Casein kinase I (CKI) was recently reported as a positive regulator of Wnt signaling in vertebrates and Caenorhabditis elegans. To elucidate the function of Drosophila CKI in the wingless (Wg) pathway, we have disrupted its function by double-stranded RNA-mediated interference (RNAi). While previous findings were mainly based on CKI overexpression, this is the first convincing loss-of-function analysis of CKI. Surprisingly, CKI\alpha- or CKI\epsilon-RNAi markedly elevated the Armadillo (Arm) protein levels in Drosophila Schneider S2R+ cells, without affecting its mRNA levels. Pulse–chase analysis showed that CKI-RNAi stabilizes Arm protein. Moreover, Drosophila embryos injected with CKI\alpha double-stranded RNA showed a naked cuticle phenotype, which is associated with activation of Wg signaling. These results indicate that CKI functions as a negative regulator of Wg/Arm signaling. Overexpression of CKI\alpha induced hyperphosphorylation of both Arm and Dishevelled in S2R+ cells and, conversely, CKI\alpha-RNAi reduced the amount of hyper-modified forms. His-tagged Arm was phosphorylated by CKI\alpha in vitro on a set of serine and threonine residues that are also phosphorylated by Zeste-white 3. Thus, we propose that CKI phosphorylates Arm and stimulates its degradation.

**Keywords:** Armadillo/casein kinase I/proteasome/RNAi/Wnt

---

**Introduction**

Wnt signaling is essential for many aspects of development in invertebrates and vertebrates (reviewed in Cadigan and Nusse, 1997; Dale, 1998) and mutations in components of the Wnt pathway are oncogenic (reviewed in Polakis, 2000). A variety of studies in divergent organisms have set a general framework for the Wnt [Wingless (Wg) in Drosophila] pathway as well as revealing that players in this pathway are structurally and functionally conserved in various species. In this pathway, the stabilization of β-catenin/Arm in Drosophila (homolog of β-catenin, Arm) protein is a key regulatory step. The Wnt/Wg ligand binds to the receptor, Frizzled, which activates an intracellular multi-modal protein, Dvl/Dishevelled (Dsh). Several Wnt/Wg pathway components, including Dvl/Dsh, glycogen synthase kinase-3β (GSK-3β)/Zeste-white 3 (ZW3), β-catenin/Arm, adenomatous polyposis coli (APC) protein/Dapp and protein phosphatase 2A, have been shown to form a large multimeric protein complex on the scaffold protein Axin/Daxin (reviewed in Kikuchi, 1999). In the absence of Wnt/Wg signaling, GSK-3β/ZW3 phosphorylates β-catenin/Arm (Yost et al., 1996; Pui et al., 1997), targeting it to the ubiquitin–proteasome pathway for degradation (Aberle et al., 1997). Wnt/Wg inhibits GSK-3β/ZW3 function through the Dsh family proteins, thereby up-regulating β-catenin/Arm protein levels. β-catenin/Arm then forms a complex with the Tcf-LeF/D-Tcf family of transcription factors and activates transcription of Wnt/Wg target genes (reviewed in Hecht and Kemler, 2000).

Recently one isoform of the casein kinase I (CKI) family, CKI\epsilon, was identified as a positive regulator of the canonical Wnt pathway. Overexpression of CKI\epsilon in Xenopus embryos induced second axes, activated the transcription of target genes and rescued UV-treated embryos (Peters et al., 1999; Sakana et al., 1999). From epistasis analysis, CKI\epsilon appears to act between Dvl/Xenopus-dsh (Xdsh) and GSK-3β (Peters et al., 1999). Moreover, associations of CKI\epsilon, Axin and Dvl/Xdsh were also demonstrated by co-immunoprecipitation experiments (Sakanaka et al., 1999). The kinase domain of CKI\epsilon was shown to directly bind to the PDZ (PSD95, discs large, ZO-1) domain of Xdsh (Peters et al., 1999; Mckay et al., 2001b). With the yeast two-hybrid assay, CKI\epsilon was shown to directly bind to the C-terminal portion of Axin (Mckay et al., 2001b; Rubinfeld et al., 2001). However, direct binding of CKI\epsilon to the DEP (Dsh, EGL-10, Pleckstrin) domain of Dvl and the association of CKI\epsilon with Axin via another unknown protein were reported (Kishida et al., 2001). On the other hand, direct phosphorylation of Dvl/Xdsh by several CKI isoforms and their involvement in Wnt-induced phosphorylation of Dvl have been shown (Mckay et al., 2001b). In line with these findings, a synergistic interaction of Dvl and CKI\epsilon in the activation of Wnt signaling has been reported (Kishida et al., 2001; Lee et al., 2001). However, in spite of intensive study, the mode of CKI action in Wnt signaling remains unclear.

Two recent papers suggest a more complicated mechanism. CKI\epsilon was shown to mediate, at least in part, Axin-dependent phosphorylation of APC, which stimulates APC to downregulate β-catenin (Rubinfeld et al., 2001). Moreover, Lee et al. (2001) have shown in Xenopus systems that a cytoplasmic fraction of Tcf3 competes with the Axin–APC–GSK-3β complex for β-catenin...
is form-specific amino and carboxyl extensions plus highly conserved kinase domains. Recently, Mckay et al. (2001b) have reported that the CKI isoforms, α, β, γ and δ, could also activate the Wnt pathway in Xenopus embryos. The CKI family also functions in a variety of cellular processes, including cell cycle regulation, DNA repair and circadian rhythms (Santos et al., 1996; Price et al., 1998). However, the mechanisms conferring the different functions on the variety of isoforms are unknown.

