Published in final edited form as:

Biochem Biophys Res Commun. 2008 September 5; 373(4): 637–642. doi:10.1016/j.bbrc.2008.06.084.

Drosophila CK2 phosphorylates Hairy and regulates its activity in vivo

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Abstract

Hairy is a repressor that regulates bristle patterning, and its loss elicits ectopic bristles (neural hyperplasia). However, it has remained unknown whether Hairy is regulated by phosphorylation. We describe here the interaction of protein kinase CK2 and Hairy. Hairy is robustly phosphorylated by the CK2-holoenzyme (CK2-HoloE) purified from *Drosophila* embryos, but weakly by the catalytic CK2 α subunit alone, suggesting that this interaction requires the regulatory CK2 β subunit. Consistent with this, Hairy preferentially forms a direct complex with CK2-HoloE. Importantly, we demonstrate genetic interactions between CK2 and *hairy* (*h*). Thus, flies trans-heterozygous for alleles of CK2 α and *h* display neural hyperplasia akin to homozygous hypomorphic *h* alleles. In addition, we show that similar phenotypes are elicited in wild-type flies upon expression of RNAi constructs against CK2 α/β , and that these defects are sensitive to *h* gene dosage. Together, these studies suggest that CK2 contributes to repression by Hairy.

Keywords

Drosophila; neurogenesis; CK2; Hairy; repression

Introduction

Hairy is a basic helix-loop-helix (bHLH) repressor, which plays multiple functions during development [1,2]. During embryogenesis, its periodic expression drives segmentation via repression of genes such as *fushi tarazu* [3]. Later, it regulates neurogenesis by repression of *achaete* or *atonal*, two bHLH proneural transcription activators [4–6]. Hairy, the Enhancer of split and Deadpan bHLH proteins, along with their mammalian homologs, are referred to as the HES repressors. The precise patterning of gene expression, which is necessary for animal development, entails multiple mechanisms, and in this regard all HES proteins serve as transcriptional repressors [7]. However, little information has been forthcoming on whether repression by Hairy is regulated by phosphorylation in vivo, and if so the identity of the protein kinase(s) that target it.

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We describe studies that implicate protein kinase CK2 as a regulator of repression by Hairless during bristle patterning. CK2 is a highly conserved Ser/Thr protein kinase ubiquitous amongst eukaryotes (reviewed in [8]). Biochemical studies have shown that the catalytic CK2 α subunit exhibits ~20% of the activity of the (α 2 β 2) holoenzyme, while the regulatory CK2 β subunit stimulates phosphorylation and modulates specificity [9,10]. CK2 modifies residues located within micro-acidic domains [11] and targets proteins involved with transcription, cell-cycle progression and signal transduction (reviewed in [8]). *Drosophila* CK2 is structurally similar to its homologs in other organisms [12] and also plays critical roles. Its activities are essential for embryogenesis [13], and for conserved and vital signaling pathways such as Notch [14, 15]. For example, CK2 phosphorylates E(spl)M5, M7, and M8 [14], the downstream nuclear effectors of Notch signaling in neurogenesis. Consistent with multiple functions, null alleles of CK2 α or CK2 β elicit lethality [16,17].

In this report, we characterize the biochemical and genetic interaction of CK2 and Hairless. Using in vitro phosphorylation and pull-down assays, we show that Hairless is a substrate and an interacting partner of the CK2 holoenzyme. Importantly, we show genetic interaction between mutants in CK2 and *h*. Flies trans-heterozygous for alleles of CK2 α and *h* display ectopic bristles (neural hyperplasia) and closely mimic the bristle phenotypes of homozygous mutations in *h*. Similar phenotypes are also elicited in otherwise wild type flies upon simultaneous knockdown of CK2 α + β . Together, these studies identify CK2 as a new interacting partner of Hairless, and implicate a role for phosphorylation in the regulation of Hairless-mediated repression in vivo.

Materials and methods

Purification of *Drosophila* CK2 and phosphorylation of Hairless

Monomeric CK2 α was purified as described [9], and the CK2 holoenzyme (CK2-HoloE) was purified from embryos according to Glover et al [12]. The V_{max} of CK2 α is 0.4 μ mol/min/mg and that of CK2-HoloE is 1.6 μ mol/min/mg, values identical to those reported [10]. GST-Hairless fusion protein was purified to homogeneity as described [14]. Phosphorylation was carried out using 40 ng CK2 α or CK2-HoloE and 2 μ g of GST fusion protein in 50 mM Tris, pH 8.5, 100 mM NaCl, 10 mM MgCl₂, 10 μ M ATP, 5 μ Ci γ ³²P-ATP in a volume of 40 μ l, for 10 min at 25°C, and terminated with 10 μ l of 5x SDS-buffer [10]. Proteins were separated by SDS-PAGE, stained with Coomassie, and the destained gels were exposed to Kodak XAR-5 film. Kinetic constants were determined as described [10].

