Casein kinase 2 associates with and phosphorylates Dishevelled

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The *dishevelled* (*dsh*) gene of *Drosophila melanogaster* encodes a phosphoprotein whose phosphorylation state is elevated by Wingless stimulation, suggesting that the phosphorylation of Dsh and the kinase(s) responsible for this phosphorylation are integral parts of the Wg signaling pathway. We found that immunoprecipitated Dsh protein from embryos and from cells in tissue culture is associated with a kinase activity that phosphorylates Dsh *in vitro*. Purification and peptide sequencing of a 38 kDa protein co-purifying with this kinase activity showed it to be identical to *Drosophila* Casein Kinase 2 (CK2). Tryptic phosphopeptide mapping indicates that identical peptides are phosphorylated by CK2 *in vitro* and *in vivo*, suggesting that CK2 is at least one of the kinases that phosphorylates Dsh. Overexpression of *Dfz2*, a Wingless receptor, also stimulated phosphorylation of Dsh, Dsh-associated kinase activity, and association of CK2 with Dsh, thus suggesting a role for CK2 in the transduction of the Wg signal.

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Introduction

The *Drosophila* segment polarity gene *wingless* (*wg*) is a member of the *Wnt* gene family, which encodes secreted glycoproteins with diverse functions in developmental processes (reviewed in Nusse and Varmus, 1992). The biochemical analysis of the signal transduction pathway of the Wingless protein (Wg) has made significant progress in recent years with the development of an *in vitro* assay for soluble Wg protein (van Leeuwen *et al.*, 1994) and the identification of *Drosophila frizzled 2* (*Dfz2*) as a receptor for Wg (Bhanot *et al.*, 1996).

Genetic analysis of *wg* has led to the identification of several components required for the transduction of the Wg signal. Among these signal transducing molecules are the protein products of the segment polarity genes *dishevelled* (*dsh*), *zeste-white3* (*zw3*) and *armadillo* (*arm*). Genetic epistasis experiments have ordered these genes into a linear pathway that has served as a guide for the biochemical dissection of the Wg signal transduction

pathway (reviewed in Nusse and Varmus, 1992; Klingensmith and Nusse, 1994; Perrimon, 1994). In this pathway, *wg* activates *dsh* which in turn inhibits *zw3*. Inhibited *zw3* no longer inhibits *arm*, so that the net effect of *wg* signaling is the activation of *arm*.

Dsh encodes a cytoplasmic protein (Dsh) with no known biochemical function and little homology to other proteins (Klingensmith *et al.*, 1994). In the mouse, three *dsh* genes (*Dvl-1*, 2 and 3) have been cloned by homology (Sussman *et al.*, 1994). Sequence comparison of *dsh* genes reveals regions of high homology in the amino-terminus and in the central domain while the carboxy-terminus is highly divergent (Klingensmith *et al.*, 1994). The central region of Dsh contains a domain referred to as *discs-large* homology region (Dhr) or PDZ (Klingensmith *et al.*, 1994; Ponting, 1995), which is found in several other proteins, such as PSD-95, ZO-1 and Discs-large (Ponting, 1995). Structural analysis demonstrated that a carboxy-terminal four residue motif (X-Thr/Ser-X-Val) binds to the PDZ domain of PSD-95 (Doyle *et al.*, 1996) and to the PDZ domain of the human homolog of the *Drosophila discs-large* tumor suppressor gene product, DlgA (Morais Cabral *et al.*, 1996). However, no ligand for the PDZ domain of Dsh has been identified yet. On the carboxyl side of the PDZ domain in Dsh is a recently described domain referred to as the DEP domain (Ponting, 1996). DEP domains are found in a variety of proteins, many of which participate in G-protein signaling. Directly amino terminal to the PDZ of Dsh is a conserved stretch of basic residues which in many other proteins serves as a nuclear localization signal. However, cell biological studies have shown that Dsh is localized predominately in the cytoplasm of the cell (Yanagawa *et al.*, 1995) and not in the nucleus. The functions of the PDZ, DEP and basic domains in Dsh still need to be elucidated.

Stimulation of the wing imaginal disc cell line Clone 8 (C18) with Wg-conditioned medium leads to the accumulation of Armadillo protein (Arm; van Leeuwen *et al.*, 1994) and the hyperphosphorylation of Dsh (Yanagawa *et al.*, 1995). Since genetic analysis of the *wg* gene suggests that the Wg signal activates the Dsh protein (reviewed in Nusse and Varmus, 1992; Perrimon, 1994), we hypothesized that the hyperphosphorylated form of Dsh is the active form of Dsh (Yanagawa *et al.*, 1995). In this model, Wg stimulates the phosphorylation of Dsh, and phosphorylated Dsh transduces the signal onto the next signaling component, directly or indirectly leading to the inhibition of Zeste-white 3.

