Evidence for a Juvenile Hormone Receptor Involved in Protein Synthesis in *Drosophila melanogaster* *

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The larval fat body of newly eclosed adults of *Drosophila melanogaster* was found to contain a single major binding protein specific for juvenile hormone (JH). Binding to this protein was saturable, of high affinity, and specific for JH III. The protein has a subunit molecular weight (Mr) of 85,000, as determined by photoaffinity labeling. The same or similar JH-binding protein was found in larval fat body and cuticle of third instar larvae and in male accessory glands and heads of newly eclosed adults. It was not found in several other tissues in adults. Male accessory gland cytosol from wild-type flies was found to contain a single binder with a dissociation constant (Kd) of 6.7 nM for JH III; a binder in similar preparations from the methoprene-tolerant (Met) mutant had a Kd value 6-fold higher. JH III stimulated protein synthesis in Met flies as compared to wild-type flies, establishing a role for JH in this tissue. The methoprene-tolerant (Met) mutant had a KD value of 6.7 nM, which confers a high level of resistance to the effects of JH III and methoprene (19) and reduces the binding affinity of this protein. We believe that the altered binding protein results in the reduced JH III stimulation of protein synthesis seen when these glands are cultured in vitro, as well as the reduced accumulation of protein in vivo in Met glands.

EXPERIMENTAL PROCEDURES

Animals—Flies were raised at 25 ± 1 °C in uncrowded cultures on a cornmeal-agar-yeast-molasses diet supplemented with Tegosept or propionic acid to retard mold growth. Third instar larvae were selected several hours before pupariation from the walls of culture bottles. Adults were isolated 0-4 h following eclosion and used for tissue preparation.

Hormones—All juvenile hormones and analogs were racemic mixtures. [3H]-JH III (specific activity 11.9 Ci/mmol) was purchased from Du Pont-New England Nuclear. Unlabeled JH III, JH II, and JH I were from Sigma. JH III acid was prepared as described previously.

The importance of JH in the insect life cycle has focused much research in the area of JH mode of action. Despite considerable effort, the mechanism by which JH affects target cells has yet to be determined. It has been suggested that JH acts in a manner similar to that proposed for steroid hormones (7). In this model, the hormone enters the cell and binds to a cytoplasmic receptor, after which the hormone-receptor complex passes into the nucleus, where it interacts with DNA and thus regulates the transcription of specific genes. Support for this model of JH action has come from a variety of investigations. First, transcription of specific genes has been shown to be regulated by JH. For example, JH regulates vitellogenin mRNA levels in the fat body of *Locusta migratoria* (8). Second, cytosolic proteins that bind JH with high affinity and specificity have been identified in a number of JH target tissues from a variety of insects, including the ovary (9, 10), epidermis (7, 11, 12), fat body (13, 14), silk gland (15), and cells from the *Drosophila* Kc line (16). Lastly, JH-binding proteins have also been found in the nuclei of some of these tissues, including epidermis (12, 17) and fat body (13, 18). These nuclear JH-binding proteins have binding properties similar to those of their cytosolic counterparts. All these studies together provide evidence that the steroid hormone model may be appropriate for JH.

While the above studies have characterized in some detail the binding parameters of intracellular JH-binding proteins, none of them has demonstrated a correlation between JH binding to a binding protein and biological activity of the hormone. If a hormone-binding protein is to be classified as a receptor protein, it is important to establish this property. This paper reports the identity of a cytosolic JH-binding protein in *Drosophila melanogaster* that is associated with a biochemical response to JH, protein synthesis. This association is through the methoprene-tolerant (Met) mutation, which confers a high level of resistance to the effects of JH III and methoprene (19) and reduces the binding affinity of a binding protein for JH III in larval fat body. We now show that the same protein appears to be found in male accessory glands and that Met also reduces the binding affinity of this protein. We believe that the altered binding protein results in the reduced JH III stimulation of protein synthesis seen when these glands are cultured in vitro, as well as the reduced accumulation of protein in vivo in Met glands.