In Drosophila, Zilian et al. (1999) found that the discs overgrown (dco) gene, which strongly affects cell survival and growth control in imaginal discs, encodes a homolog of mammalian CKIe and is identical to the previously cloned double-time (dbt) gene, which regulates the period of the circadian rhythms. The fact that alterations in Wnt signaling leading to elevations in β-catenin promote tumorigenesis in mammals, and the finding that CKIe modulates β-catenin protein expression in vertebrates, appear to be related to the observation that certain dco mutants show hyperplastic growth of imaginal discs. On the other hand, in Drosophila circadian clock regulation, the CKIe, Double-time protein, directly binds and phosphorylates the Period protein, thereby promoting its turnover (Price et al., 1998). Surprisingly, a recent study has indicated that both shaggy/zw3 and double-time participate in circadian clock control (Martinek et al., 2001) suggesting an underlying synergism between ZW3–GSK-3β and Double-time–CKIe. However, no dbt or dco mutant has been reported that shows a phenotype closely associated with the loss or gain of wg function. The reason for this is not clear. However, it is possible that the expression level of the CKI isoform (dbt or dco) is relatively low, compared with that of other CKI isoforms, and thus loss of CKIe activity was masked by the activities of the other CKI isoforms. Furthermore, no Drosophila mutants for other CKI isoforms have been isolated. Therefore the roles of CKI in Wg signaling have not been explored extensively in Drosophila.

Here, we describe the use of a double-stranded RNA-mediated interference (RNAi) approach to study the function of Drosophila CKI in the Wg signaling pathway. Our results suggest that CKI functions as a negative regulator of Arm protein, by phosphorylating it on Ser and Thr residues in the N-terminus and targeting it for degradation.

**Results**

**CKI-RNAi leads to accumulation of Arm protein in Drosophila Schneider S2R+ cells**

Since loss-of-function studies are the key to revealing the actual function of Drosophila CKI in the Wg pathway, we used RNAi to disrupt the CKI gene expression in Drosophila Schneider S2R+ cells (Clemens et al., 2000). S2R+ cells were cultured in the presence of double-stranded (ds)RNA for CKIα, CKIe, Dα-catenin, casein kinase II catalytic (α) subunit (CKII-α) or LacZ for 3 days and then the protein levels in the cell lysates were analyzed by western blotting (Figure 1A). Addition of dsRNA for CKIe, Dα-catenin and CKII-α caused a selective decrease in the corresponding proteins. While previous studies with Xenopus, Caenorhabditis elegans and mammalian systems reported that CKI is a positive regulator of Wnt signaling,

and thereby inhibits β-catenin degradation. CKIe phosphorylates Tcf3 and thus strengthens Tcf3–β-catenin interaction, which leads to β-catenin stabilization. In addition, CKIe stimulates the binding of Xdsh to GSK-3β binding protein (GBP) (Lee et al., 2001). These results suggest that CKIe regulates Wnt signaling in vivo by modulating the β-catenin–Tcf3 and the GBP–Xdsh interactions. However, it is not clear whether this new model is applicable to other organisms, such as *Drosophila*, which has no apparent GBP counterpart. On the other hand, several CKI isoforms have also been shown to act in the non-canonical Wnt pathway. Blocking CKI function inhibits embryonic morphogenesis and activates JNK (Mckay et al., 2001a).

Several CKI isoforms are present in both vertebrates and *Drosophila*, and these CKI family enzymes contain..
both CKI\(\alpha\)- and CKI\(\epsilon\)-RNAi markedly elevated Arm protein levels, suggesting that CKI functions as a negative regulator of Arm protein in Drosophila. CKI\(\alpha\)-RNAi induced higher levels of Arm protein accumulation than CKI\(\epsilon\)-RNAi.

We next clarified whether CKI\(\alpha\)- and CKI\(\epsilon\)-RNAi were selective for each CKI isoform (Figure 1B). S2R+ cells expressing hemagglutinin (HA)-tagged CKI\(\alpha\) or CKI\(\epsilon\) were established and they were cultured with CKI\(\alpha\)- or CKI\(\epsilon\)-dsRNA. CKI\(\alpha\)- and CKI\(\epsilon\)-dsRNA induced the selective disappearance of the corresponding protein isoform, indicating that isoform-RNAi is specific. This is consistent with the result of CKI\(\alpha\) protein blotting (Figure 1A). These results indicate that depletion of either CKI\(\alpha\) or CKI\(\epsilon\) protein alone can induce the Arm protein elevation, suggesting that CKI family proteins in general function in down-regulating Arm protein. Double-RNAi for both CKI\(\alpha\) and CKI\(\epsilon\) isoforms led to a significantly higher level of Arm than that induced by CKI\(\alpha\)- or CKI\(\epsilon\)-RNAi alone (data not shown). It should be noted that CKI\(\alpha\)- and CKI\(\epsilon\)-RNAi did not affect the protein levels of Dsh (Figure 1A) or ZW3 (see Figure 5), indicating that the CKI-RNAi-mediated Arm elevation is not caused by modulating the protein levels of Wg signaling components upstream of Arm. Northern analysis revealed that CKI\(\alpha\)- and Dsh-catenin-dsRNA caused a selective reduction of the corresponding mRNA but did not affect Arm mRNA levels (Figure 1C). As CKI\(\alpha\)-RNAi induced a more prominent Arm accumulation than CKI\(\epsilon\)-RNAi, CKI\(\alpha\) was mainly used for later analyses.

