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Targeted mutagenesis and functional analysis of adipokinetic hormone-encoding gene in *Drosophila*☆

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ABSTRACT

Adipokinetic hormones (Akhs) are small peptides (8–10 amino acid [aa] residues long) found in insects that regulate metabolic responses to stress by stimulating catabolic reactions and mobilizing energy stores. We employed Transcription activator-like effector nuclease (TALEN) mutagenesis and isolated an *Akh*^{1} mutant carrying a small deletion in the gene that resulted in a truncated peptide; the second aa (Leu) was missing from the functional octapeptide. This null *Dmel/Akh* mutant is suitable to study Akh function without any effect on the C-terminal associated peptide encoded by the same gene. The mutant flies were fully viable and compared to the control flies, had significantly low levels of hemolymph saccharides including trehalose and were resistant to starvation. These characteristics are similar to those obtained from the flies carrying targeted ablation of Akh-expressing neurons (reported earlier). We also found that the *Akh*^{1} mutants are slightly heavy and had a slow metabolic rate. Furthermore, we showed that the ectopic expression of *Dmel|Akh* reverses the *Akh*^{1} phenotype and restores the wild-type characteristics. Our results confirmed that Akh is an important regulator of metabolic homeostasis in *Drosophila*.

1. Introduction

Maintenance of a constant internal environment, homeostasis, is a major role of the neuroendocrine system that allows organisms to adapt to perturbations in the external environment (e.g., temperature, danger, or toxins) as well as in their internal milieu (e.g., high physical activity, injury, disease, or malnutrition) (Storey, 2004). Animals exposed to various adverse conditions protect themselves via stress responses, which involve suppression of feeding and reproduction, an increase in heart rate and gas exchange, mobilization of stored energy sources and redirection of oxygen and nutrients to the stressed body parts. The humoral stress response in mammals is controlled by the hypothalamic–pituitary–adrenal axis with the help of a variety of specific hormones. The responses to stress conditions in insects are controlled at biochemical, physiological and behavioral levels predominantly by adipokinetic hormones (Akhs) (Gade and Goldsworthy, 2003; Kodrik, 2008).

Akhs are small peptides (8–10 amino acids) implicated in the adjustment of insect homeostasis via metabolic control and a number of additional pleiotropic effects (Gade and Goldsworthy, 2003; Gade et al., 1997; Kodrik, 2008; Krishnan and Kodrík, 2012). Generally, the Akh effects can be subdivided into 3 major categories: (1) metabolic activities (lipid and carbohydrate mobilization as well as stimulation of proline synthesis, via activation of the pertinent enzymes), (2) additional biochemical activities (inhibition of lipid, protein, and RNA syntheses and activation of antioxidant defense mechanisms), and (3) physiological activities (stimulation of heart beat, an increase in muscle tone and general locomotion, enhancement of the immune response, regulation of starvation-induced foraging behavior, stimulation of digestive processes, and inhibition of egg maturation).

The evolutionarily closest peptides found in vertebrates are gonadotropin-releasing hormones (Lindemans et al., 2011). At the functional level, insect Akhs rather resemble mammalian glucagon,

☆ Note: Several acronyms are used for the *Drosophila* adipokinetic hormone in literature: Drome-AKH, Drm-AKH and Dmel\Akh; the latter one is used in this paper.

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a peptidic hormone produced by α-islets of the vertebrate pancreas. The main function of glucagon in the vertebrate body is the mobilization of energy reserves, mainly glucose, and thereby participation in the control of the glucose blood level under both physiological and stress conditions (Jones et al., 2012). Nevertheless, the control of carbohydrate level in the insect hemolymph is less accurate and insects can tolerate substantial fluctuations in the concentrations of these metabolites (Chapman, 1998). There is some immunological evidence that suggests the presence of endogenous glucagon-like peptides in several insect species (Alquicer et al., 2009; Kramer et al., 1980). In spite of some earlier promising indications of their influence on the whole-body glycogen level (Tager et al., 1976), these metabolic effects were not confirmed.

There is a single *Drosophila melanogaster Akh* gene (*Dmel|Akh*) that encodes the peptide hormone precursor of 79 amino acid residues (aa) including the N-terminal signal peptide (22 aa), the active Akh octapeptide, and the 49-aa carboxy-terminal Akh-associated peptide (function unknown) (Noyes et al., 1995). The sequence of the mature Akh octapeptide is pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH_2, where pGlu is pyroglutamic acid, and Trp-NH_2 is tryptophan carboxyamide (Schaffer et al., 1990). The Akh peptides are produced and stored in the corpus cardiacum (CC) or in the corresponding CC cells of dipteran ring glands from where they are released into the hemolymph when necessary.

The single *Drosophila* Akh receptor (*Dmel|AkhR*) was cloned and its ligand specificity was determined (Park et al., 2002; Staubli et al., 2002). The importance of individual aa in the Dmel\Akh octapeptide for the AkhR signaling was recently elucidated; it was shown that the aa at positions 2, 3, 4, and 5 are crucial for receptor activation (Caers et al., 2012). The Dmel\AkhR belongs to the G-protein coupled receptor family (GPCRs) and is located in the plasma membrane of fat body adipocytes (Caers et al., 2012). It was shown recently that *Dmel|AkhR* uses G protein αq subunit (Gαq) and evokes Ca^{2+} release as a second messenger system in a *Drosophila* adult fat body (Baumbach et al., 2014).