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1 The abbreviations used are: JH, juvenile hormone; EFDA, epoxy-farnesyl diazoacetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid.
Concentrations of the unlabeled hormones and analogs were determined by measuring absorption at 290 nm of stock solutions in methanol using the extinction coefficients 13,830 for JH homologs and 26,000 for ZR-515 (21). All JH homologs and analogs were added to the binding reactions in 10 μl of dimethyl sulfoxide as the solvent.

Preparation of Tissue Homogenates for Photoaffinity Labeling—All tissues for photolabeling were dissected at 2 °C and stored in LS buffer (20 mM Tris, pH 8.0, 10 mM α-thioglycerol, 1 mM EDTA, 9 mM NaCl, 0.5 mM methionine, 1% glycerol) with 5× 10−6 M 3-octyl-1,1,1-trifluoro-2-propanone as an esterase inhibitor at −80 °C for 2–4 weeks. Larval fat body cells from 0–4-h-old adults were collected as described previously. Larval cuticle was prepared using a modification of a procedure for mass collection of salivary glands (22). In this procedure, larvae were placed in monolayer on a glass plate, and a glass vial was rolled over them. This action resulted in vascularized cuticles free of internal organs as viewed under a dissecting microscope. Adult heads were prepared by decapitation. All other tissues were dissected with forceps in ice-cold LS buffer.

All tissues were homogenized in 1 ml of LS buffer in a glass homogenizer (Radnoti Glass, Monrovia, CA) on ice. Cytosol (100,000 × g supernatant) was prepared as previously described for larval fat body. All tissue cytosols were extracted with dextran-coated charcoal to remove endogenous JH. They were then divided into 0.38-ml aliquots and stored at −80 °C until used for photoaffinity labeling.

Binding Assay—The hydroxyapatite binding assay was performed as described previously for all binding studies. The binding incubation time was 30 min because previous data had shown that binding equilibrium is reached by this time. Under these conditions, hormone metabolism was less than 10%. All the binding studies were carried out with cytosolic extracts of female larval fat body and male accessory glands from 0–4-h-old flies prepared as described above.

Polyacrylamide Gel Electrophoresis and Fluorography—0.38 ml of cytosolic extracts was incubated with 3 × 10−6 M [3H]EFD with or without a 100-fold excess of unlabeled JH III in quartz tubes that had been pretreated with 1% polyethylene glycol (M, 20,000 from Fluka). After 1 h at 4 °C, the samples were irradiated with four 30-s bursts of UV light from a hand-held Mineralight source emitting light at a wavelength of 254 nm. They were then extracted with dextran-coated charcoal2 to remove unbound photoaffinity label. The photo-labeled samples were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) using the Laemmli protocol (23). The gel was stained with Coomasie Blue to detect proteins and exposed to fluorography (24) to identify radiolabeled bands. After drying, the gel was exposed to blue-sensitive prefilled x-ray film (Kodak X-Omat XAR-5) for 4–5 weeks at −80 °C.

Molecular weight standards (Sigma) were run together with the cytosolic extracts. The standards were myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000).

Protein Synthesis and Accumulation in Male Accessory Glands—Male accessory glands from 0–4-h-old flies were dissected free of testes and gut in MOPS buffer containing 0.1 mM CaCl2 (6). Three pairs of glands were incubated with 5 μCi of [35S]methionine (ICN) in 50 μl of MOPS buffer with 0.1 mM CaCl2 that had been preincubated for 30 min at 25 °C with different concentrations of JH III in 0.5-ml microtubes. After 3 h at 25 °C, the glands were lyophilized and assayed for protein concentration.

RESULTS

The two JH target tissues examined were larval fat body and male accessory glands from 0–4-h-old flies. JH has been shown to regulate the histolysis of larval fat body during the first 2 days of adult life (25) and to stimulate protein synthesis in male accessory glands (6). The laboratory balancer strain First Multiple Seven (FM7, described in Ref. 26) was used as the wild-type methoprene-sensitive strain in these studies as in previous studies.

Saturation of JH III Specific Binding—Larval fat body cytosol from 0–4-h-old females was exposed to increasing concentrations of [3H]JH III. As Fig. 1 shows, specific binding reached saturation at 15 nM [3H]JH III. Previous analysis of these specific binding data has revealed a single JH-binding protein with a dissociation constant (Kd) of 4.5 nM, well within the range of JH binding affinities established from various insects (27).

