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Regulation of hexamerin receptor phosphorylation by hemolymph protein HP19 and 20-hydroxyecdysone directs hexamerin uptake in the rice moth *Corcyra cephalonica*

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Abstract

Hexamerins are stage specifically sequestered during the non-feeding stages mainly by the fat body cells from hemolymph through ecdysteroid regulated receptor-mediated endocytosis. 20-Hydroxyecdysone (20E) stimulates the tyrosine kinase-mediated phosphorylation of the 120 kDa hexamerin receptor in the rice moth, *Corcyra cephalonica*. Here, we demonstrate that phosphorylation of the hexamerin receptor by HP19-regulated-20E-dependent-tyrosine kinase is a critical regulator for its activation, and is required for hexamerin uptake. Hexamerin receptor is phosphorylated only in the hexamerin sequestering tissues. The receptor phosphorylation is a prerequisite for hexamerin uptake and both phosphorylation and concomitant uptake are developmentally regulated. In addition, endogenous fat body tyrosine kinase activity is also developmentally and hormonally regulated. 20E induces the tyrosine kinase activity both *in vivo* as well as *ex vivo,* and the receptor phosphorylation is likely an extra-cellular event. The hemolymph protein, HP19 inhibits the 20E-induced phosphorylation by inhibiting tyrosine kinase activity. These inhibitions are rapid in homogenate preparations and are unaffected by the inhibitors of transcription and translation. We propose that hexamerin sequestration is negatively regulated by active HP19 at the feeding larval stage, thus preventing the uptake. During the non-feeding pupal stage, high ecdysteroid titer and negligible HP19 activity, positively regulates the receptor phosphorylation resulting in hexamerin uptake. These studies are therefore the first evidence of hexamerin uptake regulated by the orchestration of 20E and HP19 at a nongenomic level.

Keywords: 20-Hydroxyecdysone; Hexamerin; Hexamerin receptor; Hemolymph protein; Tyrosine kinase; Nongenomic ecdysteroid action

1. Introduction

Post-translational modifications of proteins by reversible protein phosphorylation are critical regulator of a large number of cellular actions (Graves and Krebs, 1999). These phosphorylation events are often regulated by stimuli such as hormones (Weigel and Moore, 2007). In insects, ecdysteroids are known to stimulate phosphorylation of a few fat body proteins whose significance has been discussed (Itoh et al., 1985; Sass, 1988; Arif et al., 2003). Studies from our as well as other groups suggest a post-translational processing mechanism for hexamerin receptor activation, which is regulated by ecdysteroids (Burmester and Scheller, 1999).

Hexamerins are multimeric proteins that are synthesized stage specifically mainly by the fat body cells of actively feeding larval stage and released into hemolymph (Telfer and Kunkel, 1991; Haunerland, 1996). The fat body tissue also sequesters these proteins during the non-feeding prepupal (PP) and pupal stages to meet the energy requirements of metamorphosis (Ismail and Dutta-Gupta,

Abbreviations: 20E, 20-hydroxyecdysone; HP19, 19 kDa hemolymph protein; MARG, male accessory reproductive gland; ELI, early-last instar; MLI, mid-last instar; LLI, late-last instar; PP, prepupae; SDS-PAGE, Tris–glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis; ATP, adenosine triphosphate.

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1990a; Haunerland, 1996). This uptake occurs through a unique receptor-mediated endocytosis (Wang and Haunerland, 1994a). Although the hexamerin-receptor remains largely uncharacterized, it is known that the receptor does not belong to the low-density lipoprotein receptor super family (Burmester and Scheller, 1999). The hexamerin receptor, which acquires the ability to sequester hexamerin under the influence of 20-hydoxyecdysone (20E), has been identified both in dipteran as well as lepidopteran insects (Burmester and Scheller, 1999). In the blow fly, *Calliphora vicina*, a 130 kDa precursor hexamerin receptor sequesters hexamerin after activation by a three step post-translational proteolytic cleavage (Burmester and Scheller, 1997). In addition, an anterior fat body protein interacts with the hexamerin receptor of the fat body cells to regulate hexamerin uptake in *Calliphora* (Hansen et al., 2002, 2003). Characterization of hexamerin receptor in *Helicoverpa zea* revealed a single basic 80 kDa receptor protein and likely to be glycosylated (Wang and Haunerland, 1994b; Persaud and Haunerland, 2004). In the flesh fly, *Sarcophaga peregrina,* a 120 kDa protein sequesters hexamerin under the influence of 20E (Ueno and Natori, 1984; Chung et al., 1995). In *Drosophila melanogaster*, fat body protein-1 (*Fbp-1*) was identified as the receptor to sequester hexamerin in response to rising titer of ecdysteroid (Burmester et al., 1999). In the fat body membrane of the rice moth *Corcyra cephalonica*, ligand-binding studies identified a 120 kDa hexamerin binding protein referred to as the hexamerin receptor (KiranKumar et al., 1997). This receptor is present at maximal concentration in the PP stage and is shown to stimulate precocious hexamerin uptake in late-last instar (LLI) larval fat body under the influence of 20E.