In this paper, we show that a kinase activity is associated with Dsh protein in cultured cells and in embryos, and that this kinase phosphorylates Dsh *in vitro*. We purified this protein kinase and demonstrated it to be identical to Casein Kinase 2 (CK2). Tryptic phosphopeptide mapping strongly suggests that CK2 phosphorylates Dsh both

in vitro and *in vivo*. The phosphorylation of Dsh is regulated by expression of *Dfz2*, a gene encoding a Wg receptor (Bhanot *et al.*, 1996). These results demonstrate that CK2 phosphorylates Dsh in response to *Dsh* and *Dfz2* expression and suggest a role for CK2 in Wg signaling.

Results

Characterization of a Dsh-associated kinase

As demonstrated previously, treatment of the wing imaginal disc cell line Clone 8 with Wingless protein leads to the phosphorylation of the Dsh protein (Yanagawa *et al.*, 1995). Furthermore, this phosphorylation can be promoted by overexpression of the Dsh protein itself (Yanagawa *et al.*, 1995).

To identify a kinase responsible for the phosphorylation of Dsh, we asked whether a kinase activity was associated with Dsh. Schneider 2 (S2) cells were transfected with a *dsh* cDNA under the control of the metallothionein promoter (*S2dsh*). *Dsh* overexpression can be induced in *S2dsh* cells by the addition of copper ions (Cu^{2+}). Untransfected S2 cells display a single band of Dsh protein, while transfection of *dsh* into S2 cells leads to a lower mobility of the Dsh protein band (Figure 1A, top panel), an effect due to the phosphorylation of Dsh. Further overexpression of Dsh by incubating the cells in Cu^{2+} leads to an additional shift in mobility due to further phosphorylation of Dsh. As shown previously (Yanagawa *et al.*, 1995), overexpression of *dsh* also leads to the accumulation of the Armadillo (Arm) protein (Figure 1A, bottom panel).

When Dsh protein is immunoprecipitated from *S2dsh* cells induced to overexpress *dsh* ($+\text{Cu}^{2+}$) and the immune complex is subjected to an *in vitro* kinase reaction, a protein with the molecular weight of 70 kDa (the size of Dsh) becomes phosphorylated (Figure 1A, middle panel). *In vitro* kinase reactions on Dsh immunoprecipitates from parental S2 cells and uninduced *S2dsh* ($-\text{Cu}^{2+}$) cells do not display this predominant phosphoprotein. This kinase activity also co-immunoprecipitated with Dsh from embryo lysates (Figure 1A), and from cell lysates of the wing imaginal disc cell line, Clone 8, overexpressing the *dsh* gene tagged with a myc epitope (Figure 1B).

When a carboxy-terminally deleted Dsh protein, $\Delta\text{C-term}$ Dsh (Yanagawa *et al.*, 1995), is overexpressed and subjected to the *in vitro* kinase reaction described above, the detected phosphorylated protein is smaller (Figure 1B), corresponding to the reduction in molecular weight of $\Delta\text{C-term}$ Dsh relative to wild-type Dsh. This demonstrates that the phosphoprotein produced in the *in vitro* kinase reaction is Dsh itself.

The kinase activity present in Dsh immunoprecipitates may be due either to a Dsh-associated kinase or to an intrinsic kinase activity of Dsh. Since Dsh is not related to any protein kinases on the basis of amino acid sequence, it is likely that the kinase activity is associated with Dsh, rather than Dsh itself. To address this, we attempted to disrupt and reconstitute the kinase activity by changing the ionic strength of the buffer in the immunoprecipitation. When immunoprecipitated Dsh protein was washed with a high salt buffer and then subjected to an *in vitro* kinase reaction, much of the kinase activity was removed from the complex (Figure 2A, lane 2). The kinase activity on Dsh could be restored by diluting the high salt wash

Fig. 1. A Dsh specific kinase activity is associated with Dsh. (A) A kinase activity co-immunoprecipitates with Dsh. S2 and *S2dsh* cells were either treated (+) or not treated (–) with 0.5 mM CuSO_4 to modulate expression of the transfected *Dsh* gene. Equal amounts of total cell lysates from these cells and from embryos were immunoblotted with anti-Dsh (top panel) or anti-Arm antibody (bottom panel). Dsh protein was immunoprecipitated from equal amounts of total cell extracts of S2, *S2dsh* cells and embryos with anti-Dsh antibody, and the immune complexes were incubated with [$\gamma\text{-}^{32}\text{P}$]ATP for an *in vitro* kinase reaction (middle panel). A longer exposure of the anti-Arm blot reveals the presence of equal amounts of Arm protein in S2 and in uninduced *S2dsh* cells. (B) The kinase activity co-immunoprecipitating with Dsh phosphorylates Dsh. Dsh was immunoprecipitated from lysates of either Clone 8 *dsh-myc* or Clone 8 $\Delta\text{C-term dsh-myc}$ cell lysates with anti-Myc antibody (9E10) and the immune complexes (Dsh-myc and $\Delta\text{C-term}$ Dsh-myc) were used in *in vitro* kinase reactions. Cells were either treated (+) or not treated (–) with 0.5 mM CuSO_4 to modulate expression of the transfected genes.

to lower salt concentrations and adding it back to the immunoprecipitated Dsh protein (Figure 2A, lane 3). Thus, the protein kinase activity can be specifically eluted from Dsh and reconstituted on Dsh.

