RESEARCH ARTICLE

The insulator protein CTCF regulates Drosophila steroidogenesis
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ABSTRACT

The steroid hormone ecdysone is a central regulator of insect development. In this report we show that CTCF expression in the prothoracic gland is required for full transcriptional activation of the Halloween genes spookier, shadow and noppera-bo, which encode ecdysone biosynthetic enzymes, and for proper timing of ecdysone-responsive gene expression. Loss of CTCF results in delayed and less synchronized larval development that can only be rescued by feeding larvae with both, the steroid hormone 20-hydroxyecdysone and cholesterol. Moreover, CTCF-knockdown in prothoracic gland cells leads to increased lipid accumulation. In conclusion, the insulator protein CTCF is required for Halloween gene expression and cholesterol homeostasis in ecdysone-producing cells controlling steroidogenesis.

KEY WORDS: CTCF, Ecdysone, Halloween genes, Developmental timing

INTRODUCTION

The proper development of multicellular organisms requires accurate timing of specific developmental programs of gene expression. These timed programs involve signaling systems that respond to nutritional and environmental cues to direct coordinated developmental responses throughout the animal, such as transitions in morphology. In insects, these transitions include molting and metamorphosis which occur at regularly defined intervals and depend on pulses of the steroid hormone ecdysone (Yamanaka et al., 2013). During larval and pupal stages of insect development, the prothoracic gland (PG) is the tissue responsible for the synthesis of ecdysone that is secreted and converted into the active steroid molting hormone 20-hydroxyecdysone (20E) in target tissues, where it induces the activation of target genes. The neuropeptide protoracicotropic hormone (PTTH) is produced by two pairs of lateral neurosecretory cells in the insect brain and is released to signal to the PG (McBrayer et al., 2007). PTTH, through activation of its receptor torso and the mitogen-activated protein kinase (MAPK) cascade (Rewitz et al., 2009), leads to increased activation of its receptor torso and the mitogen-activated protein kinase (MAPK) cascade (Rewitz et al., 2009), leads to increased ecdysone biosynthetic enzymes encoded by the Halloween gene family (Gilbert, 2004). Transcriptional regulation of these genes is still poorly understood, although some transcription factors and chromatin modifiers have been recently identified for some of the biosynthetic genes (Danielsen et al., 2014; Deng and Kerppola, 2013; Moeller et al., 2013; Pankotai et al., 2010; Parvy et al., 2005). Halloween genes trigger the production of ecdysone from dietary cholesterol or phytosterols, since arthropods are unable to synthesize cholesterol. In recent years, evidence has accumulated that other factors in addition to PTTH, especially nutritional signals, act on the PG to control ecdysteroidogenesis (Layalle et al., 2008).

The insulator protein CTCF is a highly conserved zinc finger protein that has been shown to play an essential role in regulation of chromatin organization and gene expression during development both, in Drosophila and mammals (Herold et al., 2012). Recent data supports a role for insulator proteins in the organization of developmentally regulated intra- and interchromosomal contacts that are responsible for the diverse functions of CTCF in gene regulation, including context-dependent transcriptional activation, repression, insulation and imprinting (Phillips and Corces, 2009).

In this paper we show that CTCF is required for Halloween gene expression and cholesterol homeostasis in the prothoracic gland controlling steroidogenesis in Drosophila.

RESULTS AND DISCUSSION

CTCF controls the timing of larval development

The analysis of Drosophila developmental timing in CTCF<sup>y+6</sup> null mutant background (Gerasimova et al., 2007) revealed that larval development was prolonged by two days on average in those mutant animals that progressed to the larval/pupal transition (Fig. 1A). We confirmed that the developmental delay in these animals was due to the loss of CTCF by performing rescue experiments using the UAS/Gal4 system. It has been reported that expression of UAS-CTCF with ubiquitous drivers lead to embryonic lethality while a single heat-shock during larval development of animals containing a hsp70-Gal4 transgene (hs>+) was sufficient to phenotypically rescue the majority of CTCF mutant flies (Mohan et al., 2007). Our analyses showed that a short pulse of CTCF significantly rescued the developmental delay in CTCF<sup>y+6</sup> mutants (supplementary material Fig. S1). Indeed, although 90% of CTCF<sup>y+6</sup> larvae died before pupariation after a 20 min heat-shock, some larvae did reach the pupal stage but pupariation was delayed by more than 3 days. CTCF expression was able to partially rescue both, lethality (around 75% of mutant larval pupariate, data not shown) and, importantly, developmental delay (pupariation was clearly advanced by CTCF expression in the CTCF<sup>y+6</sup> mutant background, supplementary material Fig. S1).