Specificity for JH III—The JH-binding protein in larval fat body was able to discriminate between JH III, one of the two natural juvenile hormones in Drosophila melanogaster (28–30) and other competitors (Fig. 2). JH III competed for binding with [3H]JH III far better than did the two JH homologs, JH I and JH II. Methoprene (isopropyl-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate), a chemical analog of JH III, was also a potent competitor (Fig. 2). These data have previously appeared in a Scatchard plot. Each point is the average of one to three replicates.

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2 L. Shemshedini and T. G. Wilson, unpublished results and observations.

FIG. 1. Binding of JH III by larval fat body cytosol. Cytosol was incubated with increasing concentrations of [3H]JH III for 30 min at 4 °C and assayed for specific binding. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled JH III. These data have previously appeared in a Scatchard plot. Each point is the average of one to three replicates.

FIG. 2. Competition by JH homologs and methoprene (ZR-515) for binding of JH III by larval fat body cytosol. Cytosol was incubated with 10 nM [3H]JH III with or without various concentrations of unlabeled competitors for 30 min at 4 °C and then assayed for [3H]JH III binding.
of JH III with potent biological activity (31), and JH III acid, an inactive metabolite, displayed poor competition.

**Tissue Specificity Examined by Photoaffinity Labeling**—To identify the specific protein(s) of the larval fat body that bind JH, a photoaffinity analog of JH III, EFDA (epoxy farnesyl diazoacetate), was used. Separation of photolabeled cytosolic proteins from larval fat body by SDS-PAGE showed that two major proteins bind a racemic mixture of [3H]EFDA (Fig. 3). An excess of unlabeled racemic JH III inhibited the binding of [3H]EFDA to one of these, having a molecular weight (Mr) of 85,000 ± 1600 (±S.E.M.; n = 6); the other major protein, with a Mr of ~80,000, still labeled in the presence of unlabeled JH III and thus appears to nonspecifically bind EFDA (Fig. 3, protein A). A minor band, of Mr ~ 63,000, was also specifically labeled by EFDA (Fig. 3, protein B). This protein appears on the fluorograms with variable intensity, estimated to be 5–20% of that of the Mr = 85,000 specific binder. (The intensity of the Mr = 85,000 JH-binding protein in Fig. 4 is more typical of our results than that in Fig. 3.) Recently, the biological active enantiomer (R isomer) of [3H]EFDA has become available (G. Prestwich). We have found it to label almost exclusively the Mr = 85,000 JH-binding protein, suggesting that the other two JH-binding proteins may not have biological significance. Therefore, no further characterization of the Mr = 80,000 and 63,000 JH-binding proteins has been carried out.

Other tissues were also examined using [3H]EFDA for the presence of JH-binding proteins (Fig. 4). A JH III-specific binder of the same Mr (85,000) was found in two suspected JH target tissues, larval fat body and cuticle of third instar larvae, as well as in the head of newly eclosed adults. The protein was not found in two JH nontarget tissues, thoracic muscle (Fig. 4) and gut. This protein was not present in ovaries of 15–24-h-old or 44–48-h-old flies (Fig. 4). Samples having more protein were used for the head, cuticle, and ovary lanes, and it is apparent that additional nonspecific JH-binding proteins were detected. In addition, two lower Mr specific JH-binding proteins were found in the cuticle sample. The sole band in muscle appears to be spillover from the adjacent well since it was not detected when this experiment was repeated. Thus, the JH-binding protein in the larval fat body exhibits a tissue-specific distribution in Drosophila.

Cytosol from male accessory glands, a known JH target tissue (6) that was subjected to photoaffinity labeling and SDS-PAGE, gave no visible bands on fluorograms; this result was obtained even with a tissue preparation having 3 times the number of flies that were used for the other adult tissues and a film exposure twice as long as that used for the other tissues. However, when gel slices from SDS-PAGE of male accessory glands were counted, a major peak of JH III-competable [3H]EFDA was located at a Mr of ~85,000 (Fig. 5); this peak co-migrated with the JH-binding protein from larval fat body. Thus, it appears that the same JH-specific binding protein is found in both larval fat body and male accessory glands.
protein appears to be found in both fat body and male accessory glands for FM7 flies (8.1 ± 1.3). These binding data and the affinity for JH III in Met glands is reduced to 37.5 fM. Thus, we believe that the M, = 85,000 JH-binding protein is essentially absent from these tissues.