To determine which domain(s) of Dsh are phosphorylated, a series of deletions in Dsh were constructed. Bacterially expressed fusion proteins between glutathione *S*-transferase (GST) and various fragments of Dsh were purified, incubated with the eluted Dsh-specific kinase activity, and subjected to an *in vitro* kinase reaction. Full-length Dsh fused to GST (Full) was efficiently phosphorylated (Figure 2B). GST fusions with either the amino- or the carboxy-terminus of Dsh (G1 and G12) or GST alone failed to become significantly phosphorylated while GST fusions with the central domain of Dsh (G4, G5, G7) were efficiently phosphorylated. Thus, the central domain,

Fig. 2. Dsh kinase is associated with Dsh and phosphorylates GST-Dsh *in vitro*. (A) The Dsh-associated kinase can be disrupted from Dsh by an increase in ionic strength and can be reconstituted on Dsh. Shown are *in vitro* kinase reactions on Dsh immunoprecipitations from Clone 8 *dsh-myc* cells induced for Dsh overexpression. Prior to the *in vitro* kinase reaction the Dsh immunoprecipitations were treated as follows. Lane 1: wash with physiological salt concentrations (150 mM NaCl). Lane 2: wash with high salt concentrations (1 M NaCl). Lane 3: wash with high salt concentrations (1 M NaCl) and then incubation with the high salt wash of a Dsh immune complex that was diluted to 150 mM NaCl. (B) The central domain of Dsh associates with the kinase and is phosphorylated by the kinase. The high salt wash that removes the kinase activity from Dsh (panel A, lane 2) was diluted to 150 mM NaCl and added to GST-Dsh fusion proteins bound to glutathione-Sepharose beads. After incubation to allow association between kinase and GST-Dsh, the beads were washed and used in an *in vitro* kinase reaction; the products of the kinase reactions were resolved by SDS-PAGE. The slowly migrating bands represent full-length proteins while the lower bands are breakdown products. The arrow indicates the position of GST alone. (C) Summary of the data in (B).

which includes the basic domain and the PDZ domain, contains the region required for the association with the Dsh-specific kinase and most, if not all, of the phosphorylation sites on Dsh (summarized in Figure 2C).

Identification of the Dsh-associated kinase as CK2

We used the specific binding of the kinase to Dsh as a first step in the purification of this enzyme. A Triton X-100 soluble protein extract from *S2dsh* cells was applied to an anti-Dsh antibody affinity column; the column was then extensively washed with lysis buffer prior to the application of a linear salt gradient. Individual fractions from this column were assayed for kinase activity towards a GST-Dsh fusion protein (G6, a more highly expressed variant of G5 with an additional 22 amino acids at the amino-terminus). At a salt concentration of 400 mM, a Dsh kinase activity eluted from the affinity column (Figure

Fig. 3. Dsh kinase is CK2. (A) Purification of Dsh kinase. A total cell lysate from *S2dsh* cells was passed over an anti-Dsh antibody affinity column. After extensive washing, a linear NaCl gradient (150 mM to 1 M) was applied, individual fractions were assayed for their kinase activity on a GST-Dsh (G6), and products were resolved on an SDS-polyacrylamide gel that was subsequently silver-stained. The arrows mark the position of the bands co-purifying with the kinase activity. (B) The 38 kDa protein co-purifying with the Dsh Kinase activity is CK2α. The 38 kDa protein band co-purifying with the Dsh kinase activity was excised from the gel and digested with trypsin. Individual peptides were purified and one peptide (peak 88) was sequenced. The peptide sequence of this peptide is shown and lined up with the corresponding sequence of CK2α. (C) CK2 is associated with overexpressed Dsh. Dsh protein was immunoprecipitated from equal amounts of total protein of *S2*, *S2dsh* cells and embryos, and the co-immunoprecipitating proteins were immunoblotted with anti-CK2 antibody. This antibody recognizes both the α and β subunits of CK2. Cells were either treated (+) or not treated (−) with 0.5 mM CuSO_4 to modulate expression of the transfected *dsh* gene.

3A, middle panel). Fractions were also separated by SDS-PAGE, and the gel was silver-stained. The kinase active fractions contained two bands with apparent molecular weight of 38 and 28 kDa (Figure 3A, bottom panel).

Kinase active fractions were combined and re-fractionated on an anion exchange column. Fractions containing the kinase activity again contained the 38 and 28 kDa proteins (data not shown), suggesting that these two proteins comprised the Dsh-associated kinase. Very few other proteins were visible in the kinase active fractions on a silver-stained gel (data not shown), implying that the material from the anti-Dsh affinity and anion exchange column was relatively pure.