There is a long-standing problem with genetic analysis of peptidic hormones in *Drosophil*a because of the lack of mutants. Progress in molecular genetic methods allows researchers to carry out targeted ablation of Akh-expressing neurons to obtain Akh-cell-deficient (Akh-CD) flies; those flies were shown to have significantly lower levels of hemolymph trehalose than the control flies (Lee and Park, 2004). In addition, ectopic *Dmel|Akh* expression was also performed (Lee and Park, 2004). These experiments yielded the first data on the functional analysis of *Dmel|Akh*, including physiological and behavioral phenotypes. Nonetheless, the specificity of these phenotypes needs to be confirmed because many neurosecretory cells produce multiple neuropeptides (Kahsai et al., 2010), and thus the ablation of Akh-producing cells might lead to the loss in the production of some other neuropeptides and therefore, eliminate their functions. The recent progress in gene targeting methods using engineered nucleases allows to create precise double-stranded breaks in the genome and generate mutants fast by erroneous joining of nonhomologous ends or via homology-driven repair pathways (Beumer and Carroll, 2014).

In this study, we report the targeted mutagenesis of *Dmel|Akh* using engineered nucleases as well as characterization of the *Dmel|Akh* mutant. The germline targeting of *Dmel|Akh* was performed by means of a specific TALEN pair. We created the *Dmel|Akh* mutant Akh^1 carrying a 3-bp deletion leading to a loss of the second aa in the Dmel\Akh octapeptide, leaving the remaining carboxy-terminal Akh-associated peptide intact. The Akh^1 mutant showed similar hypotrehalosemia and resistance to starvation-induced death as *Akh-CD* flies. We were able to reverse the Akh^1 mutant phenotype via ectopic expression of Akh using the *UAS-GAL4* expression system.

2. Material and methods

2.1. Drosophila stocks and crosses

The flies were maintained on standard corn meal/yeast/sucrose/agar diet and experiments were performed at 25 °C. If not explicitly specified, all *Drosophila* strains were obtained from the Bloomington Stock Center. Flies w^{1118} were used for the gene targeting using TALEN RNA microinjection. All assays were carried out on 3-day old adult males in order to decrease age-related variability of physiological responses and for compatibility with earlier studies.

The isolation of mutants was performed as described in an earlier study (Takasu et al., 2014). Briefly, the *Drosophila* eggs were microinjected with poly(A) RNAs encoding the TALEN pair. The F_0 flies were crossed individually in separate vials to the *w*; +; *TM3, Ser e|TM6B, Hu Tb e* tester flies containing 2 balancers for the third chromosome. Fifteen to twenty late-F_1 embryos or larvae from the broods in each vial were used for DNA isolation and pilot assays of TALEN activity as described below. Five to six adult F_1 flies from the vials, which produced positive results in PCR tests, were used for further experiments. These F_1 flies were first individually crossed to the *w*; +; *TM3, Ser e|TM6B, Hu Tb e* tester flies and used for PCR genotyping after they had produced F_2 offspring (Fig S1). The broods of F_2 vials were genotyped and flies from one of the test-positive vials were selected for further analysis.

To create flies carrying the construct to rescue the Akh^1 mutant phenotype by ectopic expression of transgenic $UAS\text{-}Akh^+$, the original Akh^1 mutant was combined with the *Act-Gal4* driver and UAS-Akh transgene by crossing w^{1118}; *Act-Gal4|CyO, Act-GFP*; Akh^1 flies to w^{1118}; $UAS\text{-}Akh^+$; Akh^1 ones. We call these flies (which ectopically express Akh) *EE-Akh*. We selected the *Akh*-expressing flies (w^{1118}; *Act-Gal4|UAS-Akh*$^+$; Akh^1) based on the absence of green fluorescence (or the *Cy* marker in adults) among the F_1 progeny. The source of $UAS\text{-}Akh^+$ was the Bloomington line #27343, y^1 *w*; *P{UAS-Akh.L}2*. The *Act-Gal4* insertion line (*w*; *Act-Gal4|CyO, tub-GFP*) was received from Dr. T. Dolezal.

The strains *y w UAS-rpr*; +; + (from Dr. E. Hafen) and *w*; +; *Akh-Gal4* (from Drs. G. Lee and J. H. Park) were crossed to obtain *y w UAS-rpr/Y*; *Akh-Gal4*/+ male and *y w UAS-rpr*/+; *Akh-Gal4*/+ female F_1 progeny referred to below as *AKH-CD* flies.

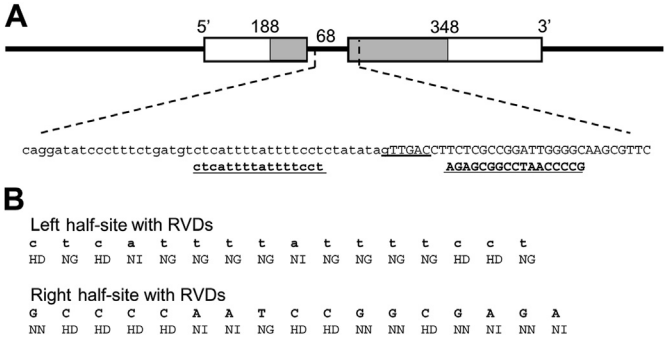

Fig. 1. TALEN target site. (**A**) Position of TALEN target in the *Dmel/Akh* gene. Rectangles and numbers above represent exons 1 and 2 and their length in bp. The empty boxes are the 5′and 3′ UTRs. The sequence below the line indicates part of intron 1 (small letters) and exon 2 (capital letters). *Hinc* II restriction site is underlined. Bold underlined sequences below represent the targeted half-sites. (**B**) The target recognition sequences (bold) are shown together with the left and right repeat-variable diresidues (RVD) specific for the nucleotides.