**CKI\(\alpha\)-RNAi stabilizes Arm protein but does not affect the rate of Arm protein synthesis**

The CKI\(\alpha\)-RNAi-mediated Arm elevation could be caused by two mechanisms: CKI\(\alpha\)-RNAi increased the rate of Arm protein synthesis or decreased the rate of Arm degradation. To distinguish between these possibilities we used pulse–chase analysis. In pulse (7 min)-labeled cells, similar amounts of Arm protein were made, irrespective of whether the cells were treated with CKI\(\alpha\)- or LacZ-dsRNA (Figure 2A). Pulse–chase analysis over 200 min indicated that the Arm protein in cells treated with CKI\(\alpha\)-dsRNA appeared to be stable, whereas in cells treated with LacZ-dsRNA, it decayed rapidly (Figure 2B and C). Hence, the Arm protein accumulation due to CKI\(\alpha\)-RNAi appears to be largely a consequence of increased stability.

**CKI\(\alpha\)-RNAi stabilizes Arm protein but not the 155 kDa Cubitus interruptus protein in clone-8 wing imaginal disc cells**

Arm normally undergoes phosphorylation by ZW3, associates with the F-box protein, Slimb, and is degraded by the ubiquitin–proteasome pathway (Jiang and Struhl, 1998). To investigate whether CKI\(\alpha\)-RNAi selectively stabilizes Arm protein, we compared the effects of CKI\(\alpha\)-RNAi on protein levels of Arm and the transcriptional regulatory protein Cubitus interruptus (Ci), a component of the Hedgehog signaling pathway. In the absence of Hedgehog signaling, the 155 kDa Ci protein is proteolytically processed by the ubiquitin–proteasome pathway to produce a 75 kDa N-terminal protein (Aza-Blanc et al., 1997). For this analysis, the clone-8 cell line, in which Arm and Ci protein levels are regulated by Wg and Hedgehog signaling, respectively, was used. In clone-8 cells, CKI\(\alpha\)-RNAi again stabilized Arm, but did not block processing of the 155 kDa Ci into 75 kDa Ci. However, treatment of this cell line with laeacystin, a specific inhibitor of the proteasome, led to stabilization of both Arm and the 155 kDa Ci proteins (Figure 3). These findings suggest that CKI\(\alpha\)-RNAi selectively protects Arm from degradation.
Disruption of CKIα function by RNAi produces a naked cuticle

The ventral epidermis of a wild-type Drosophila embryo is covered by a cuticle with a repeated pattern of denticle belts followed by the naked cuticle (like the embryo shown in Figure 4A). Wg signaling is required to elevate Arm protein levels and to specify the fate of the epidermal cells that secrete the smooth cuticle. Thus, a loss of Wg leads to a cuticle covered with denticles and lacking areas, while ubiquitous Wg causes a naked cuticle, without denticle structures (Noordermeer et al., 1992). Furthermore, Drosophila mutants for zw3 (Siegfried et al., 1992) or daxin (Hamada et al., 1999), as well as wild-type embryos injected with Daxin-dsRNA (Willert et al., 1999), all of which exhibit ubiquitous elevation of Arm protein, show the naked cuticle phenotype. Hence, RNAi-in vivo was performed to generate a ckIα-loss-of-function phenotype. Among 640 embryos injected with lacZ-dsRNA, only 45% survived to develop into larvae, because of damage from RNA injections. All of these larvae showed a cuticle phenotype indistinguishable from that of un.injected wild-type embryos (Figure 4A) and no larva with a naked cuticle was found. Among 820 embryos injected with CKIα-dsRNA, on the other hand, 38% survived to become larvae. Forty-five and 43% of these larvae showed completely and partially naked cuticle phenotypes, respectively, while the other 12% showed the cuticle phenotype of un.injected wild-type embryos (Figure 4B). Thus, we concluded that injection of CKIα-dsRNA led to a naked cuticle. To ascertain that CKIα-RNAi indeed elevates Arm protein levels and leads to Wg target gene (engrailed) activation in vivo, LacZ- or CKIα-dsRNA-injected embryos developed to stage 10 were stained with anti-Arm and anti-Engrailed antibodies. The high levels of Arm protein only found in the Wg domain in LacZ-RNAi embryos (Figure 4C), have extended to the whole segment in CKIα-RNAi embryos (Figure 4D). Moreover, as in heat-shock-Wg embryos which uniformly express Wg (Noordermeer et al., 1992), Engrailed expression domain in CKIα-RNAi embryos has broadened, indicating that the Wg target gene was activated (Figure 4F). These in vivo data indicated that the loss of CKIα function leads to a phenotype associated with Wg pathway activation, which is consistent with the idea that CKIα is a negative regulator of Wg signaling.