Biochemical Response in Male Accessory Glands—An important property of receptors, correlation with a biochemical action of the hormone, could not be evaluated in larval fat body because no biochemical response to JH stimulation of larval fat body cells has been reported. Recently, Yamamoto et al. (6) have reported that JH stimulates protein synthesis in cultured male accessory glands from Drosophila. Therefore, we used this tissue to determine if a correlation exists between JH binding and a biochemical response to JH stimulation. To do this, we used the mutant Met, which has been previously shown to have an altered cytosolic JH-binding protein in larval fat body.

Since, based on molecular weight, the same JH-binding protein appears to be found in both fat body and male accessory glands, we wanted to find out if the Met locus also reduces the JH III binding affinity in the male accessory glands. When male accessory glands were dissected from 0–4-h-old adults and assayed for JH binding, a binding component specific for JH III could be detected. This binding approached saturation for FM7 flies, but not for Met flies over the same range of [3H]JH III concentrations (figure not shown). These data were transformed into a Scatchard plot and analyzed by nonlinear curve-fitting (Fig. 6). This analysis revealed in FM7 flies a single JH-binding protein with a Kd of 6.7 ± 1.9 nM (±S.E.) (Fig. 6), similar to the 4.5 nM found for the larval fat body binding protein. However, the binding affinity for JH III in Met glands is reduced to 37.5 ± 16 nM (Fig. 6), a reduction similar to that obtained with fat body. The difference in Kd between FM7 and Met flies is highly significant (F test, p < 0.05). While the Kd values are different, the binding capacities (Rf) are similar (Fig. 6). Rf for Met flies (9.7 ± 5.1 fmol/gland pair) is slightly larger than that for FM7 flies (8.1 ± 1.3). These binding data and the electrophoresis data strongly suggest that the same JH-binding protein is found in both larval fat body and male accessory glands.

One established effect of JH III is stimulation of protein synthesis in male accessory glands (6). To determine if the reduced binding affinity in Met glands results in altered protein synthesis, incorporation of [35S]methionine into proteins by male accessory glands cultured in vitro was measured. Stimulation of protein synthesis by JH III was significantly greater in FM7 glands than Met glands (Fig. 7A). Since JH seems to be required for protein accumulation in male accessory glands (32), then it might be expected that the reduced JH response in Met glands would result in lowered protein accumulation in these glands. This, in fact, was seen during the first 2 days of adult development. Total protein accumulation was lower in Met glands than in FM7 glands for all the developmental times measured (Fig. 7B). These results correlate a reduced biochemical response to the reduced JH III binding affinity in Met glands and strongly suggest that the JH-binding protein we have identified is involved in mediating JH III stimulation of protein synthesis in male accessory glands.

**DISCUSSION**

The results presented here are the first to demonstrate a correlation between JH binding to a cytosolic JH-binding protein and biological activity of the hormone. Previous studies have described both cytosolic and nuclear JH-binding proteins in several JH-target tissues from a variety of insects (7, 9–18). While these proteins have exhibited some of the classic hormone receptor characteristics (saturability, high affinity, and ligand specificity), they have not been shown to exhibit tissue specificity or correlate with biological activity. In this paper, we describe a cytosolic JH-binding protein in Drosophila melanogaster that has all the above characteristics and thus may be classed a JH receptor.

Initial studies to characterize the binding parameters of this Drosophila JH receptor were done with larval fat body from newly eclosed adults, a known JH target tissue (25). When the cytosol of this tissue was examined for binding of [3H]JH III, specific binding was obtained that was saturable, of high affinity, and of high specificity. A previous analysis of this saturable binding has shown the presence in fat body cytosol of a single JH binder with a Kd value of 4.5 nM for JH III in FM7 flies. This high affinity is comparable to Kd values that have been obtained with JH-binding proteins from other insects (27).