The predominant bands of 38 and 28 kDa were isolated and subjected to peptide sequencing. Briefly, ~1.5 μg of the 38 kDa protein was digested with trypsin, and tryptic

peptides were purified and subjected to automated peptide sequencing. One tryptic peptide (peak 88) was sequenced, and the amino acid sequence revealed a perfect match with the α subunit of *Drosophila melanogaster* CK2 (Figure 3B; Saxema *et al.*, 1987).

To confirm that the Dsh-associated kinase is indeed CK2, an antibody raised to the α and β subunits of *D. melanogaster* CK2 (kindly provided by C.V.C. Glover; described in Dahmus *et al.*, 1984) was used in Western blotting. Cell extracts from embryos, Clone 8 and S2 cells contained the α and β subunits of CK2 (data not shown). An anti-Dsh immunoprecipitate from S2 cells contained very low levels of CK2. In contrast, Dsh immunoprecipitates from lysates of S2dsh cells induced to overexpress the transfected *dsh* gene contained significantly higher levels of CK2 (Figure 3C). Furthermore, CK2 co-immunoprecipitated with Dsh in lysates prepared from an overnight collection of embryos (Figure 3C), demonstrating that CK2 is associated with Dsh in the *Drosophila* embryo.

Phosphoamino acid analysis of *in vivo* and *in vitro* phosphorylated Dsh

To determine the nature of the individual phosphoamino acids in Dsh, we performed a phosphoamino acid analysis. Consistent with CK2 being a serine/threonine kinase, phosphoamino acid analysis of hydrolyzed Dsh protein demonstrates that most of the phosphorylation occurs on serine, some on threonine, and none on tyrosine (Figure 4). *In vitro* labeled Dsh contains more phospho-threonine than *in vivo* labeled Dsh.

Dsh is phosphorylated on similar sites *in vitro* and *in vivo*

In vitro, kinases are known to phosphorylate several substrates promiscuously. We used two dimensional maps of tryptic phosphopeptide fragments of the substrate to assess whether similar sites on Dsh are phosphorylated *in vivo* as *in vitro*, as a first estimate of whether the same enzyme phosphorylates Dsh *in vivo* and *in vitro*.

As shown in Figure 5, the tryptic maps of *in vitro* and *in vivo* phosphorylated Dsh are very similar, with three reproducible phosphotryptic products (spots A, B and C) common between *in vivo* and *in vitro* labeled Dsh. When tryptic peptides from *in vivo* and *in vitro* labeled Dsh are mixed and resolved on TLC cellulose plates, the same number of spots (A, B and C) are observed. This demonstrates that identical tryptic peptides are phosphorylated *in vivo* as *in vitro* and suggests that similar sites are being phosphorylated. Furthermore, *in vivo* labeled Dsh from S2dsh and S2Dfz2 overexpressing cells (see below) both produced similar tryptic phosphopeptide maps (data not shown), suggesting that the same kinase is responsible for the phosphorylation of Dsh in these two cell lines.

CK2 is associated with Dsh in cells overexpressing Dfz2

Recently, it has been demonstrated that Dfz2 protein acts as a receptor for Wg (Bhanot *et al.*, 1996). To determine whether overexpression of Dfz2 has an effect on the phosphorylation state of the Dsh protein and on the Dsh-CK2 complex, S2 cells were transfected with a Dfz2 cDNA under the control of the metallothionein promoter (S2Dfz2, described in Bhanot *et al.*, 1996). The Dfz2

Fig. 4. Phosphoamino acid analysis of *in vivo* and *in vitro* labeled Dsh protein reveals that Dsh is phosphorylated on serine and threonine residues. Tryptic digestion products of *in vivo* and *in vitro* labeled Dsh were acid hydrolyzed to produce individual amino acids and then resolved in two dimensions on TLC cellulose plates. To mark the position of individual phosphoamino acids, 2 mg of each phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr) were included in the electrophoresis. The position of the marker phosphoamino acids was visualized by staining with ninhydrin. The diagram illustrates the direction and conditions of the two-dimensional electrophoresis.

transgene can be induced to high levels by addition of Cu^{2+} to the medium.

In parental S2 cells, which do not express Dfz2 protein (Bhanot *et al.*, 1996), Dsh migrates as a single band of 70 kDa (Figures 1A and 6, first panel). Uninduced S2Dfz2 cells contain low, but detectable, levels of Dfz2 protein and display a series of Dsh bands that migrate more slowly than Dsh in S2 cells. Addition of Cu^{2+} to the medium induced high levels of Dfz2 protein (Figure 6, second panel) and converted the series of Dsh bands to one main band of protein with an apparent molecular weight of 80 kDa. Treatment of cell lysates of S2Dfz2 cells with potato acid phosphatase converts the slowly migrating Dsh bands to a single band with the same mobility as the Dsh protein in S2 cells (data not shown), demonstrating that the increase in molecular weight is the result of phosphorylation. In contrast to S2 cells, Clone 8 cells express Dfz2, and Dsh migrates not as a single band but as a series of differently phosphorylated forms (data not shown). This observation further supports our finding that Dfz2 expression regulates the phosphorylation state of Dsh. Dfz-2 overexpression had no effect on the expres-

Fig. 5. Phosphotryptic maps of Dsh labeled *in vivo* and *in vitro* are identical. *In vivo* and *in vitro* labeled Dsh protein was resolved by SDS-PAGE and transferred to nitrocellulose. The Dsh bands were then digested with trypsin and proteolytic fragments were resolved in two dimensions on TLC cellulose plates. The diagram represents the direction of electrophoresis and chromatography. ϵ -DNP-lysine and phosphotyramine (p-Tyn) were used as markers.

sion of *dsh* as determined by Northern blotting (data not shown).

