Casein kinase 2 associates with and phosphorylates Dishevelled

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Introduction

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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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 predominantly 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 (Cl8) 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 (Cu²⁺). 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 Cu²⁺ 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 (+Cu²⁺) 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 (–Cu²⁺) cells do not display this predominant phosphoprotein. This kinase activity also co-immunoprecipitated with Dsh from embryonic 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, ΔC-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 ΔC-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 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,
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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 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.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 vitro 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 transgene can be induced to high levels by addition of Cu²⁺ 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 Cu²⁺ to the medium induced high levels of Dfz2 protein (Figure 6, second panel) and converted the series of Dsh bands to two main bands 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. Dfz2 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 phosphorylamine (p-Tyn) were used as markers.

Fig. 6. CK2 is associated with phosphorylated Dsh in cells overexpressing Dfz2. S2 and S2Dfz2 cells were either treated (+) or not treated (−) with CuSO4 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 [γ-32P]ATP for an in vitro kinase reaction (fifth panel).

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 CK2α 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 CK2α and β (Saxema et al., 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 IκB (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 metallothionine 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 (C8), was grown in Shields and Sang M3 insect medium (Sigma) supplemented with 2% FBS, 2.5% By extract, 5 μg/ml insulin and penicillin and streptomycin. S2dsh, Cl8Dsh-MyC and Cl8Δterm 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 ~2.5×10^10 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 10 000 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 pBlueScriptIKS‘‘dsh by S.Yanagawa as follows:

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

S1 primer: TAGGATCCGGGCGGAGAGAAGTTGT.
3′ primer: CCGGATCCATACCTGGATTAGCGA.

3′ primer: TTTAGATCTGAAATATTAGTATACGG.

The PCR product was digested with BamHI and EcoRI and ligated into the BamHI and EcoRI sites of pGEX-2TK.

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

S1 primer: TAGGATCCCGGGCGGAGAGAAGTTGT.
3′ primer: CCGGATCCATACCTGGATTAGCGA.

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

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

S1 primer: same as for G7.
3′ primer: same as for Full.

The PCR product was digested with BamHI and Pwal and cloned into the BamHI and Smal sites of pGEX-2TK.

G5 (amino acids 174–394): The PCR product used for G4 was digested with Xhol, blunted, digested with BamHI and cloned into BamHI and Smal sites of pGEX-2TK.

G6 (amino acids 152–394): pBlueScriptIKS‘‘dshmyc was digested with Pwal and Xhol to generate a 727 bp fragment which was cloned into the Smal and Xhol sites of pGEX4T-2.

G12 (amino acids 394–623): pBlueScriptIKS‘‘dshmyc was digested with Xhol and NotI to generate a 1216 bp fragment which was cloned into the Xhol and NotI sites of pGEX4T-2.

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 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., 1994) and anti-Arm (7A1, Peifer, 1993) antibodies, respectively.

Preparation of anti-Dsh affinity column

The anti-Dsh antisera, anti-Dsh, was obtained by immunizing rabbits with the bacterially produced fusion protein G12. The antisera 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

Lysates of 2.5×10^10 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 0.5 ml/min. 15 ml 150–1000 mM NaCl gradient in 20 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 M urea, 1 M NaCl and regenerated by washing with lysis buffer. The second half of the lysate (1.5 g) was applied 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 HiTranTMQ (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 Trithionanamine, pH 7.3, 150 mM NaCl. A 15 ml 150 mM to 1000 mM NaCl gradient in 20 mM Trithionanamine, 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. Peptide 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 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 0.1% 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 (Schleicher and Schuell) in 25 mM Trizma, 0.192 M glycerine 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.Glover), anti-Arm and anti-Drif2 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 S2/Dfz2 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: 50/16000×100 = 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 S2/Dsh–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 mM [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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