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Wingless Signaling at Synapses Is Through Cleavage and Nuclear Import of Receptor DFrizzled2

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Abstract

Wingless secretion provides pivotal signals during development by activating transcription of target genes. At *Drosophila* synapses, Wingless is secreted from presynaptic terminals and is required for synaptic growth and differentiation. Wingless binds the seven-pass transmembrane DFrizzled2 receptor, but the ensuing events at synapses are not known. We show that DFrizzled2 is endocytosed from the postsynaptic membrane and transported to the nucleus. The C terminus of DFrizzled2 is cleaved and translocated into the nucleus; the N-terminal region remains just outside the nucleus. Translocation of DFrizzled2-C into the nucleus, but not its cleavage and transport, depends on Wingless signaling. We conclude that, at synapses, Wingless signal transduction occurs through the nuclear localization of DFrizzled2-C for potential transcriptional regulation of synapse development.

Members of the WNT signaling family function in synapse formation and maturation (1–4). In *Drosophila*, the WNT homolog Wingless (Wg) is secreted from presynaptic cells at glutamatergic larval neuromuscular junctions (NMJs) (1). The Wg receptor DFrizzled2 (DFz2) is present in both pre- and postsynaptic cells and is required for synaptic Wg function (1). Wg secretion from the presynaptic cell is crucial for both the formation of active zones (regions where synaptic vesicles accumulate adjacent to the presynaptic membrane) and postsynaptic specializations that are assembled during proliferation of synaptic boutons in larval development (1). How these Wg-dependent signaling events coordinate synapse differentiation remains unknown. To investigate the effect of Wg signaling on the distribution of its receptor and subsequent signal transduction, we used antibodies to the extracellular amino acids 1 to 114 (DFz2-N), and to the intracellular amino acids 600 to 694 (DFz2-C) (fig. S1). Staining of body wall muscles from third instar larvae showed that DFz2-C antibodies labeled the same NMJs as DFz2-N (Fig. 1, A and B) (1).

Antibodies against DFz2-C also labeled spotlike structures within each of the multiple nuclei in each muscle cell (Fig. 1, C to F; fig. S1). Quantification of the number of DFz2-C spots in each nucleus revealed that spots were more numerous in nuclei close to the NMJ

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Supporting Online Material

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Materials and Methods

Figs. S1 to S5

References and Notes

than in those more distal (fig. S1). DFz2-N immunoreactive puncta were observed near the nucleus, but unlike those seen from DFz2-C immunoreactivity, these puncta were much smaller, were localized outside the nuclear boundary, and were never observed inside the nucleus (Fig. 1, G to J). Smaller DFz2-C immunoreactive puncta were also observed at the perinuclear area, but their abundance was low (arrowheads in Fig. 1C).

Intranuclear localization of DFz2-C was confirmed by double-labeling with propidium iodide (PI) and antibodies against the chromatin remodeling protein OSA (5, 6), which associates with chromosomal DNA (Fig. 2, A and B). We also labeled preparations with antibody against HP-1, which labels hetero-chromatin (7). Regions of the nuclei containing HP-1 had either no DFz2-C spots (Fig. 2C) or only marginal coincidence (Fig. 2, D and E), which suggests that DFz2-C spots are mostly excluded from regions of transcriptionally inactive DNA. Nuclear localization of DFz2-C appeared to be cell-type-specific. Although DFz2-C spots were always observed in the nuclei of larval muscles, DFz2-C was not observed in epithelial cells (fig. S1E) or neurons.

To test whether DFz2 might be cleaved, as are some other membrane receptors such as Notch and β -amyloid precursor protein (APP) (8), we transfected *Drosophila* Schneider-2 (S2) cells with full-length DFz2 or with DFz2 fragments containing the N-terminal region (amino acids 1 to 605) or C-terminal region (amino acids 606 to 694). On Western blots of lysate from S2 cells transfected with DFz2, two protein bands were detected, an 83-kD band (full-length DFz2) and an 8-kD band (Fig. 2F). The 83-kD band was recognized by both the DFz2-N and the DFz2-C antibodies, but only the DFz2-C antibody recognized the 8-kD band, which suggested that full-length DFz2 may be cleaved to produce a C-terminal fragment. In extracts of wild-type body wall muscle, full-length DFz2 was detected at very low levels by Western blots, but the 8-kD band was not detected. However, if full-length DFz2 was overexpressed in muscle cells, an 8-kD fragment was detected (Fig. 2G).