Unlike overexpression of *dsh*, which leads to the accumulation of Arm protein (Figure 1A), overexpression of *Dfz2* does not (Figure 6, third panel). Thus, the phosphorylation of Dsh stimulated by *Dfz2* overexpression is not sufficient for the transduction of the signal to Arm. Overexpression of *Dfz2* also leads to an increase in the amount of CK2 associated with Dsh (Figure 6, fourth panel) and to an increase in kinase activity associated with immunoprecipitated Dsh protein (Figure 6, fifth panel). These results demonstrate that CK2 associates with endogenous Dsh protein when the phosphorylation of Dsh protein is elevated by the overexpression of *Dfz2*, a receptor for the Wingless protein.

Discussion

In this paper we show that CK2 associates with Dishevelled (Dsh) and phosphorylates Dsh *in vitro*. This association between CK2 and Dsh occurs in the embryo and is greatly promoted when *dsh* or *Dfz2*, which encodes a receptor for Wingless (Wg), are overexpressed. Furthermore, phosphoamino acid analysis and tryptic phosphopeptide maps suggest that CK2 phosphorylates Dsh *in vivo*.

CK2 is a highly conserved protein kinase with homologs found in every tested organism from yeast to humans. In yeast, the CK2 α gene is essential for viability (Bidwai *et al.*, 1992), and the high degree of conservation suggests an essential role in all organisms. No mutations have been identified for *Drosophila* CK2 α and β (Saxema *et al.*,

Fig. 6. CK2 is associated with phosphorylated Dsh in cells overexpressing *Dfz2*. S2 and *S2Dfz2* cells were either treated (+) or not treated (–) with CuSO_4 to modulate expression of the transfected genes. Equal amounts of total cell lysates were immunoblotted with anti-Dsh antibody (first panel), anti-Dfz2 antibody (second panel) or anti-Arm antibody (third panel). Dsh immunoprecipitates from equal amounts of protein of S2 and *S2Dfz2* cell lysates were immunoblotted with anti-CK2 antibody, which recognizes both the 38 kDa α -subunit and the 28 kDa β -subunit of CK2 (fourth panel), or incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for an *in vitro* kinase reaction (fifth panel).

1987) which map to the chromosomal locations 80A and 10E, respectively (Lindsley and Zimm, 1992).

CK2 has been studied extensively at the biochemical level but very little is known about its functions *in vivo* (reviewed in Issinger, 1993; Allende and Allende, 1995). The kinase activity of CK2 appears to be constitutively active, and no significant regulation by external stimuli has been reported. Our finding that expression of a receptor for Wingless, *Dfz2*, controls the association of CK2 with Dsh and the phosphorylation of Dsh, provides one of the first examples of regulation of CK2 association with a substrate. A vast number of *in vivo* substrates have been identified for CK2 (reviewed in Marshak and Carroll, 1991), and, as is the case with Dsh, the function of these phosphorylation events has not been elucidated. It has, however, been demonstrated that phosphorylation by CK2 leads to the breakdown of I κ B (McElhinny *et al.*, 1996).

The optimal motif for CK2 phosphorylation is a serine or threonine residue in an acidic context with a minimal requirement of one acidic residue at the +3 position (Marshak and Carroll, 1991; Rihs *et al.*, 1991; Songyang *et al.*, 1996). There are two serines and two threonines that fit this consensus within the minimal Dsh fragment (G5, see Figure 2) that is efficiently phosphorylated by CK2 *in vitro*. Site-directed mutagenesis of these sites is in progress.

We show here that CK2 associates with Dsh and phosphorylates it, but we have not determined which of these events occurs first in the cell. Unphosphorylated Dsh clearly has some affinity for CK2 since a bacterially produced GST-Dsh fusion protein (which is most likely

not phosphorylated to a significant level) can associate with CK2 *in vitro*. However, *in vivo* phosphorylated Dsh (either by *dsh* or *Dfz2* overexpression) is associated with more CK2 than is underphosphorylated Dsh. This suggests a model in which CK2 can bind to underphosphorylated Dsh with low affinity and phosphorylate it. The phosphorylated Dsh then has a higher affinity for CK2 leading to an increase in the amount of Dsh–CK2 complex. This type of interaction may be similar to the interaction of an SH2 or PTB domain with a phosphotyrosine (reviewed in Pawson, 1995) or the interaction between 14-3-3 proteins and phosphoserine (Muslin *et al.*, 1996). Whether phosphorylation of Dsh increases the binding of CK2 to Dsh still remains to be shown. Phosphorylation of Dsh may also induce a conformational change that results in a stronger association between Dsh and CK2. Alternatively, overexpression of *dsh* or *Dfz2* may promote the association of CK2 with Dsh, leading to an increase in Dsh phosphorylation.

