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Protein kinase CK2 is required for Wntless internalization and Wnt secretion

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ABSTRACT

Wnt proteins are lipid modified signaling molecules that have essential functions in development and adult tissue homeostasis. Secretion of Wnt is mediated by the transmembrane protein Wntless, which binds Wnt and transports it from the endoplasmic reticulum to the cell surface for release. To maintain efficient Wnt secretion, Wntless is recycled back to the Golgi and the endoplasmic reticulum through endocytosis and retromer dependent endosome to Golgi transport. We have previously identified protein kinase CK2 (CK2) in a genome-wide screen for regulators of Wnt signaling in *Caenorhabditis elegans*. Here, we show that CK2 function is required in Wnt producing cells for Wnt secretion. This function is evolutionarily conserved, as inhibition of CK2 activity interferes with Wnt5a secretion from mammalian cells. Mechanistically, we show that inhibition of CK2 function results in enhanced plasma membrane localization of Wls in *C. elegans* and mammalian cells, consistent with the notion that CK2 is involved in the regulation of Wls internalization.

1. Introduction

Wnt proteins are members of an evolutionarily conserved family of signaling proteins that have important functions in development and adult tissue homeostasis [1]. The mechanism of Wnt signaling has been the focus of intense investigation for over 30 years. This research has yielded a detailed understanding of the molecular mechanisms of Wnt signal transduction [2]. Wnt signaling can be categorized into a β-catenin dependent Wnt signal transduction route that revolves around the central effector protein β-catenin, which interacts with TCF transcription factors to control the expression of Wnt target genes. Next to this 'canonical' Wnt signal transduction cascade, Wnt proteins can induce β-catenin independent signal transduction pathways [3]. How Wnt proteins are produced and secreted from Wnt sending cells is however much less well understood [4].

Wnt proteins are cysteine-rich secreted proteins with a molecular mass of approximately 40 kDa. Wnts are lipid modified in the endoplasmic reticulum (ER) by the acyl transferase Porcupine [5] and require a specialized secretion pathway that depends on the transmembrane protein Wntless (Wls) to be released from Wnt producing cells [6–8]. It has recently been shown that Wls binds Wnt in the ER and escorts it through the Golgi network to the plasma membrane for release [9]. Next, Wls is retrieved back to the Golgi and the ER to take part in new rounds of Wnt secretion through a retrograde trafficking pathway that involves internalization of Wls from the plasma membrane and retromer dependent transport from endosomes to the trans-Golgi network (TGN) [9–15]. The abrogation of retrograde trafficking and the concomitant lysosomal degradation of Wls cause defects in Wnt secretion and Wnt signaling.

Genetic screens in *Caenorhabditis elegans* have identified genes that are involved in retrograde trafficking of Wls [13,16–18]. The endocytosis of Wls requires the AP2 clathrin adaptor complex, while the transport of Wls from endosomes to the TGN requires the sorting nexin SNX-3 and the cargo selective subcomplex of the retromer complex, which consists of subunits encoded by the genes *vps-29*, *vps-26* and *vps-35*.

We previously identified the β-subunit of the serine–threonine protein kinase CK2 in a genome-wide RNAi screen for novel regulators of Wnt signaling in *C. elegans* [17]. Protein kinase CK2 is implicated in a plethora of biological processes, ranging from the regulation of apoptosis and proliferation to circadian rhythms and signal transduction (reviewed in [19]). Importantly, CK2 has been shown to positively regulate Wnt signaling. CK2 phosphorylates the TCF transcription factor LEF1 [20] and in this way regulates β-catenin dependent transcription. Furthermore, CK2 has been shown to modulate the proteasomal degradation of β-catenin by the phosphorylation of Thr292 of β-catenin [21]. In addition, CK2 can phosphorylate the cytoplasmic Wnt pathway component Disheveled (Dvl) [22]. Dvl is the bifurcation point between β-catenin dependent and β-catenin independent Wnt signal transduction routes and CK2 dependent phosphorylation of Dvl has been proposed to influence both β-catenin dependent and β-catenin independent Wnt signaling [22,23]. In this study, we show that CK2 also has an evolutionary conserved role in Wnt producing cells, where it acts at the level of Wls internalization at the plasma membrane.