We compared the putative amino acid sequence of DFz2 with those of its most related Frizzled counterparts from different species (9), because regions of functional significance are highly conserved across phylogenies. A sequence in the cytoplasmic domain proximal to the transmembrane domain (VWIWSGKTLESW) (10) is virtually identical in all species, from flies to humans, and contains a glutamyl-endopeptidase cleavage site (fig. S2). In eukaryotes, glutamyl-endopeptidase activity is observed in peptidases of the ADAM (a disintegrin and metalloprotease) family (11), and ADAM members have also been implicated in APP (12) and Notch (13) receptor cleavage. Although, in the case of APP and Notch, ADAM proteases cleave the extracellular domain of the proteins, ADAM proteases are also observed intracellularly (14).

We used site-directed mutagenesis to construct three mutants: two deleting the coding sequences for KTLES, which contains the glutamyl endopeptidase cleavage site (Δ KTLES and Δ SGKTLESW), and another mutating the adjacent upstream sequence VWIWSG (DFz2- Δ VWIWSG). The amount of cleavage product was reduced in DFz2- Δ KTLES-expressing S2 cells, and no cleavage product was detected in DFz2- Δ SGKTLESW cells, but DFz2- Δ VWIWSG cells had normal amounts (Fig. 2H). Thus, KTLES is apparently contained in the cleavage site or required for cleavage.

Localization of DFz2-C and DFz2-N fragments into different compartments within and around the nucleus may occur immediately after DFz2 biosynthesis, or DFz2 fragments may translocate to the nucleus through a retrograde pathway after integration into the plasma membrane (fig. S3). To distinguish between these possibilities, we tested whether cell surface DFz2 was internalized and transported to the nucleus. Larvae were dissected, and body wall muscles were incubated in situ in physiological saline containing antibody against

DFz2-N. Under these conditions, the antibody was expected to label only surface DFz2 (15). Then, unbound antibody was washed away, the preparations were fixed, and a secondary antibody conjugated to a blue fluorescent marker (Alexa-647) was added under nonpermeabilizing conditions to detect surface DFz2. To determine whether any cell surface DFz2 had been internalized during the initial incubation, the preparation was permeabilized and then incubated with secondary antibody conjugated to a green fluorescent marker (FITC) (15). A prerequisite for such an experiment is that the antibody should label the extracellular region of DFz2 in situ, and indeed, we found that anti-DFz2-N could label NMJs in situ (Fig. 3A). A fraction of surface-labeled DFz2 was internalized into the muscle and appeared as puncta at the NMJ (Fig. 3, B and C).

To determine whether nuclear DFz2 was derived from receptors that were internalized at the postsynaptic membrane, we conducted an antibody pulse-chase experiment in living preparations. The primary antibody-binding step was done at 4°C to inhibit internalization during antibody incubation. Unbound antibody was washed away, and samples were shifted to room temperature for various time intervals before fixation (Fig. 3; fig. S3). In samples that were fixed after a 5-min shift at room temperature, most of the internalized DFz2 was observed close to the NMJ (Fig. 3, B and C), but after 60 min, little internalized DFz2 was observed at the NMJ (Fig. 3, D to F). There was a comparatively small decrease in surface DFz2 over time at the NMJ, suggesting that only a fraction of labeled DFz2 was internalized (Fig. 3, A and D). Parallel with the changes in DFz2 internalization at the NMJ, at 5 min, minimal internalized DFz2 was observed at the periphery of nuclei (Fig. 3G), whereas at 60 min, the amount of internalized DFz2 at the nuclear periphery was increased (Fig. 3H). Thus, cell surface DFz2 appears to be transported from the plasma membrane to the nucleus.

If cell surface DFz2 is endocytosed and transported to the nucleus, then blocking endocytosis or retrograde vesicle transport should block the nuclear localization of DFz2. Therefore, in a subset of muscle cells, we expressed dominant-negative transgenes that block endocytosis [dominant-negative form of the *Drosophila* Dynamin, Shibire (Shi-DN) (16)] or retrograde transport [dominant-negative form of Glued, a component of the dynein-dynactin complex (17)]. In both cases, the number of DFz2-C spots per nucleus was reduced (Fig. 4A; fig. S4). These results, together with the *in vivo* internalization assays, indicate that DFz2 is internalized from the plasma membrane and is carried by retrograde transport to the nucleus.

