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Sequences that direct subcellular traffic of the *Drosophila* methoprene-tolerant protein (MET) are located predominantly in the PAS domains

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ABSTRACT

Methoprene-tolerant protein (MET) is a key mediator of antimetamorphic signaling in insects. MET belongs to the family of bHLH-PAS transcription factors which regulate gene expression and determine essential physiological and developmental processes. The ability of many bHLH-PAS proteins to carry out their functions is related to the patterns of intracellular trafficking, which are determined by specific sequences and indicate that a nuclear localization signal (NLS) or a nuclear export signal (NES) is present and active. Therefore, the identification of NLS and NES signals is fundamental in order to understand the intracellular signaling role of MET. Nevertheless, data on the intracellular trafficking of MET are inconsistent, and until now there hasn't been any data on potential NLS and NES sequences. To analyze the trafficking of MET we designed a number of expression vectors encoding full-length MET, as well as various derivatives, that were fused to yellow fluorescent protein (YFP). Confocal microscopy analysis of the subcellular distribution of YFP–MET indicated that while this protein was localized mainly in the nucleus, it was also observed in the cytoplasm. This suggested the presence of both an NLS and NES in MET. Our work has shown that each of the two PAS domains of MET (PAS-A and PAS-B, respectively) contain one NLS and one NES sequence. Additional NES activity was present in the C-terminal fragment. The NLS activity located in PAS-B was dependent on the presence of juvenile hormone (JH), the potential ligand for MET. In contrast to this, JH didn't seem to be required for the NLS in PAS-A to be active. However, on the basis of current knowledge about the function of PAS-A in other bHLH-PAS proteins, we suggest there might be other proteins that control the activity of the NLS and possibly the NES located in the PAS-A of MET. Thus, the intracellular trafficking of MET seems to be regulated by a rather complicated network of different factors.

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1. Introduction

In contrast to the complexity of vertebrate hormone signaling pathways, *Drosophila melanogaster* has only two known physiologically active lipophilic hormones, the steroid hormone 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH). They regulate insect development, reproduction, and other important biological processes (Dubrovsky, 2005; Gruntenko and Rauschenbach, 2008). Furthermore, the fly genome contains much fewer nuclear receptors genes than the human genome. Nevertheless, all the main nuclear receptor subfamilies are represented in *D. melanogaster* nuclear receptors (King-Jones and Thummel, 2005). This together with the well-established genetic and genomic tools for studying the biology of *D. melanogaster* makes this insect an ideal model system for characterizing nuclear receptor function and regulation. The molecular mechanisms of 20E action have been extensively studied (Beckstead et al., 2007). In contrast, the mechanisms of JH signaling are poorly understood and many basic questions remain unanswered (Davey, 2000; Dubrovsky, 2005; Flatt et al., 2005; Restifo and Wilson, 1998; Wilson et al., 2006a,b). At the molecular level JH is known to modify or suppress the expression of genes involved in 20E signal transduction (Dubrovsky et al., 2000; Restifo and Wilson, 1998). Recently, the JH response element has been identified (Li et al., 2007) as well as genes whose expression is directly influenced by JH (Zhou et al., 2002). Additionally, it has been shown that the response for JH regulation can be

Abbreviations: 20E, 20-hydroxyecdysone; CFP, cyan fluorescent protein; EcR, ecdysteroid receptor; GFP, green fluorescent protein; GCE, germ cells expressed; NES, nuclear export signal; NLS, nuclear localization signal; Usp, ultraspiracle; YFP, yellow fluorescent protein; JH, juvenile hormone; MET, methoprene-tolerant; bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim (period-aryl hydrocarbon receptor nuclear translocator-single-minded); FKBP39, 39 kDa FK506-binding nuclear protein; Chd64, calponin-like protein Chd64; SRC, steroid receptor co-activator.

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modified by 20E. Thus, the interplay between JH and 20E is more complex than previously thought (Beckstead et al., 2007).

Many attempts have been made to identify JH receptors. One of the candidates for this function is methoprene-tolerant protein (MET) (Ashok et al., 1998; Miura et al., 2005; Shemshedini et al., 1990). The *Met* gene was identified by screening mutagenized *D. melanogaster* larvae for both the toxic and morphogenetic effects of methoprene (Shemshedini and Wilson, 1990; Wilson and Ashok, 1998; Wilson and Fabian, 1986). It has been shown that MET binds JH in physiological concentrations (Miura et al., 2005; Shemshedini et al., 1990). Initially, it was difficult to believe that MET was a JH receptor because of the fact that *D. melanogaster* MET null mutants were viable (Wilson, 1996; Wilson et al., 2006a,b). However, Konopova and Jindra (2007) have documented that MET is an essential mediator of antimetamorphic JH signaling in *Tribolium castaneum*, which supports its putative role as a JH receptor (Parthasarathy and Palli, 2009; Parthasarathy et al., 2008). It has been shown that MET is able to interact with its homolog, germ cell expressed (GCE) protein (Godlewski et al., 2006). Additionally, MET interacts with proteins involved in the signal transduction of ecdysteroids, i.e., ecdysteroid receptor (EcR), ultraspiracle (USP), 39 kDa FK506-binding nuclear protein (FKBP39), calponin-like protein Chd64 (Chd64) (Bitra and Palli, 2009; Li et al., 2007) and steroid receptor co-activator (SRC) (Li et al., 2011; Zhang et al., 2011).

