binding or lack of

binding to microtubules. In the absence of Hh signaling, binding to microtubules could be constitutive, and this would lead to processing of Ci. Some sort of processing protein or protein complex might move down the microtubules or in some other way be targeted to the Fu/Cos2 microtubule-bound complex where it could then cleave Ci. Fu induced phosphorylation would release this complex from microtubules which would prevent the processing protein or complex from acting on Ci. Disruption of the microtubule binding of the complex through any means would then simulate Hh signaling. Elimination of the microtubule binding domains of Cos2 or the substitution of an acidic amino acid at Ser 572 of Cos2 to mimic phosphorylation of Ser 572 could then prevent binding of the complex to microtubules and cause constitutive activation of the Hh signaling pathway.

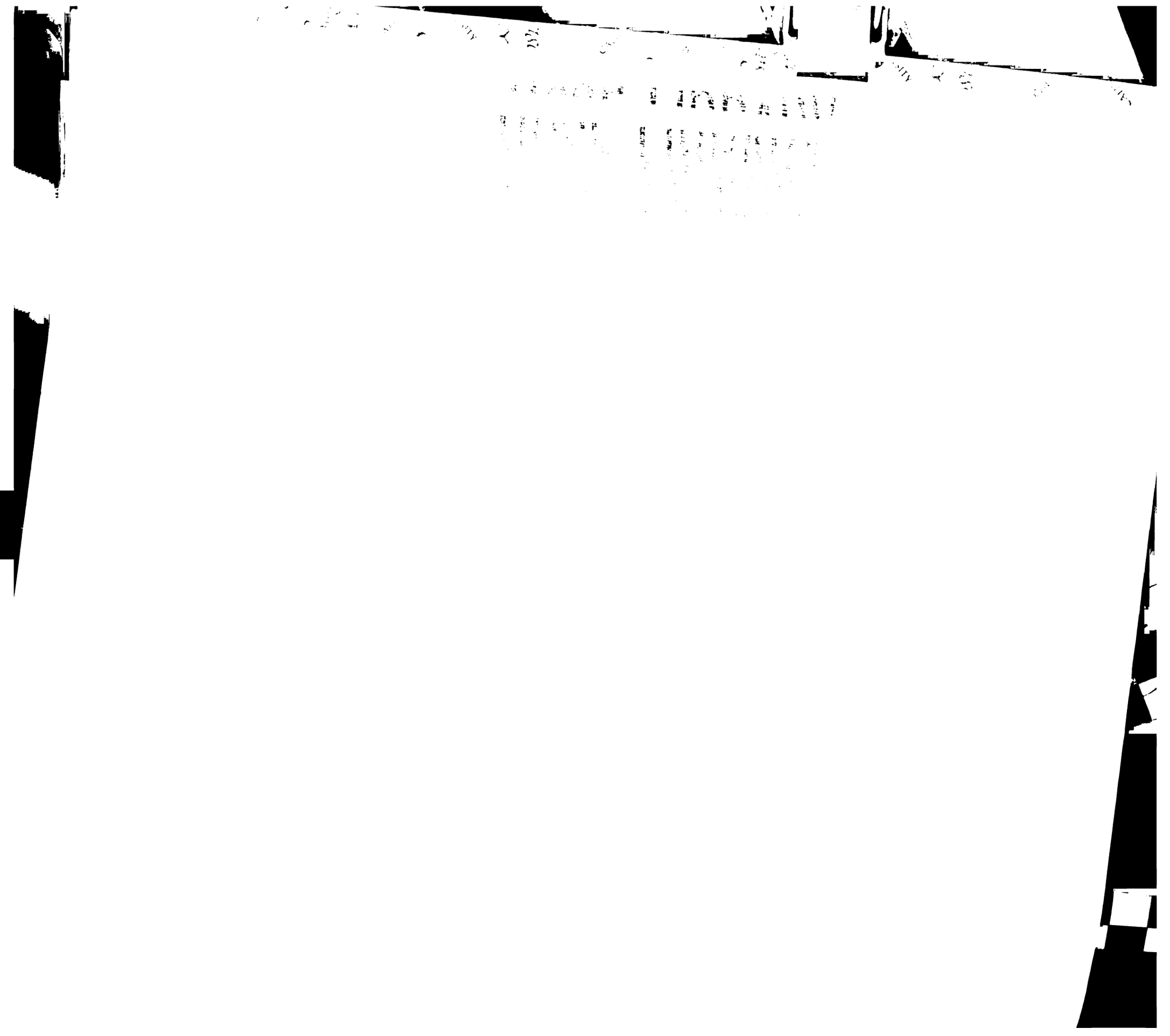
It is also not known if Ci is the only cargo carried by the Fu/Cos2 complex. Assuming that the globular C terminus of Cos2 binds to some sort of cargo, as most kinesin like molecules have beeen shown to do, then the Fu/Cos2 complex could carry cargo other than Ci, especially since it is not known how Ci interacts with Cos2. Su(fu) binds to both Ci and the C terminal 2/3 of Fu in yeast two hybrid assays, and so Cos2 might only bind Ci indirectly, through a bridge formed by Fu and Su(fu) (Monnier et al., 1998). If this bridge were the mechanism of Ci binding to Cos2 and if Fu is not bound to the cargo binding domain of Cos2, then another vesicle or protein complex could be bound by Cos2. This model would allow for Fu/Cos2 to carry Ci and also interact with the multivesiculate bodies in which Hh and Ptc are found. Cargo binding by the C terminus of Cos2 might even be linked to Ci processing. Development of especially gentle lysis and immunoprecipitation protocols would help test these hypothesis. Sedimentation assays combined