We also tested whether Wg signaling was required for DFz2 transport to the nucleus. To decrease Wg signaling, we used a temperature-sensitive *wg^{ts}* mutant, as well as two conditions that disrupt Wg-dependent DFz2 signaling: overexpression of full-length DFz2 in muscles (1, 18) and expressing a DFz2 dominant-negative DFz2 construct (DFz2-DN) (19). We also overexpressed Wg in the pre-synaptic cells, which caused those cells to increase Wg secretion (1, 16, 18). Disrupting Wg signaling caused a decrease in the number of DFz2-C spots inside muscle nuclei (Fig. 4A). In contrast, when presynaptic secretion of Wg was increased, there was an increase in the number of nuclear spots (Fig. 4A; fig. S4).

We also expressed transgenic DFz2 variants in muscles, full-length DFz2, DFz2ΔSGKTLESW, and Myc-NLS-DFz2-C [consisting of a Myc-tagged DFz2-C fragment alone (20) or fused to a nuclear localization sequence]. When DFz2 was overexpressed in muscle, bright DFz2-C immunoreactivity accumulated just outside the nucleus (Fig. 4B; fig. S4), which suggests that overexpression of DFz2 does not disrupt retrograde transport of DFz2, but rather, the nuclear import of DFz2-C. To further test the model that the DFz2 pool transported to the nucleus is derived by endocytosis from the plasma membrane, and not from an internal pool, we simultaneously expressed DFz2 and the Shi-DN in muscle cells. In the presence of Shi-DN, no accumulation of DFz2-C at the perinuclear area was observed

(Fig. 4C; fig. S4). Mutations in the DFz2 cleavage site did not alter the endocytosis of DFz2, because expression of transgenic DFz2ΔSGKTLESW in muscles did not suppress the accumulation of perinuclear DFz2-C spots (although it did not enter the nucleus), and the perinuclear spots had a distribution that was indistinguishable from that of cells expressing transgenic wild-type DFz2. Muscle cells expressing the DFz2-C transgenes showed diffuse Myc immunoreactivity in the cytoplasm and nuclei.

We also tested whether expressing DFz2, DFz2ΔSGKTLESW, or DFz2-C could rescue the synaptic phenotypes of a mutant of the *dfz2* gene (*dfz2^{C1}/Dfz2*) (1). Interfering with DFz2 function prevents the proliferation of synaptic boutons and the formation of pre- and postsynaptic specializations in many boutons (1). Like *wg^{ts}* mutants, *dfz2^{C1}/Dfz2* NMJs had irregular and tightly spaced boutons and a reduced number of boutons (Fig. 4D; fig. S5).

Expression of DFz2 in a *dfz2^{C1}/Dfz2* mutant background completely rescued the decrease in bouton number and partially restored the abnormal morphology of the boutons (Fig. 4D; fig. S5). It also restored the presence of nuclear spots in the mutant larvae. In contrast, only a slight rescue was observed when DFz2ΔSGKTLESW was expressed, and no rescue was detected when Myc-NLS-DFz2-C was expressed (Fig. 4D; fig. S5). Thus, cleavage of DFz2 appears to be needed for DFz2 signaling at the NMJ, and DFz2-C is necessary but not sufficient for DFz2 function. The slight rescuing activity observed in *dfz2^{C1}/Dfz2* mutants expressing DFz2ΔSGKTLESW may indicate that not all of DFz2's function at the NMJ is accomplished through DFz2 cleavage and nuclear import (21).

To test whether Wg is required for nuclear import of DFz2, we treated DFz2-transfected S2 cells with conditioned medium containing soluble Wg (20, 22). In the presence of Wg, prominent immunoreactive spots were detected inside the nucleus of DFz2-transfected cells, but not in DFz2-ΔSGKTLESW-transfected cells or in transfected cells not exposed to Wg-conditioned medium (Fig. 4, E to G; fig. S4).