GST pull-down assays

Purified GST or GST-Hairless (2 μ g) was bound to 25 μ l of glutathione-Sepharose 4B beads overnight at 4°C. The beads were washed with 1.5 ml Wash-buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 5% glycerol, 1 mM PMSF, and 0.1% Triton X-100) to remove unbound proteins. The beads, containing immobilized GST-fusions or beads alone as a mock control, were incubated with 100 ng of purified CK2 α or CK2-HoloE for 3 hrs at 4°C. Unbound CK2 was recovered, and the beads were washed 3 times 5 min each with 0.5 ml of Wash-buffer. Bead-bound and -unbound CK2 was analyzed by Western blots using anti *Drosophila* CK2 antibody (1:1000, [18]) and anti-rabbit IgG coupled to alkaline phosphatase (1:3000).

Phenotypic Analysis

The Gal4 lines and *hairless* alleles were obtained from the Bloomington Stock Center. *UAS-CK2 α -RNAi* and *UAS-Tik* flies have been previously described [19], while *UAS-CK2 β -RNAi* flies were a gift of Rob Jackson (Tufts). Crosses were performed at 25°C, and bristle images were acquired and collated in Adobe Illustrator.

Results

CK2 phosphorylates Hairy

We first investigated the phosphorylation of Hairy using two purified isoforms of CK2. These are, the $\alpha 2\beta 2$ holoenzyme from *Drosophila* embryos (CK2-HoloE), and monomeric CK2 α (Fig. 1A). We find that GST-Hairy is robustly phosphorylated by CK2-HoloE, but weakly by CK2 α (Fig. 1B). The weak phosphorylation of Hairy by CK2 α does not reflect lack of activity, because this preparation efficiently phosphorylates the CK2-peptide (RRREEETEEE, [11]), and is potently stimulated by the activator poly(DL)lysine (not shown). We tested for specificity and found that GST was not modified by CK2 α or CK2-HoloE (Fig. 1B). The preferential phosphorylation of Hairy by CK2-HoloE, suggests that the regulatory CK2 β subunit is important for this interaction.

To rule out the possibility that CK2-HoloE is contaminated with a kinase, other than CK2, and that this enzyme was responsible for phosphorylation of Hairy, we exploited the observations that CK2 is inhibited by Heparin, and that ATP or GTP can serve as phosphoryl donors [12]. We find that phosphorylation is strongly inhibited by 0.25 μ g/ml of Heparin (Fig. 1C). Similarly, a 50% reduction in radiolabeling was observed with 50 μ M cold-GTP (not shown), a concentration that correlates with the K_m of CK2 (65 μ M) for this nucleotide [12]. Together, these results suggest that Hairy is a bona fide in vitro substrate of CK2.

Hairy directly interacts with CK2-HoloE

We next assessed if Hairy forms a direct complex with CK2. Immobilized GST or GST-Hairy were used to pull-down CK2 α or CK2-HoloE, and the bound and unbound fractions were analyzed by Western blots. As expected, neither CK2 α nor CK2-HoloE interacted with free beads (Fig. 1D, lanes 1–4) or with GST-beads (Fig. 1D, lanes 5–8), consistent with our finding that neither isoform phosphorylates this affinity tag (see Fig. 1B). The faint bands marked with an asterisk in lanes 5 and 7 (see Fig. 1D) reflect weak crossreactivity of the CK2 antibody with GST, because their mobility is distinct from CK2 β . Consistent with the phosphorylation analysis, CK2 α weakly interacted with GST-Hairy beads (Fig. 1D, lanes 9 and 10). On the other hand, incubation of GST-Hairy beads with CK2-HoloE (Fig. 1D, lanes 11 and 12) resulted in significant amounts of CK2 in the pellet fraction, demonstrating that Hairy and CK2-HoloE form a direct complex. We estimate (by densitometry) that $\geq 60\%$ of the available CK2-HoloE interacted with Hairy. Since CK2 was present at a low concentration (<5 μ g/ml) and binding assays were conducted at physiological ionic strength (150 mM NaCl), complex formation appears to be efficient. These results show direct and preferential interaction of Hairy with CK2-HoloE. Consistent with a positive role for CK2 β , we find that CK2-HoloE displays ~ 20 -fold higher V_{max}/K_m for phosphorylation, as compared to CK2 α (Fig. 1E).