with chromatographic purification might also allow isolation of intact vesicle populations with bound Fu/Cos2 complexes.

The uncertainty surrounding the binding interactions of Fu, Cos2, Ci, and Su(fu) is another issue that needs to be resolved. As pointed out above, it is particularly important that the role of Su(fu) in binding the complex be worked out. If Su(fu) does bind the complex, then the binding domains of Su(fu) and its binding partner(s) need to be identified and then removed to determine how loss of Su(fu) binding affects Hh signaling. Ultimately, a crystal structure of the complex would provide the most definitive information on the interactions of the complex members.

In the long term, the most important question still to be answered is how does the Hh signal transit through the Ptc and Smo proteins to the Fu/Cos2/Ci complex? As mentioned above, the Fu/Cos2/Ci complex could be involved in vesicular trafficking through direct or indirect interactions with Ptc and/or Smo. Ptc or Smo may interact with Fu, Cos2, and/or Ci in a weak or transient fashion that is difficult to detect directly. In this case, more extensive yeast two hybrid analysis may be required to reveal interactions between these five proteins. Certainly, it is possible that there are as yet unidentified intermediary molecules that interact with both Ptc and Smo, or a Ptc/Smo containing vesicle and Fu/Cos2/Ci. Such molecules should be identifiable in yeast two hybrid screens or suppressor/enhancer screens of mutants of Fu, Cos2, or Ci.

A related avenue of investigation is the time delay between stimulation of cells by Hh and hyperphosphorylation of Fu and Cos2 (and the subsequent release of Fu/Cos2 from microtubules). The 15 to 30 minute delay between stimulation and hyperphosphorylation indicates that the signaling



step between Hh binding to its receptors and hyper-phosphorylation of Fu is quite slow (assuming Hh binding to its receptors is reasonably speedy). This delay could be explained by a need to endocytose Ptc/Smo containing vesicles or by the presence of some sort of feedback mechanism. Any such feedback mechanism, however, could not require new protein synthesis, as it has been shown that inhibition of protein synthesis does not affect Fu hyperphosphorylation (Therond et al., 1996). It is possible that this delay has evolved as a fidelity mechanism to ensure small pertubations in signaling to not change the all-important delineation of the compartment boundaries and subsequent development of the organizing center.

A closer examination of how Ci enters and/or exits the nucleus to affect transcription is certainly warrented. More convincing demonstrations (i.e. non-pharmacologic) of Ci's presence in the nucleus will be necessary before the mechanism of its shuttling in and out of the nucleus can be examined. Identification of the factors involved in this shuttling, their localization, and the strength and timing of their interactions with Ci are all topics that need to be examined (and almost certainly are being examined).

In summary, the investigation of the intracellular mechanisms of transduction of the Hh signal has already provided a wealth of data and insight into several novel mechanisms of signal transduction utilized by the Hh pathway. Future studies promise to further clarify these novel mechanisms and perhaps identify even more unique mechanisms of Hh signaling.