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2. Materials and methods

2.1. C. elegans strains and culture

C. elegans strains were cultured at 20 °C using standard conditions as described [24]. Mutant alleles and transgenes used were: *vps-29(tm1320), mig-14(mu71), muIs32[Pmec-7::gfp]* [25], *huIs60[Pegl-20::egl-20::protA]* [16], *huIs72[Pmig-14::mig-14::gfp]* [15], *huSi2[Pmig-14::mig-14::gfp]* [17] and *huEx442[Pmig-14::kin-3(RNAi)]*.

2.2. C. elegans RNAi, transgenesis and imaging

Systemic RNAi by feeding and tissue-specific RNAi by transgene mediated expression of double-stranded RNA (dsRNA) were performed as described [26–28]. To express *kin-3* dsRNA from the *mig-14* promoter, 500 base pair (bp) fragments of coding sequence were PCR amplified from genomic DNA. After PCR fusion to the *mig-14* promoter (in the sense as well as the antisense orientation), the final PCR products were injected in *vps-29(tm1320); muIs32* animals at a concentration of 7 ng/μl with 7 ng/μl *Pmyo2::mCherry* injection marker and 150 ng/μl pBluescript plasmid DNA, yielding the transgene *huEx442[Pmig-14::kin-3(RNAi)]*.

The QL.d migration phenotype was determined when the animals reached the young adult stage. The final position of the QL descendant PVM was scored relative to the vulva in young adult animals as described [25]. ALM polarity was determined in L4 larvae. Animals that displayed at least one ALM with reversed polarity were scored as defective. EGL-20::protA staining was performed as described [16]. MIG-14 protein levels were determined as described [17]. MIG-14 localization was imaged in young adult animals as described [15]. The subcellular localization of MIG-14 was scored blind by 5 lab members and the results were consistent among the different scorings. Antibodies used were anti-goat-Alexa647 (Life Technologies), anti-GFP (BD Livingcolors), anti-alpha-tubulin (Sigma) and anti-mouse-HRP (GE Healthcare).

2.3. Wnt5a secretion

Wnt5a expressing L cells and control L cells were cultured in DMEM with 10% FBS, 5% L-glutamine and 5% penicillin/streptomycin and grown to confluence in 12 well plates. Cells were washed with PBS and culture medium before incubation in culture medium supplemented with 50 μM TBB (Sigma) or DMSO for the indicated time. Conditioned medium was collected and centrifuged for 4 min at 2400 rpm before analysis by standard Western blotting techniques. Antibodies used: anti-Wnt5a (Cell signaling) and anti-rabbit-HRP (GE Healthcare).

2.4. Wls cell surface labeling

HEK293T cells were transfected with Wnt3a and cultured in DMEM with 10% FBS, 5% L-glutamine and 5% penicillin/streptomycin in 15 cm dishes. The culture medium was supplemented with 50 μM TBB (Sigma) or DMSO for 4 h prior to cell surface protein biotinylation using a commercially available kit (Pierce/Thermo) according to the manufacturer's instructions. Cell surface proteins were analyzed by Western blot. Antibodies used: anti-Wls (ab72385-500, Abcam), anti-transferrin-receptor (236-15375, Invitrogen), anti-GAPDH (GAPDH71.1, Sigma), anti-mouse-HRP (GE Healthcare), and anti-chicken-HRP (Abcam).