dsh and zw3 slightly influence the CKIα-RNAi-induced elevation in Arm

We used epistasis analysis to explore where CKI functions in the Wg pathway. In this analysis, the effects of Dsh- or ZW3-dsRNAs on CKIα-RNAi-mediated Arm elevation were analyzed (Figure 5). Consistent with the accepted notion of dsh and zw3 functions, Dsh- and ZW3-RNAi resulted in a slight decrease and increase in Arm protein levels, respectively. Compared with CKIα-RNAi, ZW3-RNAi induced the accumulation of much lower levels of Arm. As western blotting revealed that ZW3-RNAi led to a significant reduction in protein levels, this result was rather surprising, but the reason for it is unknown. Notably, both Dsh- and ZW3-RNAi had little effect on the CKIα-RNAi-induced Arm elevation, suggesting that dsh and zw3 play a minor role (Figure 5). Assuming that dsh and ckIα function in a linear pathway, these results suggest ckIα is downstream of dsh.

Overexpression of CKIα leads to hyper-phosphorylation of Arm and Dsh proteins

The RNAi-based loss-of-function analyses of CKIα described so far are all consistent with the notion that CKIα negatively regulates Arm protein levels. As a complementary approach, we examined the effect of overexpressing the wild-type or kinase-negative form of CKIα, as well as wild-type ZW3, on protein levels and biochemical properties of Arm, Dsh and ZW3 proteins. S2R+ cells transfected with pMK3 vector were used as the control. As Arm is rapidly turned over, analyses were performed in the presence and absence of lactacystin (Figure 6A).

In the absence of lactacystin, overexpression of the wild-type CKIα or kinase-negative form had little effect on the total Arm protein levels. Similar experiments with CKIε gave the same results (data not shown). In addition, overexpression of wild-type CKIα or CKIε could not block the Wg-induced Arm accumulation (data not
shown). Drosophila CKI functions as a negative regulator of Arm (Figures 1A and 2) and the kinase-dead form of CKI was shown to be dominant-negative in Xenopus (Peters et al., 1999). Therefore, these results were contrary to our expectations that overexpression of wild-type CKI or the kinase-negative CKI would lead to a decrease or increase of Arm protein levels, respectively. The reason for this is not clear, but it is possible that the endogenous CKI activity is already sufficiently high to exert the maximum rate of Arm degradation in normal S2R+ cells. However, this failed to explain why the overexpressed kinase-negative forms of CKI could not appear to compete effectively with endogenous CKI activity.

Nevertheless, as shown in Figure 6A, overexpression of wild-type CKI could induce the accumulation of low levels of hyper-phosphorylated (showing less electrophoretic mobility) forms of Arm (open arrow) and Dsh (closed arrow), but that of the kinase-negative form of CKI or wild-type ZW3 did not. These mobility shifts were eliminated by treating the immunoprecipitates with phosphatase prior to electrophoresis, indicating that they were mainly due to phosphorylation (data not shown). On the other hand, overexpression of wild-type CKI or the kinase-negative form did not affect ZW3 protein levels or shifted it on SDS–PAGE (Figure 6A). These results, together with CKI-RNAi data (Figure 5), indicated that modulation of CKI protein levels has little effect on ZW3 protein in S2R+ cells.

In the presence of lactacystin, several Arm species with lower electrophoretic mobility (representing various phosphorylated and poly-ubiquitylated forms) were detected in all samples. Notably, lactacystin accentuated the increase in phosphorylated forms of Arm (Figure 6A, showing unique electrophoretic mobility different from other phosphorylated and poly-ubiquitylated forms, indicated by an arrowhead), which was induced by expression of wild-type CKI but not the kinase-negative form. In addition, overexpression of wild-type ZW3 led to slight increases in highly modified forms of Arm. The Arm species induced by CKI-RNAi and ZW3-overexpression in the presence of lactacystin showed distinct mobility profiles on SDS–PAGE, suggesting that this CKI-induced modification of Arm was due to its own kinase activity and not mediated by that of ZW3 (Figure 6A).

To demonstrate that endogenous CKI could, at least in part, participate in phosphorylation of Arm and thus induces its subsequent modification in intact S2R+ cells, we analyzed whether CKI-RNAi decreased the amount of modified forms of Arm in the presence of lactacystin (Figure 6B). CKI-RNAi again led to a marked Arm elevation in the absence of lactacystin. While western blots from lactacystin-treated cells showed that various modified forms of Arm were detected in the LacZ-RNAi cells as in pMK33 transfectants (Figure 6A). In the CKI-RNAi cells, however, the amount of these modified forms was decreased, although some clearly remained. At the same
time, the level of non-modified Arm (showing the highest mobility) was elevated. These results suggest that, in normal circumstances, a significant fraction of Arm is modified under the control of CKIα, further supporting the idea that CKIα downregulates Arm protein levels by phosphorylation.