CK2 genetically interacts with *hairy*

We next tested for genetic interactions between CK2 and *hairy* (*h*), as this would provide compelling in vivo evidence, and, perhaps, uncover the consequences of this phosphorylation. Aside from its roles in embryogenesis, Hairy regulates specification of the macrochaetes (MC's, the large bristles) and microchaetes (mc's, the small bristles), components of the fly peripheral nervous system (PNS). In this regard, the name-giving phenotype reflects the specification of excess MC's and mc's, as attenuated *h* activity elicits a greater density of these two bristle types [2]. For these studies, we employed three alleles, i.e., h^1 , h^2 and h^{41} . Of these, h^1 and h^2 are hypomorphic and, accordingly, $\sim 70\%$ of h^1 and h^2 animals are homozygous viable. In contrast, h^{41} , which terminates Hairy at Cys¹¹⁴, is a strong loss of function and is homozygous lethal [20]. As previously reported, h^1/h^1 or h^2/h^2 animals display ectopic MC's and mc's on the thorax, scutellum and the wing veins, phenotypes that are hallmarks of partial

loss of *h* function (Fig. 2 and see below). We employed *Timekeeper* (*Tik*), a catalytically inactive allele of *CK2α* [16], which is homozygous lethal.

The *Drosophila* wing is characterized by six veins (L0–L6), and bristles at the anterior or posterior wing margins (AWM and PWM, Fig. 2B). In the wild type, bristles are devoid from the wing veins or the inter-vein region (Fig. 2B). In contrast, $\geq 99\%$ of h^1/h^1 or h^2/h^2 flies display bristles along the L2 vein (Fig. 2C, D), to a lesser extent along L5, and interspersed in the inter-vein regions (not shown). These bristles are not seen in flies heterozygous for these alleles of *h* or *Tik* (Fig. 2E–H).

We conducted crosses between *Tik* and *h* alleles (Fig. 2A), and assessed for bristles on the wing veins. As Fig. 2 shows, bristles are manifest with high penetrance (85–89%) along the L2 vein in flies trans-heterozygous for any one of the three alleles of *h* in combination with *Tik* (Fig. 2I–K). In addition, these flies also displayed ectopic bristles (mc's) on the head and halteres (not shown). These phenotypes mimic those of h^1/h^1 or h^2/h^2 animals (Fig. 2C–D, and [2]). In all crosses, the percent viability of trans-heterozygous progeny (h^1/Tik , h^2/Tik , or h^{41}/Tik) was similar to the controls, suggesting that halved dosage of CK2 is not rate limiting for embryonic functions of *Hairy*. No adult eye defects were observed, even though *Hairy* plays a role in eye development [4]. The genetic interactions between *CK2α* and *h* suggest that modification of *Hairy* by CK2 is physiologically relevant for neurogenesis.

Compromising CK2 elicits MC defects that mimic partial *hairy* loss of function

We next assessed whether compromising CK2 levels/activity in wild type flies will phenocopy the bristle defects observed in hypomorphic *h* backgrounds. We employed the Gal4-UAS system [21] to knockdown CK2, and assessed effects on the MC's using three UAS-constructs. Two are RNAi-constructs (*UAS-CK2α-RNAi* and *UAS-CK2β-RNAi*), while the third (*UAS-Tik*), encodes catalytically dead CK2α that behaves as a dominant-negative, i.e., a CK2-DN [19]. Transgenes were expressed with the drivers, *hGal4* or $h^{H10}Gal4$. Importantly, the latter driver is homozygous lethal because the Gal4 insertion has rendered *h* non-functional [22]. Thus *hGal4* enabled us to assess the effects of compromised CK2 in a background wild type (+/+) for *h*, whereas $h^{H10}Gal4$ assessed effects in a background with reduced (halved) *h* dosage.

