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Regulation of wingless signaling by the CKI family in *Drosophila* limb development

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Abstract

The Wingless (Wg)/Wnt signaling pathway regulates a myriad of developmental processes and its malfunction leads to human disorders including cancer. Recent studies suggest that casein kinase I (CKI) family members play pivotal roles in the Wg/Wnt pathway. However, genetic evidence for the involvement of CKI family members in physiological Wg/Wnt signaling events is lacking. In addition, there are conflicting reports regarding whether a given CKI family member functions as a positive or negative regulator of the pathway. Here we examine the roles of seven CKI family members in Wg signaling during *Drosophila* limb development. We find that increased CKIε stimulates whereas dominant-negative or a null CKIε mutation inhibits Wg signaling. In contrast, inactivation of CKIα by RNA interference (RNAi) leads to ectopic Wg signaling. Interestingly, hypomorphic CKIε mutations synergize with CKIα RNAi to induce ectopic Wg signaling, revealing a negative role for CKIε. Conversely, CKIα RNAi enhances the loss-of-Wg phenotypes caused by CKIε null mutation, suggesting a positive role for CKIα. While none of the other five CKI isoforms can substitute for CKIα in its inhibitory role in the Wg pathway, several CKI isoforms including CG12147 exhibit a positive role based on overexpression. Moreover, loss of Gilgamesh (Gish)/CKIγ attenuates Wg signaling activity. Finally, we provide evidence that several CKI isoforms including CKIα and Gish/CKIγ can phosphorylate the Wg coreceptor Arrow (Arr), which may account, at least in part, for their positive roles in the Wg pathway.

Keywords: Wg; Wnt; CKI; Kinase; β_catenin; Limb development; Signaling; Cancer

Introduction

The Wnt family of secreted growth factors controls many key developmental processes, including cell proliferation, cell fate determination, tissue patterning, and planar cell polarity in a wide variety of organisms (Logan and Nusse, 2004). Mutations in Wnt signaling components lead to many types of cancers including colon and skin cancers (Moon et al., 2004). The *Drosophila* Wingless (Wg), a founding member of the Wnt family, controls embryonic segmental polarity and patterning of adult appendages such as wing, leg, and eye. Wg exerts its biological influence through the canonical Wnt/β-catenin pathway, which is evolutionarily conserved from invertebrates to vertebrates.

Genetic and biochemical studies in several organisms have suggested a model for Wnt/Wg signal transduction (Logan and Nusse, 2004). Binding of Wnt/Wg proteins to their cognate receptors, members of the Frizzled (Fz) family of seven transmembrane proteins, and coreceptors, LRP5/6/Arrow (Arr), activates a cytoplasmic signaling component Dishevelled (Dsh), which counteracts the activity of a destruction

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complex composed of Axin, APC, and the Ser/Thr kinase GSK3β/Shaggy (Sgg)/Zest White 3 (Zw3), leading to the accumulation and nuclear translocation of the transcriptional effector β-catenin/Armadello (Arm). β-Catenin/Arm forms a complex with the DNA-binding protein Lef1/TCF to activate Wnt/Wg target genes.

A cohort of studies have provided evidence that CKI family members participate in many aspects of the Wnt/Wg signaling pathway (Price, 2006). CKIε was first identified as a positive regulator of the canonical Wnt pathway (Peters et al., 1999; Sakanaka et al., 1999). Overexpression of CKIε in *Xenopus* embryos induced ectopic dorsal axis formation, activated Wnt-responsive genes, and rescued the axial formation of UV-treated embryos. Dominant-negative forms of CKIε and a pharmacological inhibitor of CKI blocked the responses to ectopic Wnt signaling in *Xenopus*. Biochemical and epistasis study suggested that CKIε binds Dsh and acts between Dsh and GSK3β (Peters et al., 1999; Sakanaka et al., 1999). In vivo and in vitro kinase assays showed that CKIε can phosphorylate Dsh and a pharmacological CKI inhibitor can block Wnt induced Dsh phosphorylation, suggesting that Dsh is a target of CKIε (Peters et al., 1999). However, the role of CKIε appears to be more complex than it was originally anticipated. For example, it has also been shown that CKIε interacts with Axin, and Axin-bound CKIε phosphorylates APC and modulates its ability to regulate β-catenin (McKay et al., 2001; Peters et al., 1999; Rubinfeld et al., 2001; Sakanaka et al., 1999). What makes the picture even more complicated is the finding that, in a reconstituted system of *Xenopus* extracts, CKIε can phosphorylate Tcf3 and enhance Tcf3-β-catenin association and β-catenin stability, implying that CKIε may also exert a positive influence downstream of GSK3β (Lee et al., 2001).