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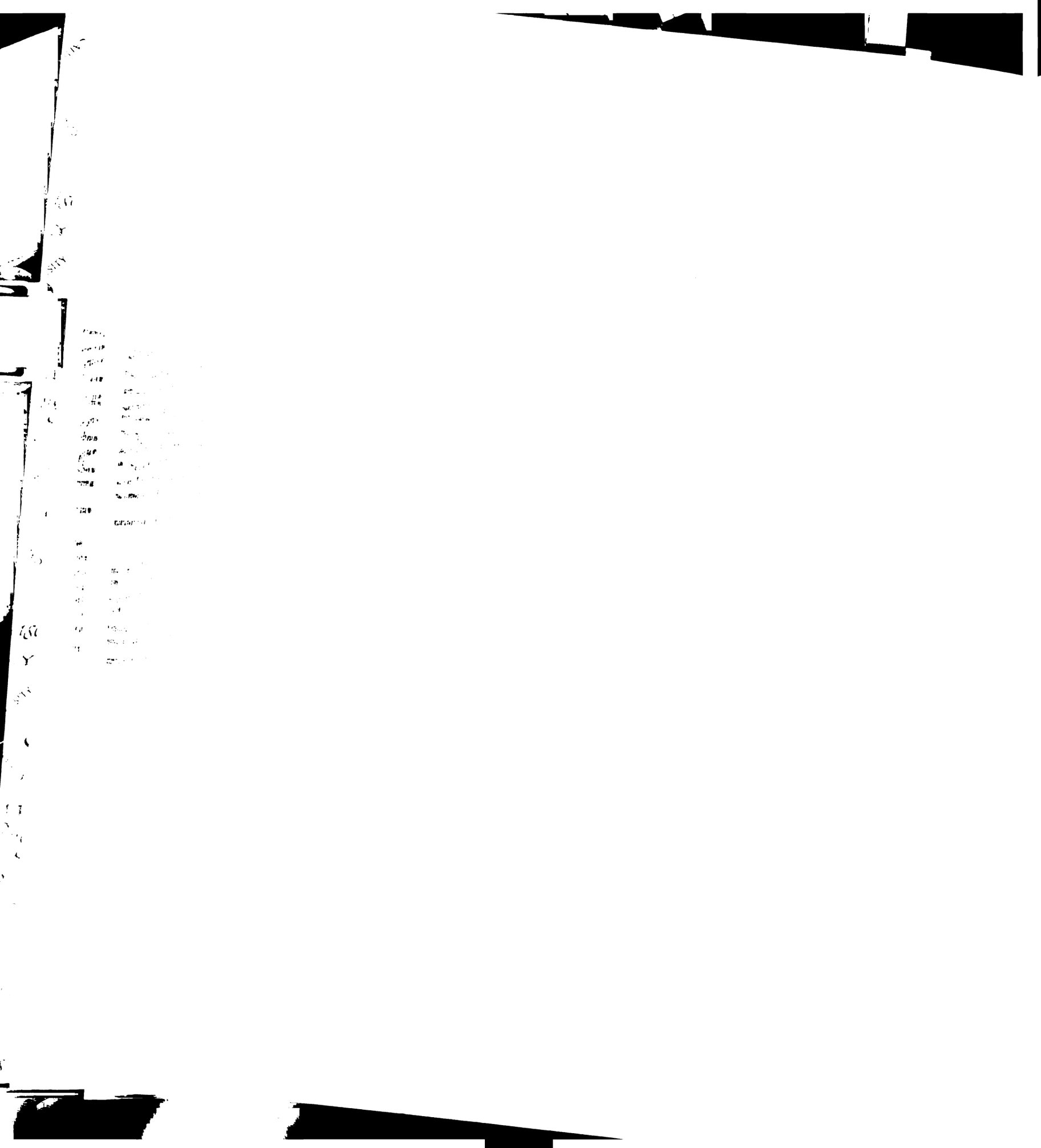