In a recent paper, we demonstrated that Wingless signaling leads to the increased phosphorylation of Dsh, and that this phosphorylation of Dsh correlated with the accumulation of the Arm protein (Yanagawa *et al.*, 1995). However, we now find that overexpression of a receptor for Wg, *Dfz2*, leads to the phosphorylation of Dsh but fails to induce elevated levels of Arm protein. Thus, Dsh phosphorylation alone is not sufficient for the transduction of the Wg signal to Arm. It remains possible, however, that the phosphorylation of Dsh by CK2 and by perhaps other unidentified kinases is required but not sufficient for the transduction of the Wg signal. In this model, Dsh is phosphorylated on multiple sites, some of which are regulated by CK2. Wg signaling would lead to yet other, presently unidentified, changes in Dsh that allow the signal to be transduced to downstream effectors. In this respect, it is noteworthy that the two Wg responsive cell lines, Clone 8 and S2 cells overexpressing *Dfz2*, contain phosphorylated Dsh, while the Wg non-responsive S2 cells contain unphosphorylated Dsh. Alternatively, the phosphorylation of Dsh is not required for the transduction of a Wnt signal but is only a consequence of the signal. Such phosphorylation events may be involved in down-regulating the Wg signal.

Clearly, the function of the phosphorylation of Dsh by CK2 is not yet established, and further experiments, such as site-directed mutagenesis of potential phosphorylation sites and assessment of the activity of such mutant alleles in embryos, are necessary.

Materials and methods

Cell culture

Schneider 2 (S2) cells and S2 cells overexpressing *dsh* or *Dfz2* under the control of the metallothionein promoter (*S2dsh* and *S2Dfz2*, respectively) were grown in Schneider's *Drosophila* medium (Gibco BRL) supplemented with 12.5% fetal bovine serum (Sigma), 50 units/ml penicillin and 50 µg/ml streptomycin (Gibco BRL) at 25°C. The wing imaginal disc cell line, Clone 8 (Cl8), was grown in Shields and Sang M3 insect medium (Sigma) supplemented with 2% FBS, 2.5% fly extract, 5 µg/ml insulin and penicillin and streptomycin. *S2dsh*, *Cl8Dsh-myc* and *Cl8ΔC-term Dsh-myc* cells are described elsewhere (Yanagawa *et al.*, 1995; ΔC-term Dsh-myc is referred to as D1). The generation of the cell line *S2Dfz2* is described in Bhanot *et al.* (1996). These cells were induced to overexpress the transfected genes by adding 0.5 mM CuSO₄ for 4–16 h.

Preparation of cell lysates

For the purification of CK2/Dsh-associated kinase ~2.5×10¹⁰ *S2dsh* cells (6 l of dense culture medium) were pelleted at 1000 g and washed three times with cold phosphate buffered saline (PBS). The cell pellet was then resuspended in 130 ml ice-cold lysis buffer (1% Triton X-100, 50 mM Tris–HCl pH 8.0, 150 mM NaCl) containing protease inhibitors [1 mM Pefabloc® SC (Boehringer Mannheim), 1 mM PMSF, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin (Sigma)] and 0.2 µM phosphatase inhibitor microcystin-LR (Gibco BRL) and incubated on ice for 20 min. The cell lysate was pre-cleared by spinning at 1000 g for 10 min at 4°C. The supernatant was centrifuged at 100 000 g for 1 h at 4°C. The protein concentration was determined by the method of Bradford (Bio-Rad Protein Assay). The lysate from an overnight collection of embryos was obtained by douncing on ice dechorionated embryos in lysis buffer containing protease inhibitors in a tight fitting dounce. The lysate was then centrifuged at 20 800 g for 10 min to remove insoluble cell debris.

Generation of GST–Dsh fusion proteins

The fusion constructs between GST and fragments of Dsh, Full, G4, G5 and G7 were constructed from pBluescriptIIKS⁺*dsh* by S. Yanagawa as follows:

Full (amino acids 7–623): PCR using the primers:

5' primer: TAGGATCCGGCGGGCAGGAGACGAAGGTGT.

3' primer: CCGAATTCCAATACGTAATTAGATACGGA.

The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of pGEX-2TK.

G7 (amino acids 174–372): PCR using the primers:

5' primer: TAGGATCCCCGCCACCGCTCACATATCAA.

3' primer: CCGAATTCTGTGAGGGCCTGAGTGTGCGC.

The PCR product was cloned into pGEX-2TK as for Full.

G4 (amino acids 174–494): PCR using the primers:

5' primer: same as for G7.

3' primer: same as for Full.

The PCR product was digested with *Bam*HI and *Pvu*II and cloned into the *Bam*HI and *Sma*I sites of pGEX-2TK.