The scutellum of wild type flies displays four MC's and the absence of mc's (Fig. 3A). In contrast, $\sim 32\%$ of h^1/h^1 animals display ectopic MC's and mc's on the scutellum (Fig. 3B). Similarly, expression of *CK2α-RNAi* or *CK2β-RNAi* by *hGal4* (Fig. 3C–D) elicited ectopic MC's in 31–42% of the flies. Expression of *UAS-Tik* by *hGal4* led to similar effects, but with lower penetrance (Fig. 3E). These phenotypes are sensitive to *h* dosage, because expression with $h^{H10}Gal4$, a background with reduced *h* activity (see above), elicited ectopic MC's with greater severity (42–62%, Fig. 2F–H). The bristle defects in flies heterozygous for *hGal4* or $h^{H10}Gal4$ were ~ 5 –9% (Fig. 3), indicating that these insertions do not elicit significant levels of ectopic MC's. None of the UAS-insertions, by themselves, displayed ectopic MC's (Fig. 3), demonstrating that their expression (by *hGal4* or $h^{H10}Gal4$) was required for the MC phenotypes. The RNAi-constructs are target-specific; their effects are neutralized by co-expression of the corresponding CK2 subunits [19], and are mimicked by the CK2-DN construct (*UAS-Tik*) that is not expected to have non-target effects.

Knockdown of CK2α+β elicits ectopic mc's on the scutellum and wing veins akin to *h* mutants

While RNAi against CK2α or CK2β elicited ectopic MC's, these flies did not exhibit ectopic mc's as in h^1/h^1 animals (compare Fig. 3B, F, G). We thus tested whether RNAi against CK2α+β would elicit ectopic mc's on the scutellum and the L2 vein, and mimic h^1/h^1 (Fig. 4A, Fig 2C), but not h^2/h^2 , $h^2/Tm3$ or $h^1/Tm3$ flies (Fig. 4B and inset). Similar to h^1/h^1 flies,

RNAi against CK2 α + β elicited ectopic mc's on the scutellum and wing veins (Fig. 4C–E). Moreover, these flies displayed mc's along the L2 and L5 veins, both also affected in *h*⁴¹/*Tik* trans-heterozygous flies (Fig. 2K, Fig 4F). Thus, knockdown of CK2 α + β elicits ectopic mc's on the scutellum and along the L2 and L5 wing veins, phenotypes that closely mimic mutations in *h*. Together, these results provide strong evidence that attenuated levels of CK2 elicits phenotypes that mimic *h* loss of function in vivo.

Discussion

The biochemical and genetic studies make a strong case that CK2 and Hairy interact directly, that the latter is a substrate for CK2 phosphorylation, and that reduction of CK2 elicits phenotypes akin to *h* mutants. Importantly, these MC/mc phenotypes are sensitive to *h* gene dosage. The correspondence of these defects to established phenotypes of *h* alleles strongly suggests that CK2 regulates repression by Hairy in vivo. This regulation involves interaction of this repressor with both subunits of CK2. This is the first demonstration that repression by Hairy is regulated by CK2. While this interpretation is consistent with the biochemical and genetic studies, direct evidence for phosphorylation of Hairy in vivo is currently lacking. The restricted developmental context and the absence of suitable antibodies, preclude analysis of this modification in developing tissues, i.e., the wing imaginal disc, where specification of MC's and mc's occurs (see below).

Hairy contains CK2 recognition motifs

A number of substrates of CK2 that have been identified demonstrate that the recognition consensus is S/T-x-x-D/E [23]. Hairy contains two motifs (Fig. 4), both of which conserve an acidic residue at the critical n+3 position. It is noteworthy that both sites are virtually identical in the Hairy protein from *virilis*, a *Drosophila* species that diverged from *melanogaster* ~40–50 Myr ago [24]. We, however, note that Site-1 appears sub-optimal for phosphorylation by CK2, given the presence of a Pro at the n+2 position. Nevertheless, phospho-amino acid analysis shows that Hairy is phosphorylated by CK2 only at Ser residues (data not shown).

Repression by Hairy

As stated above, the roles played by Hairy in specification of the mc's are well known. During the pupal stage, Hairy functions as a pre-pattern factor to restrict expression of proneural transcription factors of the *Achaete-Scute Complex* (*ASC*), whose activities are necessary for specification of the mc's and MC's. This restriction is an important feature of neural patterning.