Arm is phosphorylated in vitro by CKIα but its kinase activity is not affected by Wg signaling

To demonstrate that Arm is a substrate for CKIα and to examine the effect of Wg signaling on CKIα kinase activity, in vitro kinase assays were performed. HA-tagged CKIα was immunoprecipitated from S2R+ transfectants treated with or without Wg (Figure 6C). Western blots from these cell lysates revealed that the same amounts of HA-tagged CKIα were immunoprecipitated. Incubation of the immunoprecipitates with His-tagged Arm or a CKI peptide substrate in the presence of γ-32P-labeled ATP indicated that Arm is a good substrate for CKIα and that Wg signaling has little effect on its kinase activity. However, it is possible that in vivo Wg signaling regulates CKIα-mediated Arm phosphorylation by modulating a physical interaction between CKIα and Arm. Therefore, it is not clear in vivo whether Wg signaling affects CKI-mediated phosphorylation of its substrates, including Arm and Dsh.

Identification of the sequence in Arm protein which is responsible for CKIα-RNAi-mediated Arm accumulation

To search for the sequence in Arm that responds to CKIα-RNAi, stable S2R+ cell lines expressing wild-type and various mutant forms of myc-tagged Arm were established and the effects of CKIα-RNAi (LacZ-RNAi was used as the control) on accumulation of these Arm mutant proteins were examined by western blotting (Figure 7A). Similar to endogenous Arm, wild-type Arm with the myc-tag was markedly stabilized by CKIα-RNAi. As phosphorylation of Arm at the N-terminus is known to determine its stability, we first analyzed Arm mutants lacking the N-terminal 58 or 138 amino acids. These two mutants, which are more stable than the wild-type, no longer responded to CKIα-RNAi, indicating that the target sequence for CKIα-RNAi resides in the N-terminal 58 amino acids. Therefore, we made a series of N-terminal mutants (Figure 7B). In the S/T to A mutant, the Ser and Thr residues originally identified as phosphorylation target sites for ZW3 (S at codon 44, 48, 56 and T at 52) were changed to Ala. In S56A and S58A, the Ser at 56 and 58, respectively, was changed to Ala. In the ED to QN mutant, a stretch of acidic amino acids (E and D) was replaced with Q and N (E at 61, 63, 64, 66 to Q and D at 62 to N). This mutant was produced because CKI is known to phosphorylate a Ser or Thr residue close to the acidic residues and this stretch of acidic amino acids is also conserved in β-catenin and plakoglobin.

Analyses with this series of Arm mutants revealed that protein levels of the S58A mutant were somewhat elevated even without CKI-RNAi, but this mutant responded to CKI-RNAi similarly to the wild-type Arm, while the S56A mutant responded slightly less than the wild-type Arm. The S/T to A mutant no longer responded to CKIα-RNAi, while the ED to QN mutant responded much weaker than the wild-type Arm. These results suggest that CKIα directly or indirectly stimulates phosphorylation of Ser44, 48 and 56, as well as Thr52, thereby destabilizing Arm and that the stretch of acidic amino acids may facilitate this process. If so, we would expect the ED to QN mutant to be more stable than the wild-type Arm. Hence, the stabilities of the wild-type, S/T to A, S56A and ED to QN forms of Arm were compared (Figure 8). To confirm that the steady-state levels of each Arm protein reflected the stability of each protein, the transfection efficiency and Arm mRNA levels were monitored in transient and stable expression experiments, respectively. Both experiments demonstrated that the S/T to A mutant was the most stable with the S56A mutant second. The ED to QN mutant was more stable than the wild-type Arm, but less stable than the S/T to A mutant.

CKIα phosphorylates the same Ser and Thr residues in the N-terminal portion of Arm as ZW3

Next, we examined whether CKIα directly phosphorylates a set of Ser and Thr residues in the N-terminal region of Arm as ZW3 does. To this end, we generated a series of glutathione S-transferase (GST)–Arm fusion proteins in which the N-terminal 39 amino acids (from codon 37 to 75) from the wild-type or mutant forms of Arm described
A Transient expression
IB: myc
Dc-catenin
β-gal activity (%) 100 102 98 96 94

B Expression in the stable lines
IB: myc
Dc-catenin

Fig. 8. The Arm ED to QN mutant protein is stable, compared with the wild-type. (A) Transient expression experiment: 0.2 µg of pAcLacZ, together with 0.2 µg of pMK33 vector or various pMK-Arm-myc constructs were introduced into S2R+ cells, and after 36 h CuSO4 was added for 12 h. Expression levels of myc-tagged Arm were analyzed by western blotting. Note relative β-galactosidase activities in the cell lysates were almost the same. (B) Stable expression experiments. Stably transfected cells (5 × 10⁶) were plated and expression of various myc-tagged Arm was induced with CuSO4. Expression levels of myc-tagged Arm and Dc-catenin were analyzed (upper two panels). Total RNAs from these cells were subjected to northern blot analysis with Arm or RP49 probe (lower two panels).

above were fused to GST (our initial attempt using the whole Arm failed). CKIα-HA or ZW3-HA immunoprecipitated were used as enzyme preparations. 32P incorporation into these GST–Arm fusion proteins was analyzed by autoradiography (Figure 9). While naive GST was not phosphorylated by either CKIα or ZW3, the GST proteins containing the sequence from the wild-type Arm or S58A mutant were phosphorylated by CKIα to the same levels, indicating that S58 may not be phosphorylated. Fusion proteins with the S56A or the S48A and T52A double (data not shown) mutation were phosphorylated by CKIα at levels of 9 and 15%, respectively, of the fusion protein containing the wild-type Arm sequence. However, fusion proteins with the S/T to A or the ED to QN mutation were not phosphorylated by CKIα. These results indicate that the phosphorylation sites for CKIα are Ser44, 48 and 56, as well as Thr52 residues (among these, S56 seems to be the major phosphorylation site, whose phosphorylation affects those of the other three sites). A cluster of acidic amino acids is also required for this phosphorylation.