The delayed pupariation of CTCF<sup>y+6</sup> mutants suggested that the ecdysone signaling pathway might be affected in these animals. Since the insulator protein CTCF plays an essential role in regulation of gene expression, we analyzed the transcript levels of components acting at different steps in ecdysone signaling pathway. It has been previously reported that the ecdysone-induced protein 75B locus contains a CTCF site involved in recruitment of the insulator protein CP190 in response to the hormone and proper activation of gene expression (Wood et al., 2011). Thus, we analyzed the expression of the ecdysone-responsive gene E75B during larval development. We also
analyzed the transcript levels of the Ecdysone receptor (EcR), which is activated by hormone to regulate expression of ecdysone-responsive genes, PTTH, which is the peptide hormone that initiates steroid production, and the Halloween genes spookier (spok) (Ono et al., 2006) and disembodied (dib) (Chavez et al., 2000), two ecdysone biosynthetic enzymes expressed in the PG. Our analyses showed no changes in EcR transcriptional levels in CTCF mutant animals and only a delay in the induction of the expression of E75B and PTTH, each of which eventually showed transcript levels similar to controls (Fig. 1B). However, we observed a reduction in the transcriptional induction of two genes involved in ecdysone biosynthesis, spok and dib. These results suggest that CTCF might regulate induction of certain Halloween genes necessary for ecdysone synthesis.

**Loss of CTCF in ecdysone-producing cells impairs Halloween gene transcriptional activation**

In order to analyze whether CTCF is required autonomously in the ecdysone-producing cells themselves to regulate Halloween gene expression we knocked down CTCF specifically in the PG using the UAS/Gal4 system. A UAS-CTCF-RNAi construct combined with UAS-dicer2 was expressed in the prothoracic cells using a PG-specific phantom-Gal4 (phm>) driver. We then measured the transcript levels of the Halloween genes spok, dib, shadow (sad) (Warren et al., 2002), neverland (nvd) (Yoshiyama et al., 2006), phantom (phm) (Niwa et al., 2004; Warren et al., 2004), noppera-bo (nobo) (Enya et al., 2014) and shroud (sro) (Niwa et al., 2010). Control larvae that only express phantom-Gal4 in a wild type background exhibit a clear expression increase of these genes during the early third-instar stage and then rises, as previously reported (McBrayer et al., 2007). In phm>CTCFRNAi background the transcript levels of dib, sad and nobo were 2-5 fold lower in phm>CTCFRNAi larvae at the time points of maximal induction (Fig. 2) and we observed almost no induction of spok, which is one of the genes of the so-called Black-Box that has been suggested to be a rate limiting step in ecdysone synthesis (Warren et al., 2009). On the other hand, no clear differences were observed for the Halloween genes nvd, phm and sro indicating that CTCF is required specifically in ecdysone-producing cells for activation of several, but not all of the ecdysone biosynthetic enzymes.

We investigated the possibility that loss of CTCF in the PG might indirectly affect ecdysone production by modifying PTTH signaling. First we analyzed whether the gene is expressed normally and found that PTTH transcripts are only found in the PG neurons in each brain hemisphere as in controls and the transcript levels were not decreased. In fact they were actually increased, during the prolonged larval stage (supplementary material Fig. S2A and B). We also analyzed the expression of the receptor torso and found similar transcript levels in phm>CTCFRNAi knockdown as in control larvae (supplementary material Fig. S2C). Therefore, our data indicate that loss of CTCF in the PG does not likely interfere with PTTH signaling to the PG.