As revealed from a comparison of the MET sequence to the sequences of proteins deposited in databases, MET has been classified as a member of the family of bHLH-PAS transcription factors (Ashok et al., 1998). The domains of both bHLH (basic helix-loop-helix) and PAS (named for Per-Arnt-Sim, the first three proteins from this family) play important roles in the transcriptional activity of these kinds of transcription factors (Gu et al., 2000; Kewley et al., 2004). Interestingly, the MET sequence exhibits higher homology to vertebrates than other *D. melanogaster* bHLH-PAS proteins (Ashok et al., 1998).

The bHLH-PAS proteins are critical regulators of the gene expression networks that are responsible for many essential physiological and developmental processes in invertebrates (Furness et al., 2007). For example, the aryl hydrocarbon receptor (AhR) regulates transcriptional responses to environmental pollutants (Hahn, 1998) and has been shown to be a modulator of anti-viral immunity (Head and Lawrence, 2009). The hypoxia inducible factor (HIF) is a bHLH-PAS receptor for low oxygen tension (Bracken et al., 2003; Déry et al., 2005), whereas single-minded (Sim) is responsible for neuronal development (Chrast et al., 1997; Nambu et al., 1991). Many aspects of the functional control of bHLH-PAS proteins are analogous to those defined for nuclear receptor signaling (Kewley et al., 2004). The bHLH-PAS proteins usually dimerise to form functional DNA binding complexes. The PAS domain shows the specificity of the complex formation and the distinct recognition of target genes (Chapman-Smith and Whitelaw, 2006; Zelzer et al., 1997).

The ability to localize and translocate proteins to specific cellular compartments is fundamental to the organization and functioning of all living cells. For a number of transcription factors that mediate inducible gene regulation in response to extracellular signals, translocation from the cytoplasm to the nucleus is an important event, enabling the transcription factors to recruit co-activators (Kallio et al., 1998; Lee and Hannink, 2003). For example, it has been shown that steroid/nuclear receptors continuously shuttle between the cytoplasm and the nucleus and that their localization at any given point in time is a consequence of the fine balance between the operational strength of the sequences for the nuclear localization signal (NLS) and the nuclear export signal (NES) (Kumar et al., 2006). In the case of AhR, which belongs to the bHLH-PAS family, its subcellular localization is differentially regulated by one NLS and two NESs and depends on the interaction of other proteins (Berg and Pongratz, 2001; Ikuta et al., 1998; Kawajiri and Fujii-Kuriyama, 2007). Current knowledge about the subcellular traffic of MET has been very limited. The first study on the localization of the MET protein was done by analyzing JH binding in larval fat bodies, and localization was determined to be in the fat body cytosol (Shemshedini et al., 1990). The photoaffinity labeling technique made it possible to localize MET both in cytoplasmic (Shemshedini and Wilson, 1990) and nuclear (Shemshedini and Wilson, 1993) fractions of *D. melanogaster* cells. In contrast, immunolocalization studies of MET in a variety of *D. melanogaster* tissues showed this protein to be exclusively nuclear (Pursley et al., 2000). Miura et al. (2005) confirmed this using *Drosophila* Schneider cells and GFP (green fluorescent protein)-labeled MET.

In order to better determine the presence of NLS and NES signals in the MET protein we decided to use mammalian cells, which do not produce juvenile hormone and 20-hydroxyecdysone. These cells are also devoid of some insect cell-specific endogenous proteins like GCE and the EcR/USP complex, which could influence results obtained in insect cells.

Recently, mammalian cells have been successfully used to analyze the subcellular trafficking of EcR and Usp, nuclear receptors from *D. melanogaster* (Dutko-Gwóźdź et al., 2008; Gwóźdź et al., 2007; Nieva et al., 2005, 2007, 2008). Similarly, as was described for EcR and Usp, we decided to investigate the subcellular distribution of MET in living cells using yellow and cyan fluorescent proteins (YFP, CFP), which have been shown to be useful tags for monitoring the subcellular distribution of various proteins in living cells (Chalfie et al., 1994).

This study is the first detailed characterization of the subcellular traffic of *D. melanogaster* methoprene-tolerant protein. We have shown that MET contains both NLS and NES activities which are localized in the PAS-A and PAS-B regions of MET. For the first time, we have documented that MET can translocate from the cytoplasm to the nucleus, and this process seems to be mediated through a mosaic of elements in a JH-dependent and/or JH-independent manner.

2. Materials and methods

2.1. Plasmid construction

MET cDNA from *D. melanogaster* was a kind gift from Prof. Thomas G. Wilson (Department of Entomology, Ohio State University, USA). Full-length cDNA, encoding amino acid residues 1–716, was amplified by PCR and cloned into the *Eco*RI and *Sma*I restriction sites of the MCS of the pEYFP-C1, pEYFP-N1, pECFP-C1 and pECFP-N1 vectors. Deletion mutants of MET were cloned analogically in the pEYFP-C1 vector. Primers used for PCR are listed in Table 1 (Supplementary data). DNA constructs: YFP–MET/R98A/K102A, YFP–MET34–190/R98A/K102A and YFP–MET98–508/R98A/K102A, were obtained analogically to the full-length and deletion mutants, and for the PCR reaction, the mutated template MET/R98A/K102A was used thanks to a gift from Jakub Godlewski (Department of Entomology, Ohio State University, USA). The point mutants listed in Table 2 (Supplementary data) were obtained by the PCR of point mutation insertions according to Ko and Ma (2005) and cloned with *Lgu*I, *Eco*RI and *Sma*I restriction enzymes. All constructs were verified by DNA sequencing.

2.2. Cell culture and DNA transfection

Chinese hamster ovary cells (CHO-K1), (ATCC CCL-61) were maintained in Ham's F12 medium. African green monkey kidney fibroblasts COS-7 (ATCC CRL-1651) and human cervix adenocarci-

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