3. Results and discussion

3.1. CK2 is required for β-catenin dependent and β-catenin independent Wnt signaling in C. elegans

We identified *kin-10* in a genome-wide RNAi screen aimed at identifying novel regulators of Wnt signaling in *C. elegans* [17]. *kin-10* encodes an ortholog of the regulatory β-subunit of protein kinase CK2. To characterize the function of *kin-10* in β-catenin dependent Wnt signaling, we analyzed the effect of *kin-10* knockdown on the Wnt dependent migration of the QL neuroblast descendants (QL.d). During the first stage of larval development, QL and its three descendants (QL.d) migrate from a position in the midbody to distinct positions in the posterior (Fig. 1A). This migration is dependent on the Wnt protein EGL-20, which induces a β-catenin dependent Wnt signal transduction cascade in QL, which results in the expression of the Hox gene *mab-5* [29,30]. *mab-5* in turn directs the migration of the QL.d towards the posterior. When EGL-20 signaling is inhibited, *mab-5* expression fails to be induced and as a consequence, the QL.d migrate in the opposite, anterior direction.

Since *kin-10* is an essential gene [31], we had to rely on partial knockdown to study the function of *kin-10* during post-embryonic development. For this reason, we used a sensitized genetic background to enhance Wnt signaling phenotypes. The background that we used is a mutation in the retromer subunit gene *vps-29*. In *vps-29* mutants, the secretion of EGL-20 is reduced, resulting in a partially penetrant defect in QL.d migration [15,32]. Interfering with Wnt pathway components, or components of the Wnt secretion machinery such as the retromer component *vps-35*, strongly enhances this phenotype (Fig. 1B) [17,32]. We found that the knockdown of *kin-10* also resulted in a significant increase in the percentage of animals with anteriorly displaced QL.d (Fig. 1B). When we subjected *vps-29* mutants to *kin-3* RNAi, which targets the catalytic α-subunit of CK2, we observed a comparable increase in the QL.d migration phenotype (Fig. 1B), suggesting that the CK2 holoenzyme is required for β-catenin dependent Wnt signaling.

Next, we asked if knockdown of CK2 also inhibits β-catenin independent Wnt signaling in *C. elegans*. To this end, we investigated the polarity of the ALM neurons. The ALM neurons direct a long protrusion towards the anterior and a short protrusion posteriorly (Fig. 1C). This process is regulated by the Wnt proteins EGL-20 and CWN-1, but is independent of β-catenin [33,34]. Using a partial loss of function mutation of *mig-14*/Wls as a sensitized genetic background [15], we found that *kin-10* RNAi induces a significant increase in the percentage of animals with defects in ALM polarity (Fig. 1D). Taken together, these results show that *kin-10* is required for β-catenin dependent as well as β-catenin independent Wnt signaling in *C. elegans*.

3.2. CK2 is required for Wnt secretion in C. elegans and mammalian cells

The requirement of CK2 for both β-catenin dependent and β-catenin independent Wnt signaling indicates that CK2 acts upstream in the Wnt pathway, either at a proximal level in the Wnt signal transduction cascade or at the level of Wnt production and secretion in Wnt sending cells. To investigate whether CK2 is required in Wnt producing cells, we specifically knocked down *kin-3* in Wnt producing cells using transgene mediated RNAi [26] and investigated whether this affects the EGL-20 dependent migration of the QL.d in the *vps-29* sensitized genetic background. As shown in Fig. 2A, we observed a mild, but significant increase in the percentage of animals with anteriorly displaced QL.d, indicating that *kin-3* is required in Wnt producing cells.

To investigate whether CK2 is necessary for EGL-20/Wnt secretion, we visualized the EGL-20 gradient using a fusion of EGL-20 with the immunoglobulin binding region of protein A. Staining with fluorescently tagged IgG revealed a punctate gradient of EGL-20 that ranges from the producing cells in the tail to the midbody region [16]. We found that the EGL-20 gradient was markedly reduced when we subjected the animals to *kin-10* RNAi (Fig. 2B) indicating that CK2 is required for the secretion of the Wnt protein EGL-20 in *C. elegans*.

To investigate if the function of CK2 in Wnt secretion is evolutionary conserved, we chemically inhibited CK2 function in Wnt5a producing mouse L cells [35] using the specific CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) [36]. We found that the inhibition of CK2 resulted in a significant reduction in the amount of Wnt5a that is