The onset of neurogenesis in *Drosophila* involves expression of the *ASC* activators, which confer neural potential upon groups of cells called the proneural clusters, PNC's [25–28]. Later, a single cell from each PNC is selected as the sensory organ precursor (SOP), and undergoes asymmetric divisions to give rise to the cell types that characterize these sensory organs [29]. In this context, Hairy restricts *ASC* expression to precise regions of the wing disc, the primordial tissue for the adult notum and wings. Consequently, loss of Hairy leads to expanded expression of *ASC* and ectopic MC's/mc's. Our observations that hetero-allelic combinations of CK2 α and *h*, or CK2 α + β RNAi, mimic the bristle phenotypes of hypomorphic *h* alleles, provides strong evidence that repression by Hairy is sensitive to CK2 dosage.

While CK2 plays roles in the cell cycle [30], it is unlikely that ectopic bristles would manifest in the event of cell cycle arrest. As stated above, bristle development requires three cell divisions of the SOP's. In this case, cell cycle arrest in the PNC's would elicit loss of the SOP's, and manifest as missing bristles. Thus the ectopic MC's/mc's cannot be reconciled with loss of cell viability. More likely, compromised CK2 leads to hypo-phosphorylation of Hairy and impaired restriction of *ASC* expression. This interpretation is consistent with the ectopic MC/

mc phenotypes that we describe (Fig. 2, Fig. 3 and Fig. 4), and that increased dosage of ASC elicits excess PNC's, from which the SOP's and bristles arise.

Implications on repression by Hairy

Our previous studies on the related bHLH repressor E(spl)M8 provide a potential answer. In the case of M8, phosphorylation by CK2 accentuates repression of the bHLH activator Atonal [15]. We have previously proposed that phosphorylation stabilizes a conformation that is permissive for this antagonism. Although the three dimensional structures of E(spl) proteins remain unknown, the CK2-sites are in close proximity to the C-terminus, as is also the case with Hairy. The possibility thus arises that a conformational switch might exert a similar influence on Hairy-mediated repression. Our studies thus expand on the repertoire of binding partners of Hairy, and further implicate a role for CK2 in Hairy-mediated repression in vivo.

Acknowledgements

Supported by National Institutes of Health (EY-015718) to A.P.B. Z.P. was supported by the Jan M. and Eugenia Krol Charitable Foundation.

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Fig. 1. CK2 interacts with and phosphorylates Hairy. (A) SDS-Page gel of purified CK2 α and CK2-HoloE. (B) Phosphorylated proteins were separated (Gel) and autoradiographed (Film). (C) Hairy phosphorylation is inhibited by Heparin. (D) CK2 pull-downs; lanes 1–4 (beads alone, mock), lanes 5–8 (GST-beads), and lanes 9–12 (GST-Hairy beads). CK2 in the bound (P, pellet) and the unbound (S, supernatant) fractions was assessed by Western blot. (E) Km and Vmax are reported as μ M and nmoles/min/mg, respectively.

Fig. 2.

CK2 genetically interacts with *h*. Alleles of *h* (h^1 , h^2 and h^{41}) were tested for interactions with *Tik*, a CK2-DN allele. (A) Schematic of cross. Balanced stocks of *Tik/Tm3, Sb^1, Ser^1* were crossed to h^1/h^1 , h^2/h^2 or $h^{41}/Tm3, Sb^1$ flies. (B) Wild type wing showing the L0–L6 wing veins, anterior/posterior wing margin (A/PWM). (C–K) Wing L2 vein phenotypes. Numbers after genotypes indicate percent flies with L2-vein bristles.

Fig. 3.

Compromised CK2 elicits MC defects that are sensitive to *h* dosage. Scutellar MC and mc phenotypes of wild type (A) and *h¹/h¹* flies (B). UAS-constructs were expressed with *hGal4* (C–E) or *h^{H10}Gal4* (F–H). Numbers denote percent flies with ectopic MC's (arrows). Ectopic mc's in *h¹/h¹* flies are circled. Inset shows the bristle defects of relevant controls.

Fig. 4.

Knockdown of CK2α+β mimics bristle defects of *h* mutants. RNAi against CK2α+β elicits mc's on the scutellum (C) akin to those in *h¹/h¹* (A), but not in *h²/h²* animals (B). Similarly, CK2α+β RNAi elicits ectopic bristles along the L2 and L5 veins (D–E) that mimic *h⁴¹/Tik* flies (Fig. 2K and F). Inset shows genotypes that do not display ectopic mc's on the scutellum, or ectopic bristles along the L2 and L5 wing veins. (G) Schematic showing the CK2 sites in *D. melanogaster* (Dm) and *D. virilis* (Dv) Hairy.