On the other hand, ZW3 phosphorylated the fusion proteins containing the wild-type sequence and the S58A mutation to the same levels, but not that containing the S/T to A mutation. The fusion protein with the S56A or the ED to QN mutation was phosphorylated by ZW3 at levels of 22 and 28%, respectively, of the fusion protein containing the wild-type Arm sequence, indicating that ZW3 did not necessarily require the cluster of acidic amino acids. As far as this in vitro experiment is concerned, prior phosphorylation of Arm from other kinases (known as priming kinases) does not appear to be essential for ZW3-mediated phosphorylation of these Ser and Thr residues. These results confirmed that CKIα can phosphorylate the same series of Ser and Thr residues in the N-terminal region of Arm as ZW3 does, which is consistent with the observation that the Arm S/T to A mutant no longer responds to CKIα-RNAi.

Discussion

In this study, we have demonstrated that CKIα- and CKIε-RNAi elevated Arm protein levels in S2R+ cells by protecting Arm from degradation. In line with this, Drosophila embryos injected with CKIα-dsRNA showed a naked cuticle phenotype. In S2R+ cells, overexpression of wild-type-CKIα induced hyper-phosphorylation of Arm, while CKIα-RNAi inhibited these hyper-modifications. Moreover, the target sequence of CKIα-RNAi in Arm was found to be a series of Ser and Thr residues in its N-terminus, which was phosphorylated by CKIα in vitro. Thus, we propose that CKI phosphorylates Arm, which targets it for ubiquitin-mediated degradation in Drosophila. In vertebrates, CKIε, GSK-3β, Dvl, APC and Axin are known to form a complex (Sakakana et al., 1999; Kishida et al., 2001; Rubinfeld et al., 2001). This suggests that CKI, ZW3, Dsh, Dacp and Arm could form a complex on Daxin in Drosophila. In addition, taking into account
our finding that CKI binds to Slimb (unpublished result), we present a model of how CKI functions in the Wg pathway (Figure 10).

CKIβ was proposed as a positive regulator of Wnt signaling in vertebrates, mainly based on the observation that overexpression of CKIβ induced dorsal axis duplication in Xenopus and stimulated Tcf/β-catenin binding in mammary cells. However, it should be noted that one component of a multimeric protein complex, when overexpressed, sometimes acts as a dominant-negative inhibitor (e.g. APC; Vleminckx et al., 1997). To avoid possible artifacts of overexpression studies, we performed a loss-of-function study using the highly effective method of RNAi in Drosophila.

We have shown previously that Wg/Wnt treatment rapidly induces hyper-phosphorylation of Dsh/Dvl in both Drosophila and mammalian cells (Lee et al., 1999). Here, we showed that CKIβ overexpression led to hyper-phosphorylation of Dsh. Mckay et al. (2001b) have reported that Wnt-3a-induced Dvl phosphorylation was due to CKI, whereas PAR-1, a Dsh/Dvl-associated kinase, has been shown to phosphorylate Dsh/Dvl in response to Wg/Wnt (Sun et al., 2001). To resolve this issue, it is informative to see whether the Wg-induced phosphorylation of Dsh is affected by CKI- or PAR-1-RNAi.

A group of GSK-3β substrates are formed by prior phosphorylation from other kinases, an event known as 'priming', to generate the sequence S/T-X-X-S/T-P-O4, where S/T corresponds to Ser or Thr and X to any other residues. In the case of glycogen synthase, CKII was assumed to be a priming kinase (Picton et al., 1982). In contrast, β-catenin is not known to require a priming phosphate and may rely on high affinity interactions in a multiprotein complex with GSK-3β. Recently, two groups have reported the existence of a phosphate-binding site in GSK-3β and showed that primed substrates require this site but non-primed ones do not (Dajani et al., 2001; Frame et al., 2001). However, we found that the GSK-3β target sequence in glycogen synthase (amino acid sequence from 640 to 661: SVVPSPLSRHSSPHQSEDEEE) and the ZW3 target sequence in Arm (amino acid sequence from 44 to 68: SGIIHSGAVQAPSLSGKEDEEMGD) share a combination of S/T-X-X-S/T repeats and a cluster of acidic amino acids. CKI was shown to phosphorylate a Ser or Thr residue C-terminal to a stretch of acidic residues (Flotow et al., 1991), but it also phosphorylates sites not matching this consensus. Actually, Ser56 of Arm, which is located to the N-terminus of the acidic residues cluster, is a major phosphorylation site for CKIβ (Figure 9), and it corresponds to the residue with a priming phosphate in glycogen synthase (Ser656). In addition, CKI, ZW3 and Arm appear to form a complex. Thus, it is possible that CKI partly works as a priming kinase that phosphorylates any of the residues Ser56, Ser48 or Thr52 of Arm and thereby stimulates ZW3-mediated phosphorylation of Ser48, Ser44 or Thr52.