Competition studies indicated that this high affinity binder preferentially binds JH III, one of two native juvenile hormones in Drosophila (28–30). The specific nature of this...
A variety of insects (33-36). We used it in this study to determine if there are any JH-binding proteins in D. melanogaster. In larval fat body cytosol from newly eclosed adults, a single major binding protein specific for JH III with a $M_r$ of 85,000. Based on molecular weight, the same JH-binding protein was found in male accessory glands and head of newly eclosed adults and larval fat body and cuticle of third instar larvae; all of these tissues are known or suspected JH target tissues. No JH-binding proteins were detected in two nontarget tissues, adult gut and muscle.

The subunit molecular weight of the JH-binding protein identified in this study differs from the $M_r = 24,600$ of the JH-binding protein of the Drosophila Kc cell line (37). Since the Kc cell line was derived from embryonic tissue and the tissues examined in this study are larval and adult, these results suggest that different JH-binding proteins are used by D. melanogaster at different developmental stages.

Although no clear role for JH has been detected in preadult Drosophila, the presence of JH III during the late larval stage (28, 29) and the identity of a high affinity, high specificity JH-binding protein in larval hemolymph (20) suggest that JH does serve a function in Drosophila larvae. Thus, our discovery of a putative JH receptor in two larval tissues is consistent with a JH role in D. melanogaster larvae.

Two unexpected results were the presence of this protein in the adult head and its absence in ovaries. The head is not usually considered to be a JH target tissue, but recent evidence suggests that JH might play a role in pleuripotential synthesis, and this role might be at a site in the brain (38). Additionally, JH could interact with brain tissue to regulate neuroendocrine activity, such as the synthesis or secretion of prothoracotropic hormone. Thus, the possibility remains that JH may have an effect in the head of insects, and our results suggest that this effect may be mediated via an intracellular receptor. Because JH-binding proteins have been found in the ovaries of a few insects (9, 10), it was surprising not to find such a protein in ovaries of D. melanogaster. Possible explanations for this are: 1) the JH effect on oogenesis in Drosophila may be indirect and mediated by another molecule or 2) the newly discovered JH bisepoxide (30) may be the primary gonadotropic hormone in Drosophila, and this hormone might use a receptor in the ovaries different from the JH III receptor we have identified. Further speculation for this last possibility must await binding studies and photoaffinity labeling with the JH bisepoxide.

To examine if the JH-binding protein in larval fat body mediates biological activity, we made use of a mutation generated in our lab (19) and the recently devised in vitro assay for JH activity on protein synthesis in male accessory glands (6). The Met mutation has been previously shown to reduce JH activity on protein synthesis in these glands. JH III increased protein synthesis in FM7 glands to a capacity of JH III to stimulate protein synthesis in these glands, while Met glands the increase was barely above the control. Since Met is a single gene mutation, this finding, together with the molecular weight determinations, strongly suggests that the same JH-binding protein is found in both larval fat body and male accessory glands. This lowered binding affinity in Met male accessory glands is correlated with a reduced capacity of JH III to stimulate protein synthesis in these glands. JH III increased protein synthesis in FM7 glands to a maximum of nearly 2.5 times that of the control, while in Met glands the increase was barely above the control. Since Met glands are known to be involved in the correct development of male accessory glands in other insects (39-41), it might be expected that this reduced response of Met glands to JH III would affect the phenotype of the glands. In fact, it has been observed that Met glands are smaller than FM7 glands in newly eclosed adults. This reduced size of Met glands appears to be due to a reduced accumulation of protein and
not to a reduced number of cells. Protein accumulation in these glands was significantly lower in Met flies than in FM7 flies, while the glandular DNA content was similar for the two strains. The reduced JH-induced protein synthesis and accumulation in Met males is not due simply to moribund animals resulting from the mutation. Met flies have high fecundity, fertility, and longevity (42). All of these results are consistent with the idea that the JH-binding protein we have identified is the receptor that mediates JH III action in male accessory glands.

This study has provided evidence that a specific, high affinity JH III-binding protein mediates a JH effect in male accessory glands. Although the properties of this protein suggest that it is a receptor in the classical sense, more work will be required to unambiguously determine if this is the case.

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