G5 (amino acids 174–394): The PCR product used for G4 was digested with *Xho*I, blunted, digested with *Bam*HI and cloned into *Bam*HI and *Sma*I sites of pGEX-2TK.

G6 (amino acids 152–394): pBluescriptIIKS⁺*dshmyc* was digested with *Pvu*II and *Xho*I to generate a 727 bp fragment which was cloned into the *Sma*I and *Xho*I sites of pGEX4T-2.

G12 (amino acids 394–623): pBluescriptIIKS⁺*dsh* was digested with *Xho*I and *Nor*I to generate a 1216 bp fragment which was cloned into the *Xho*I and *Nor*I sites of pGEX4T-1.

GST fusion proteins were produced and purified as described by the vendor Pharmacia.

Antibodies

For immunoprecipitation of Dsh, a rabbit antibody (anti-Dsh) raised to the carboxy-terminus was obtained (see below). For immunoblotting, either anti-Dsh or a rat anti-Dsh antibody raised to the amino-terminus of Dsh (Yanagawa *et al.*, 1995) was used. Dsh-myc or ΔC-term Dsh-myc was immunoprecipitated with anti-myc antibody (9E10). Dfz2 was detected by immunoblotting with an anti-Dfz2 antibody raised to the amino-terminus of Dfz2 (Bhanot *et al.*, 1996). CK2 and Arm were detected with anti-CK2 (Dahmus *et al.*, 1984) and anti-Arm (7A1, Peifer, 1993) antibodies, respectively.

Preparation of anti-Dsh affinity column

The anti-Dsh antiserum, anti-Dsh, was obtained by immunizing rabbits with the bacterially produced fusion protein G12. The antiserum was affinity purified against the GST fusion protein G12. Affinity purified anti-Dsh antibody was covalently cross-linked to protein A–Sepharose with Dimethylpimelimidate using the ImmunoPure® protein A–IgG Orientation Kit (Pierce). The resulting protein A–Sepharose/anti-Dsh matrix was then used to pour a column (total bed volume ~5 ml) which was used for affinity purification of Dsh and its associated kinase.

Affinity purification of Dsh kinase

Lysis of 2.5×10¹⁰ cells yielded ~3 g of Triton X-100 soluble protein as determined by the method of Bradford (Bio-Rad Protein Assay). The

following steps were all carried out at 4°C. 1.5 g of the lysate was preincubated with Sepharose CL-4B (Sigma), and then applied to the anti-Dsh affinity column at a flow rate of 0.075 ml/min using the LKB Pump P-1 (Pharmacia). The column was washed extensively with lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 8, 150 mM NaCl) at a flow rate of 1 ml/min. A 15 ml 150–1000 mM NaCl gradient in 1% Triton X-100 and 50 mM Tris-HCl, pH 8 was applied onto the column at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected and later assayed for kinase activity and for protein content by silver-stained gels. After this first run, the column was stripped with 6.4 M urea, 1 M NaCl and regenerated by washing with lysis buffer. The second half of the lysate (1.5 g) was applied, washed and eluted as above.

Anion exchange chromatography of Dsh kinase

The kinase active fractions from the two runs of the anti-Dsh affinity column were pooled and the NaCl concentration was diluted to 150 mM. The pooled fractions were applied onto a HiTrap™Q (Pharmacia) anion exchange column at a flow rate of 0.5 ml/min. The column was washed with 10 ml lysis buffer, and then with 20 ml 20 mM Triethanolamine, pH 7.3, 150 mM NaCl. A 15 ml 150 mM to 1000 mM NaCl gradient in 20 mM Triethanolamine, pH 7.3 was applied onto the column at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected and later assayed for kinase activity (see below) and for protein content by silver-stained gels (as described in Giulian *et al.*, 1983).

TCA precipitation and gel electrophoresis of Dsh kinase

The kinase active fractions from the anion exchange column were pooled and precipitated by adding an equal volume of 24% trichloroacetic acid (TCA) and incubating on ice for 30 min. The precipitate was pelleted, washed with acetone, air dried, resuspended in 40 µl protein loading dye (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.73 M β-mercaptoethanol), boiled and run on an SDS 12.5% polyacrylamide gel. The gel was stained with 0.1% Coomassie, 40% methanol, 10% acetic acid for 30 min and then destained for 4 h with several changes of 50% methanol, 10% acetic acid. The bands of 38 and 28 kDa were cut out, frozen and sent to the Keck Foundation at Yale University for protein digestion and sequencing. Briefly, ~1.5 µg of the 38 kDa protein was eluted from the gel and digested with trypsin. Tryptic peptides were purified and subjected to automated peptide sequencing.

Kinase assays

For the *in vitro* kinase reactions in Figures 1, 2A and 6, the anti-Dsh immune complex was washed four times with lysis buffer and twice with distilled water and then incubated with 30 µl kinase buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 µM ATP, 1 mM DTT and 7 µCi [γ -³²P]ATP) for 1 h at room temperature.