The cluster of acidic amino acids described above is conserved in β-catenin (amino acid sequence from 53 to 58: EEEVDV). Notably, mutations in this region have been reported in tumors. Of 37 independent anaplastic thyroid carcinoma samples, four had mutations (one case of E54 to K, two cases of E55 to K, and one case of D58 to N; Garcia-Rostan et al., 1999). One hepatoblastoma has been reported that had a 42 base pair deletion in β-catenin exon 3, which led to deletion of amino acids from S45 to D58 (Koch et al., 1999). Clearly, CKI mutations in certain tumors remain to be explored.

**Materials and methods**

**Cell cultures and transfections**

The Drosophila S2R+ cell line (a line of Schneider S2 cells that respond to Wingless signaling; Yanagawa et al., 1998) and Drosophila-wing imaginal disc cell line clone-8 were cultured as described (van Leeuwen et al., 1994). Expression plasmids were introduced into S2R+ cells using Effectene reagent (Qiagen). The transfectants generated with pMK33-based vectors were mixtures of stable S2R+ cell clones selected with hygromycin (200 μM). Expression of the transfected genes was induced by adding 0.5 mM CuSO4. The pMK-ZW3-HA plasmid and β-galactosidase assay with pAdacZ plasmid were as described previously (Yanagawa et al., 1997, 2000).

**dsRNA production and RNAi procedures**

The RNAi experiments in Drosophila S2R+ cells were performed as described previously (1 × 10^6 S2R+ cells were incubated for 3 days with 15 μg of dsRNA in each well of a six-well plate; Clemens et al., 2000). Individual dsRNAs were generated using a Megascript T7 transcription kit (Ambion) and the DNA templates, which were generated by PCR using sets of primers with T7 RNA polymerase binding sites. Primer sequences used to generate specific dsRNA were obtained as follows: Drosophila CKIβ, DDBJ/EMBL/GenBank accession No. U55848, sense primer (S-P) 457–480, anti-sense primer (AS-P) 1138–1161; CKIγ, accession No. AF055583, S-P 65–89, AS-P 785–811; Dishevelled, accession No. L26974, S-P 240–259, AS-P 954–970; ZW-3, accession No. X53332, S-P 544–560, AS-P 1271–1292; CKI α subunit, accession No. M16534, S-P 259–285, AS-P 941–965; Der-catenin, accession No. D13964, S-P 101–126, AS-P 799–828; LacZ, accession No. EI0069, S-P 399–420, AS-P 1138–1162. For in vivo RNAi experiments, dsRNA for LacZ or CKIβ was injected anteriorly or posteriorly into wild-type Drosophila (Canton S strain) embryos at a concentration of 2 μM. After a 48 h incubation at 18°C, the injected embryos were fixed and cuticle preparations made as described elsewhere (Willert et al., 1999).
Northern analysis
The probes for Dax-catenin, Arm and CKIε were 1.5 kb Clal, 1.9 kb BamHI and 0.5 kb HincII fragments from the corresponding cDNA clones, respectively. A cDNA fragment of ribosomal protein, Rf49, was used as a probe for the RNA loading control.

Immunoblot analyses and antibodies
The laminas were subjected to western blot analysis as described previously (Yanagawa et al., 1997). Rabbit antibody against the N-terminal region of Ci (AB; Aza-Blanc et al., 1997) and affinity-purified rabbit antibody against human CKIε (Fish et al., 1995) were gifts. The other antibodies used in this study were described previously (Yanagawa et al., 1997, 2000; Lee et al., 1999).

Whole-mount antibody staining of the embryos
Embryos injected with dsRNA for LacZ or CKIε were allowed to develop until stage 10–11. The embryos were fixed with 4% formaldehyde and stained with monoclonal anti-Arm (N2-TA1) or anti-Engrailed (4D9) antibody using the Vectastain ABC kit and diaminobenzidine (Vector) as described previously (Noordermeer et al., 1992).

In vitro kinase assay
The stable PMK-CKIε-HA transfectants induced with CuSO4 for 14 h were co-cultured with S2-HS-Wg or plain S2 cells in the presence of CuSO4 for 3 h. From the lysates of these cells, HA-tagged CKIε was immunoprecipitated with the rabbit anti-HA antibody and protein A–Sepharose. The immune complexes were washed with lysis buffer and with kinase buffer (10 mM HEPES pH 7.5, 75 mM KCl, 5 mM MgCl2, 20 mM ATP, 1 mM dithiothreitol) before being suspended in 80 μl of kinase buffer supplemented with 20 μl of [γ-32P]ATP (Amersham) containing either 10 μg His-tagged Arm or 0.1 mM CKI substrate peptide (Sigma, C-2335). At 5, 10, 20 and 30 min after incubation at 30°C, 5 μl of the reaction mixture containing Arm were taken and subjected to SDS–PAGE. The 32P incorporated in Arm was detected by autoradiography. Peptide phosphorylation reactions were quantified by spotting 5 μl of reaction mixture on phosphocellulose filters (p81, Whatman). The filters were washed with 75 mM phosphoric acid and dried. Similar in vitro kinase assays were performed using ZV3- or CKIε-immunoprecipitates and the various GST–Arm fusion proteins. 32P incorporated in each substrate was quantitated using a BAS 2000 image analyzer (Fuji film).