The potential role of other CKI isoforms in Wnt signaling has also been examined in several systems. In an overexpression study using *Xenopus* embryonic explants, all other CKI isoforms, including α, β, γ, and δ, can activate Wnt signaling (McKay et al., 2001). All of these CKI isoforms with the exception of CKIγ can stimulate Dsh phosphorylation in cultured cells (McKay et al., 2001). However, subsequent studies provided evidence that CKIα plays a negative role in Wnt/Wg signaling that acts as a priming kinase for GSK3β-mediated phosphorylation of β-catenin/Arm (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). Purification of the Axin-bound kinases that can prime GSK3β-mediated phosphorylation of β-catenin identified CKIα (Liu et al., 2002). RNAi knockdown of CKIα inhibited phosphorylation at Ser45 of β-catenin and subsequent phosphorylation by GSK3β, resulting in β-catenin stabilization (Liu et al., 2002). Consistent with the vertebrate results, CKIα RNAi of *Drosophila* embryos resulted in "naked cuticle", a phenotype consistent with gain-of-Wg signaling (Liu et al., 2002). The possible role of CKIε as a priming kinase for β-catenin remained unclear. Overexpression of a dominant-negative CKIε inhibited Axin-induced phosphorylation at Ser45 of β-catenin in 293 cells (Amit et al., 2002). In addition, RNAi knockdown of CKIε stabilized Arm in *Drosophila* S2+ cells, although the effect was less dramatic than CKIα RNAi knockdown (Yanagawa et al., 2002). On the other hand, RNAi knockdown of CKIε in 293T cells had no detectable effect on Ser45 phosphorylation and stability of β-catenin (Liu et al., 2002). It remains possible that CKIε plays a minor partially redundant role in β-catenin/Arm phosphorylation and the effect of its inactivation on β-catenin/Arm phosphorylation and degradation could have been masked by CKIα.

Although CKIα RNAi in *Drosophila* embryos resulted in phenotypes consistent with "gain-of-Wg" function, the recent finding that CKIα is also a negative regulator of the Hh pathway complicated the interpretation (Jia et al., 2005; Lum et al., 2003). Because Wg and Hh cross-regulate each other during embryonic development, the "gain-of-Wg" phenotype resulted from CKIα RNAi could be attributed to ectopic Hh signaling. To further investigate the physiological roles of the CKI family members in Wg signaling In vivo, we applied overexpression, dominant-negative, genetic mutations, and RNAi approaches to study the function of CKIε, CKIα and Gish/CKIγ in *Drosophila* wing development where Wg signaling is independent of Hh. We also assessed the potential roles of other CKI family members (Fig. 1; Morrison et al., 2000) in Wg signaling using overexpression assays.

Materials and methods

Mutations and transgenes

dco^{3}, *dco*^{2}, *dco*^{P103}, and *dco*^{le88} are hypomorphic, strong, and null allele of *dco/dbt* (Zilian et al., 1999). *gish*^{e01759} is a strong allele of *gish* (Jia et al., 2005). *dsh*^{V26} is a null allele (Jiang and Struhl, 1996). *hsp-flp1*, *hsp-CD2*, *hsp-Myc-GFP*, *MS1096*, *act>CD2>Gal4*, *ap-Gal4*, *omb-Gal4* have been described (Jiang and Struhl, 1995, 1998; Pignoni et al., 1997; Wang et al., 1999). The CKIα RNAi constructs CRL and CRS have been described (Jia et al., 2004, 2005). DN-DBT (K38 to R) and DN-XCKIε (K38 to R) have been described (Jia et al., 2005; Peters et al., 1999). N-terminal flag-tagged CKI isoforms have been described (Jia et al., 2005). *UAS-XCKIε-KD* contained the coding sequence for the kinase domain of XCKIε (Peters et al., 1999) inserted into the *pUAST* vector. *UAS-Sgg*, *UAS-DN-GSK3*, *UAS-DN-dFtz2*, *UAS-P35* have been described (Hay et al., 1994; Hazelett et al., 1998; Jia et al., 2002; Zhang and Carthew, 1998).

Genotypes for generating clones are as follow.

dco clones (with CRS2 coexpression): *MS1096 UAS-FLP*/*UAS-P35*; (*CRS2*/+); *FRT82 dco*^{le88}/*FRT82 hsp-CD2*, *y*$^{+}$ *M*(*3*)*w124*.
dsh clones expressing XCKIε-KD: *hsp-FLP hsp-Myc-GFP FRT101*/*dsh*^{V26} *FRT101*; *ap-Gal4*/*UAS-XCKIε-KD*.
gish clones: *MS1096 UAS-FLP*; *FRT82 gish*^{e01759}/*FRT82 hsp-CD2*, *y*$^{+}$ *M*(*3*)*w124*.
dco gish double mutant clones: *MS1096 UAS-FLP*/*P35*; *FRT82 gish*^{e01759} *dco*^{le88}/*FRT82 hsp-CD2*, *y*$^{+}$ *M*(*3*)*w124*.

Cell culture, transfection, immunoprecipitation, and Western blot analysis

S2 cells were cultured in the Schneider's *Drosophila* medium (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Transfection was carried out using Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. An ub-Gal4 construct was cotransfected with pUAST expression vectors for all the transfection experiments. 4 μg DNA for ub-Gal4 and 2 μg DNA for each pUAST expression vector were used in a typical transfection experiment. Immunoprecipitation and Western blot analyses were performed using standard protocols. Antibodies used are mouse αHA, F7 (Santa Cruz), mouse αFlag, M2 (Sigma).