In Figure 2B, the eluted kinase activity was incubated with various purified GST fusion proteins immobilized on glutathione-Sepharose 4B (Pharmacia). The kinase-GST-Dsh-Sepharose complexes were washed four times with lysis buffer, twice with distilled water and then incubated with kinase buffer as above. Kinase reactions were stopped by adding protein gel loading dye and boiling for 5 min. The products of the kinase reactions were resolved by SDS-PAGE.

To assay for kinase activity in the fractions from the anti-Dsh affinity column and from the anion exchange column, 1 µl (1/500th) of each fraction was incubated with 10 µl Sepharose-G6 beads (a 1:1 slurry containing ~1 µg of fusion protein) in 0.5 ml lysis buffer for 1 h at 4°C to allow for association between the kinase and its substrate. The Sepharose-G6 beads were washed with 1 ml distilled water. 20 µl of kinase buffer was added to the Sepharose-G6 pellet and incubated at 25°C for 1 h. Protein gel loading dye was added, samples were boiled and resolved by SDS-PAGE. The gel was stained and fixed in 0.002% Coomassie, 20% methanol, 7% acetic acid for 30 min and then dried. Labeled protein bands were visualized by autoradiography.

Immunoblotting and immunoprecipitations

Triton X-100 soluble proteins were resolved by SDS-PAGE and electroblotted to nitrocellulose filter (Schleicher and Schuell) in 25 mM Tris, 0.192 M glycine and 20% methanol for 1.5 h in the cold room at 500 mA. Filters were blocked in 3% non-fat dry milk (Carnation), 1% bovine serum albumin (BSA) in Tris buffered saline (TBS: 20 mM Tris-HCl pH 8, 150 mM NaCl) and 0.2% Tween-20 (TBST). Rat anti-Dsh, affinity purified anti-Dsh, anti-CK2 (kindly provided by C.V.C. Glover), anti-Arm and anti-Dfz2 were added to the filters at dilutions of 1:2000, 1:2000, 1:6000, 1:1000 and 1:5000, respectively, and incubated overnight at 4°C. The filters were washed in TBST, incubated with secondary antibody conjugated to horse radish peroxidase (HRP, Bio-Rad). After

washing the filters, the chemiluminescent reagent (ECL, Amersham) was added.

For immunoprecipitations, 5 µl of anti-Dsh, 20 µl anti-Dsh coupled to protein A-Sepharose (see above under 'Preparation of anti-Dsh affinity column'), or 20 µl anti-Myc (9E10) were added to 1–5 mg total cell lysate and incubated overnight at 4°C. To precipitate the antibodies, 20 µl of protein A-Sepharose (PAS, 1:1 slurry) was added and incubated for 1 h at 4°C. The immune complexes were washed four times with lysis buffer and then boiled in sample dye prior to SDS-PAGE. Western blotting of the Dsh immunoprecipitate with anti-CK2 antibody (Figure 4) produced high levels of background signal due to the reactivity of the secondary goat anti-rabbit antibody with the rabbit anti-Dsh antibody chains on the nitrocellulose blot. To circumvent this problem, the Dsh immune complex was first washed extensively with lysis buffer containing a physiological NaCl concentration (150 mM). Associated proteins like CK2 were eluted from the immune complex by washing it five times with 150 µl lysis buffer containing 1 M NaCl; this high salt eluate was TCA precipitated by adding an equal volume of 24% TCA. Precipitated protein was boiled in sample dye prior to SDS-PAGE.

The amount of CK2 associated with phosphorylated Dsh in S2Dfz2 cells was estimated as follows: the signal strength of an anti-CK2 immunoblot on 50 µg total cell extract was equivalent to the signal strength of a CK2 immunoblot on a Dsh immunoprecipitate from 16 mg total cell extract. Since all the Dsh was immunodepleted from the cell extract, we can calculate the percentage of CK2 associated with Dsh: $50/16000 \times 100 = 0.31\%$.

In vivo and in vitro labeled Dsh protein for phosphoamino acid analysis and tryptic phosphopeptide mapping

In vitro labeled Dsh protein was obtained as described under 'Kinase assays.' *In vivo* labeled Dsh was obtained as follows: a confluent T-75 flask of C18dsh-myc cells was starved in phosphate-free M3 medium (Kemp Biotechnologies, Inc.) in the presence of 0.5 mM CuSO₄ for 1 h. The medium was removed and replaced with 5 ml phosphate free M3 medium containing 0.5 mM CuSO₄ and 5 mCi [³²P]orthophosphate (Amersham) and incubated for 5 h to overnight at 25°C. The cells were washed and lysed as described above. The Dsh protein was immunoprecipitated from whole cell extracts with α-myc antibody (9E10). Protein gel loading dye was added to the washed immune complex prior to SDS-PAGE and electroblotting to nitrocellulose. Immobilized and labeled Dsh protein (*in vitro* and *in vivo*) was visualized by autoradiography and excised from the filter. Digestions of the Dsh protein, phosphoamino acid analysis and tryptic phosphopeptide mapping were performed as described (Boyle *et al.*, 1991; Luo *et al.*, 1991).

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