Pulse–chase analysis
S2R+ cells were treated for 3 days with 15 μg of dsRNA for CKIε or LacZ in six-well dishes. To determine the total protein synthesis rate, cells were pulse-labeled for 7 min with 0.8 μCi of [35S]methionine in 1 ml of M3 medium lacking methionine (Sigma). Fluorescein isothiocyanate-conjugated cells as this assay described previously (Hsiung et al., 1996). The total cell lysate or the anti-Arm immunoprecipitates were prepared with Enhance™ solution (NEN). For kinetic analysis of Arm turnover, the cells were pulse-labeled with 0.6 μCi of [35S]methionine in 1 ml of M3 medium lacking methionine for 10 min, and incubated in 1 ml of M3 medium supplemented with 25 μM unlabeled methionine containing 15 μg of CKIε or LacZ dsRNA. At the chase times indicated, the cells were lysed in 400 μl/well lysis buffer and centrifuged. Arm was immunoprecipitated from supernatants with 30 μl of monoclonal anti-Arm and 30 μl of goat anti-mouse IgG–Sepharose 4B (Zymed) before being subjected to SDS–PAGE.

Expression constructs
To add the HA epitope to the C-terminus of full-length Drosophila CKIε, the RT–PCR cloning procedure for CKIε was amplified by RT–PCR with the single-stranded cDNA synthesized from Drosophila embryonic poly(A)+ RNA and the following set of primers: sense primer with Xhol site: 5'-TACGTCAGGACGCCTAGCCGATGACACCG-3', and anti-sense primer with SpeI: 5'-CAAGACTAGTTCGACCGATGACCGG-3'. Similarly, a CKIε cDNA fragment was amplified using sense primer with Xhol site: 5'-TACGTCAGGACGAAACACCG-3', and anti-sense primer with SpeI: 5'-TACGTCAGGACGAAACACCG-3'. The CKIε and CKIε-RT–PCR products were double-digested with Xhol and SpeI before being cloned into the Xhol–SpeI-cleaved pMK33-HA. The resulting plasmids were named pMK-CKIε-HA and pMK-CKIε-RA, respectively. From these, plasmids expressing kinase-negative mutant of CKIε and CKIε (a lysine residue in the ATP-binding region was changed to arginine) were constructed using the Transformer™ site-directed mutagenesis kit (Clontech).

From myc-tagged Arm cDNA in pBluescript II (Yanagawa et al., 1997), the various mutants described in Results were generated with QuickChange™ site-directed mutagenesis kit (Stratagene). These wild-type and mutant forms of myc-tagged Arm cDNA were digested with BamHI and the resulting 2.7 kb fragments inserted into the BamHI site of PMK33. An Arm cDNA fragment encoding myc-tagged Arm lacking the N-terminal 58 amino acids was amplified by PCR and it was cloned into the BamHI site of pmKK33. pMK-Arm-myc was double-digested with XhoI and EcoRI, then blunt and self-ligated. This plasmid, named pMK-del-Arm myc, expresses a myc-tagged Arm mutant with an N-terminal deletion that starts at the internal methionine 139.

To construct GST–Arm fusion proteins, DNA fragments encoding the N-terminal 39 amino acids (from codon 37 to codon 75) of wild-type and mutant forms of Arm were amplified using the sense primer with BamHI site: 5'-TGTGGATCAGAATTCTGATGATAGGCGAC-3', anti-sense primer with XhoI site: 5'-GAACCTGAGGTTCCAGGTCGACAT-3' and wild-type and mutant forms of myc-tagged Arm cDNA. The fragments were double-digested with BamHI and XhoI before being ligated with BamHI–XhoI cleaved pGEX5X-3. The GST–Arm fusion proteins were expressed in Escherichia coli XL1Blue and purified with glutathione–Sepharose CL4B beads (Amersham). His-tag was added to the N-terminus of the full-length Arm protein as follows: the entire coding sequence of Arm was amplified by PCR using Arm cDNA, sense primer with BamHI site: 5'-ATCCTGATCCGATGATAGGCGACATGACC-3' and an anti-sense primer with a termination codon and a SalI site: 5'-TCGTCGACCAATAACCTGGATATCGACCA-3'. After the Arm PCR products were double-digested with BamHI and SalI, it was cloned into the BamHI–SalI-cleaved pQE30 (Qiagen). Escherichia coli was transformed with this plasmid, and under denaturing conditions, the His-tagged Arm protein was purified from the lysate using Ni2+–NTA agarose (Qiagen) and then refolded.

Acknowledgements
We thank Y.Kitagawa for providing facilities. We also thank T.Tabata, C.V.C.Glover and D.Virshup for anti-Ci and anti-Engrailed, anti-CKII and anti-CKI antibodies, respectively. This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan to S.-Y.

References