**CTCF expression in the PG is required for proper timing of ecdysone-signaling gene expression**

Reduced levels of CTCF in the PG led to partial lethality at different larval stages, but many knockdown animals were able to pupariate although most died before eclosion. We analyzed developmental timing in phm>CTCFRNAi knockdown larvae and we found that larval development was prolonged by two days on average and was less synchronous than control animals (Fig. 3A). We also measured...
Developmental timing depends on CTCF-mediated cholesterol homeostasis and ecdysone synthesis

Though phm>CTCFRNAi knockdown larvae showed low transcriptional levels of the Halloween genes spok, sad and nobo, they are able to induce ecdysone-responsive genes such as E74B and most larvae still progressed to the larval/pupal transition. However, pupariation was delayed in these animals, suggesting that CTCF is needed for proper timing of high level ecdysone production. To determine if low levels of ecdysteroids are responsible for the developmental delay, we fed either 20-hydroxyecdysone (20E), which is the active form of the hormone, or cholesterol (C), which is the precursor for ecdysone biosynthesis in the PG, to phm>CTCFRNAi. Although some of the 20E-fed CTCF-knockdown larvae started to pupariate at the same time as control animals, individuals of the phm>CTCFRNAi population pupariate over a longer period of time (Fig. 4A). Since it has been recently reported that Ecdysone (E) and 20E have different effects on developmental timing of wild type larvae (Ono, 2014), we also fed E to phm>CTCFRNAi larvae but we did not observe any differences with respect to 20E-fed larvae (data not shown). On the other hand, when larvae were cholesterol-fed puparium formation and adult eclosion were normal although delayed by an average of one day. Indeed, we were only able to efficiently rescue developmental delay and adult eclosion by feeding larvae with both, 20E and C, suggesting that CTCF is needed not only to synthesize ecdysone from cholesterol but also to mediate cholesterol homeostasis in PG cells.

It has been reported that at the onset of pupariation there is an increase of lipid droplets in PG cells that can be seen by Oil Red O staining of precisely staged 120 h AEL wandering larvae (Talamillo et al., 2013). At 120 h AEL, phm>CTCFRNAi PGs had a reduced content of lipid droplets in comparison to controls (Fig. 4B, compare right and left panels), likely due to developmental delay. However, there is also an increase of lipid droplets at the end of larval development in phm>CTCFRNAi PG cells which tend to be slightly higher than in control animals (Fig. 4B, compare right and left panels and supplementary material Fig. S4). Since the subcellular lipid accumulation phenotype of phm>CTCFRNAi PG cells is similar to that of Niemann-Pick type C (npc) mutants (Huang et al., 2005), we analyzed dnpcl1a transcriptional levels but we found no changes between control and phm>CTCFRNAi larvae (data not shown). Increased lipid accumulation in the fat body in EcR knockdown larvae has been reported (Kamoshida et al., 2012), but our results show that EcR transcriptional levels do not change in
CTCF mutant animals (Fig. 1B). On the other hand, as shown above, CTCF expression in the prothoracic gland is required for transcriptional activation of the Halloween gene nobo (Fig. 2), which has been shown to be necessary for normal accumulation of cholesterol in PG cells (Enya et al., 2014). Thus, the abnormal accumulation of lipids droplets in CTCF knockdown animals are in agreement with CTCF-mediated regulation of nobo gene expression. These data along with results reported above showing partial rescue in cholesterol-fed CTCF-knockdown larvae (Fig. 4A) suggest a role of CTCF in sterol homeostasis in the PG.