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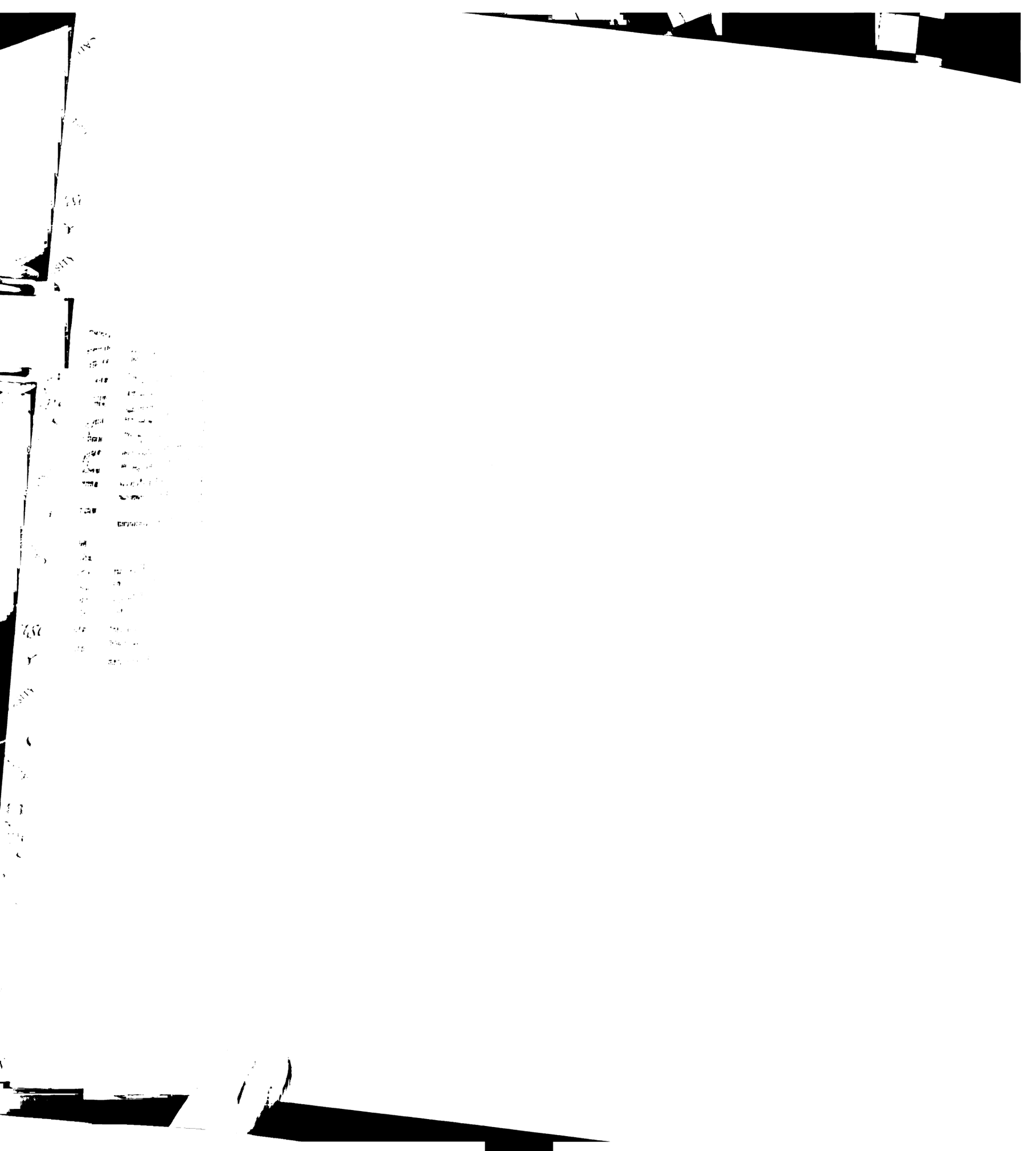

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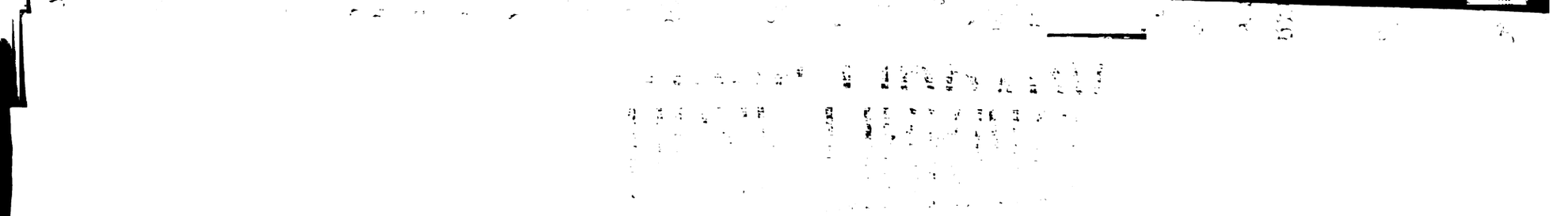

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