Studies on receptor activation in different insects were shown to be independent of gene activation (Ueno and Natori, 1984; Burmester and Scheller, 1997). We have demonstrated earlier that 20E-induced phosphorylation of the hexamerin receptor which is partly mediated by a tyrosine kinase is a pre-requisite for hexamerin uptake (Arif et al., 2003). Our studies also revealed that a 19 kDa hemolymph protein, HP19, in *Corcyra* inhibited the receptor phosphorylation (Arif et al., 2004). In addition to fat body, hexamerins are also actively sequestered into male accessory reproductive glands (MARG) of several insects including *Corcyra* and the process is steroid dependent (Ismail and Dutta-Gupta, 1990b; Ismail et al., 1993). In the present study, we have investigated the role of phosphorylation of hexamerin receptor in these hexamerin sequestering tissues. We also investigated the developmental and hormonal regulation of fat body tyrosine kinase activity and the mechanism of receptor phosphorylation. Our study suggests that hexamerin uptake is nongenomically regulated by the orchestration of 20E and HP19 during the feeding larval and non-feeding pupal stages in *Corcyra*.

2. Materials and methods

2.1. Insect rearing and sample collection

The larvae of lepidopteran insect rice moth, *C. cephalonica* (Stainton) were reared in coarsely crushed sorghum seeds at 26 ± 1 °C, 60 ± 5% relative humidity and 14:10 h light: dark photoperiod. Classification of larval stages was based on their body weight and head capsule size (Lakshmi and Dutta-Gupta, 1990). In the present study, early-last instar (ELI), late-last instar (LLI) larvae, prepupae (PP), pupae and freshly eclosed adults were used. For thorax-ligation, LLI larvae were ligated with a loop of silk thread behind the first pair of prolegs and maintained on moistened filter paper for required time (Ashok and Dutta-Gupta, 1991). Tissue homogenates and membrane fractions were prepared as described (Arif et al., 2003; KiranKumar et al., 1997).

2.2. Electrophoresis, immunoblotting and immunoprecipitation

Tris–glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried according to the procedure of Laemmli (1970). For immunoblotting, electrophoretically separated polypeptides were transferred to nitrocellulose membrane (Towbin et al., 1979) and analyzed using anti-hexamerin antibody (Arif et al., 2001; Nagamanju et al., 2003) or anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Specific cross-reactivity was observed using anti-rabbit IgG coupled to alkaline phosphatase followed by nitrotetrazolium blue/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color reaction. Immunoprecipitation of *in vitro* phosphorylated LLI fat body membrane protein was performed using anti-phosphotyrosine antibody and Seize primary immunoprecipitation kit (Pierce, Rockford, IL).

2.3. In vitro phosphorylation of fat body proteins

In vitro phosphorylation was carried as described previously (Arif et al., 2003). For most studies, equal quantity of homogenate/membrane protein was phosphorylated using 4 μCi of [γ-^{32}P] ATP (~3000 Ci/mmol, BRIT, India), resolved on 10% SDS-PAGE, vacuum dried and exposed to Kodak X-Omat AR film for autoradiography. For phosphorylation of proteins of cultured tissues, fat bodies from 24 h post-thorax-ligated LLI larvae were, dissected, rinsed and kept in culture in TC-100 insect culture medium (JRH Biosciences, Inc., USA) with streptomycin sulfate (1 μg/ 200 μl) for 2 h. The tissues were transferred to fresh medium and incubated in absence or presence of 20E (80 nM), HP19 (40 ng) and genistein (40 μM) for 4 h at 25 °C with gentle shaking. Equal volume of carrier solvent (0.05% ethanol, used to prepare 20E stock) was added to control cultures. After incubation, fat body was rinsed with insect Ringer (130 mM NaCl, 5 mM