In recent years evidence has accumulated that PTTH is a major, but not the only, developmental signal that triggers ecdysteroidogenesis; multiple factors, in particular nutritional signals reflecting the general metabolic status of the larvae, act directly on the PG to control ecdysteroidogenesis. Insulin and Target of Rapamycin (TOR) pathways have been shown to play a direct role in regulating ecdysone synthesis in the PG (Caldwell et al., 2005; Colombani et al., 2005; Layalle et al., 2008; Mirth et al., 2005). Insulin signaling has been studied extensively in Drosophila as a regulator of tissue growth and involves activation of several downstream factors including PI3K and AKT. In contrast, PTTH signals through activation of the receptor Torso and the Erk pathway (Rewitz et al., 2009). Interestingly, it has been reported that CTCF plays a role in insulin-induced cell proliferation and acts downstream of insulin-induced activation of both Erk and Akt (Gao et al., 2007; Tsui et al., 2014). In addition to insulin/TOR, recent work has shown that TGFβ/activin signaling is also essential for ecdysone synthesis and developmental transitions (Gibbens et al., 2011). It has been suggested that dSmad2, the major downstream mediator of TGFβ signals, might participate directly in regulating Halloween gene transcription. The observation that TGFβ promotes complexes between Smad proteins and CTCF on the H19 imprinting control region (Bergstrom et al., 2010), raises the possibility that these complexes could also regulate Halloween gene transcription. We searched for CTCF-binding sites in the promoter regions of Halloween genes and we found motifs at less than −1 kb from TSSs in all of them except nvd (supplementary material Fig. S5). Nevertheless, it remains to be determined whether CTCF-mediated induction of these genes is mediated through these sites and whether complexes with other transcription factors might also be involved.

Conclusions

Here we show that the insulator protein CTCF plays important roles in Drosophila steroidogenesis at two different levels: on one hand, CTCF controls cholesterol homeostasis in the PG and, on the other hand, CTCF is required for activation of the enzymes responsible for the synthesis of the hormone. Whether these two events are coupled needs further analyses but our data suggest that this is unlikely since to efficiently rescue of developmental delay in CTCF-knockdown larvae required both, ecdysone and cholesterol. Overall, our results suggest that the insulator protein CTCF might be part of a molecular mechanism that couple nutritional input and timing of juvenile maturation.
MATERIALS AND METHODS

Fly stocks

All flies were raised at 25°C on standard medium. The following transgenic and mutant flies were used: CTCFΔ6+6 (Gerasimova et al., 2007), phm-Gal4 (Rewitz et al., 2009), hsp70-Gal4 (Bloomington Drosophila Stock Center #2077), UAS-dCTCF (Mohan et al., 2007) UAS-CTCF-RNAi (Vienna Drosophila RNAi Center-VDRC #30713), UAS-CP190-RNAi (VDRC #35078), UAS-Ibf1-RNAi (VDRC #35426), UAS-Ibf2-RNAi (VDRC #42121) and UAS-dicer2 (VDRC #60008).

Staging larvae, developmental timing analyses and feeding experiments

Females were allowed to lay eggs on apple juice agar plates for 2 h. Synchronized larvae were reared on standard medium supplemented with yeast at 25°C and allowed to develop to the desired time points. For developmental timing analyses, 30 larvae were placed per vial and pupariation timing was scored periodically. A single heat shock (37°C) for 20 min at 24 h of development was performed in rescue experiments. Feeding experiments were performed by adding 20-Hydroxyecdysone (Santa Cruz) at 0.33 mg/ml in ethanol and/or Cholesterol (Sigma) at 0.35 mg/ml. Control larvae were fed with ethanol.

Quantitative RT-PCR

Total RNAs were extracted from ring gland+brain of precisely staged larvae with TRIzol Reagent (Life Technologies) and purified with RNeasy Mini Kit (Qiagen). cDNAs were prepared from 0.5 µg of RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). qRT-PCR was performed on Roche LightCycler 480 System (Roche) using LightCycler 480 SYBER Green I Master (Roche). Three independent biological replicates for each genotype at each time point were performed. Transcriptional levels were normalized to ribosomal protein RpL23. Primers used are listed in supplementary material Table S1.

Oil Red O staining

Ring gland+brain were fixed in 4% paraformaldehyde for 20 min, washed twice in PBS and stained with Oil Red O (Sigma) solution at 0.06% in isopropanol for 30 min. Samples were washed and stained with DAPI before mounting in Vectashield (Vector Laboratories). Single plane images were taken with a confocal Leica TCS SP2-AOBS microscope.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.L.E. designed the study. U.F., S.C. and M.L.E. performed the experiments. U.F., S.C., M.B.O. and M.L.E. analyzed the data. M.L.E. wrote the manuscript.
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Supplementary material
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