Our results indicate that, at the *Drosophila* NMJ, Wg secretion initiates a signaling mechanism, whereby DFz2 receptors at the post-synaptic muscle membrane are endocytosed and undergo retrograde transport to the nucleus. The C-terminal fragment is cleaved during this process and is ultimately transported into the nucleus. We propose that Wg binding to DFz2 may initiate an event that marks the DFz2 C-terminal region. Endocytosed vesicles containing the entire DFz2 receptor travel toward the nucleus. Once at the periphery of muscle nuclei, the C terminus is cleaved, and only marked C-terminal fragments are imported into the muscle nuclei, where they may regulate gene transcription. Our studies help unravel a mechanism by which pre- and postsynaptic cells communicate during the coordinated growth and maturation of synaptic specializations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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10. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Fig. 1.

Localization of DFz2-C and DFz2-N to the same NMJs, but to different subcellular compartments, inside the nucleus (DFz2-C) and at the perinuclear region (DFz2-N). (**A** and **B**) Wild-type third instar NMJs from muscles 6 and 7 (A3) stained with antibodies against (A) DFz2-C and (B) DFz2-N. (**C** through **J**) Representative muscle nuclei at muscle 6 (A3) in preparations double-stained with antibodies against (C through F) DFz2-C (green) and tubulin (red) and (G through J) DFz2-N (blue) and tubulin (Tub) (red). Note that (C to F) and (G to J) were obtained from different preparations. (C, D, G, H) show a confocal slice at a focal plane through the nuclear-cytoplasmic boundary (defined by the microtubular array; tangential), and (E, F, I, J) a confocal slice at a focal plane midway through the nucleus (medial). N, nucleus, arrowheads in C point to cytoplasmic DFz2-C. Scale bar, 9 μm in (A and B), and 8 μm in (C to J).

Fig. 2.

Localization of DFz2-C to euchromatin and evidence for cleavage of DFz2. (**A** and **B**) Colocalization of (A) DFz2-C (green) and PI (red) and (B) DFz2-C and OSA. In (**C** to **E**), muscle nuclei were double-labeled with antibodies against the heterochromatin-specific protein HP-1 (red) and DFz2-C (green). Arrows point to regions of adjacent DFz2-C and HP-1 immunoreactivity. Scale bar, 9 μm in (A to C), and 6 μm in (D and E). (**F**) Western blot of S2 cells transfected with full-length DFz2, DFz2-N, and DFz2-C. (**G**) Western blots of body wall muscle extracts from wild-type larvae and larvae overexpressing full-length DFz2. (**H**) Western blot of S2 cells and S2 cells transfected with DFz2 constructs. Blots were sequentially probed with antibodies to DFz2-C, DFz2-N, and tubulin.

Fig. 3.

In vivo transport of DFz2 from the cell surface to the nucleus. (**A to F**) show anti-DFz2-N immunoreactivity at NMJs during the in vivo DFz2 internalization assay. (**A and D**) Surface DFz2 (blue) and (**B and E**) internalized DFz2 (magenta) are shown at 5 and 60 min after pulse-labeling. (**C and F**) show the merged blue and magenta channels. (**G and H**) DFz2-N immunoreactivity around a muscle nucleus at 5 and 60 min after pulse-labeling. Scale bar, 9 μ m in (**A to F**), and 6 μ m in (**G and H**).

Fig. 4.

Role of endocytosis, retrograde transport, and Wg signaling in DFz2-C nuclear import. **(A)** Mean number of nuclear spots per nucleus in different genotypes. **(B and C)** Muscle nuclei in larva expressing **(B)** DFz2 and **(C)** DFz2 and Shi-DN in the muscles. Tissues were double-labeled with antibodies against tubulin and DFz2-C. Arrow in **(B)** points to DFz2-C spots that accumulate just outside of the nuclear perimeter. **(D)** Number of synaptic boutons in wild type and *dfz2^{C1}/Dfz2* mutants expressing various DFz2 transgenes in muscle as indicated. ****P* < 0.0001 compared with wild type [ANOVA (20)]. **(E to G)** DFz2-C and tubulin immunoreactivity in **(E)** S2 cells, and **[(F and G)]** S2 transfected with DFz2, and treated with and without Wg-conditioned medium. Scale bar, 9 μ m in **(B)** and **(C)**, and 7 μ m in **(E)** to **(G)**.