ACCESSORY GLAND DEVELOPMENT IN MEALWORM BEETLES[1]

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ABSTRACT 20-Hydroxyecdysone accelerates cell cycles
in the bean-shaped accessory glands of male pupae of
Tenebrio molitor. The hormone acts within an hour in
the G_2 phase of the cell cycle in vitro. Its action
coincides with a change in competence which becomes
expressed days later. We report the isolation of a
proline-rich adult-specific secretory protein that is
indistinguishable from a structural protein of
spermatophore.

INTRODUCTION

Insect reproduction usually involves both sexes, yet
the vast majority of the research on insect reproductive
physiology has emphasized chorion formation and
vitellogenesis in females. Male physiology is equally
important to most insect species, and disruption of male
function has great potential for control of agricultural
pests and disease vectors. Recently, increasing attention
has been paid to male physiology, especially the
endocrine regulation of maturation in testes (1) and
accessory glands (2,3). Work in our laboratory concerns
hormonal control of development in male accessory glands
of a model insect, Tenebrio molitor.

Mealworm beetles have two pairs of accessory
reproductive glands --- the smaller tubular accessory
glands (TAGs) and the larger bean-shaped accessory glands
(BAGs). Primary organogenesis of these glands takes place
in the last larval instar and the two organ rudiments can

be readily located by dissection in the newly-ecdysed pupa. Over the ensuing pupal and adult stages, the TAGs and the BAGs grow in volume (100-fold and 10-fold, respectively) and in cell numbers (15-fold and 5-fold, respectively) (4-6). In the late pupa, after ecdysteroid levels have fallen, terminal differentiation begins (5,9).

20-HYDROXYECDYSONE ACCELERATES CELL CYCLES IN THE PUPA

Mitoses in the BAGs and TAGs persist through the major pupal ecdysteroid peak (6) while at the same time there is mitotic arrest in the sternal epidermis (8). This correlation suggests that ecdysteroids might stimulate cell cycling in the accessory glands while they inhibit mitoses in the epidermis. To investigate the latter possibility, BAGs were cultured In vitro. When glands were cultured in Landureau's medium, physiological concentrations of 20-hydroxyecdysone accelerated mitoses (7).

We decided to investigate the actions of 20-hydroxyecdysone on the cell cycle in the BAGs by a variant of the classic stathmokinetic design of Puck and Steffan (10). Glands were cultured in the presence of hydroxyurea to block the cells in early S-phase, near the G_1-S boundary. At various times, the experiment was terminated and a nuclear pellet prepared from the organs. After staining with propidium iodide, the nuclei were examined by flow cytometry. In these experimental conditions, 20-hydroxyecdysone accelerated flow of cells from G_2 into G_1. (Figure 1). Calculated doubling times were 61 hrs with hormone and 151 hrs without hormone.

In the late pupa and the adult, the cells of the BAGs are almost all in G_2. Similar G_2 arrests have been associated with increased levels of ecdysteroids in cells of Drosophila (11) and epidermal cells of Tenebrio (8). To determine whether G_2 arrest in the pupal BAGs requires hormone, we cultured 0 day pupal glands with and without hormone for 3 days and determined each cell fraction. Under these conditions, the majority of the cells did not arrest in G_2. In fact, addition of 20-hydroxyecdysone increased the proportion of cells in G_1 in these cultures. From doubling times and G_1:S:G_2 fractions, we calculated the length of each phase of the cell cycle. The calculations show that addition of hormone in vitro shortened the length of G_2 from 94 hours to 24 hours. At what point in the cell cycle did ecdysteroid act? Since

the effect persisted in the presence of hydroxyurea,
20-hydroxyecdysone acts later than early S (and therefore
not in G_1). The lag between addition of hormone and the
first detectable effect (intersection of the regression
lines in Figure 1) was 5 hours. Our data suggest the
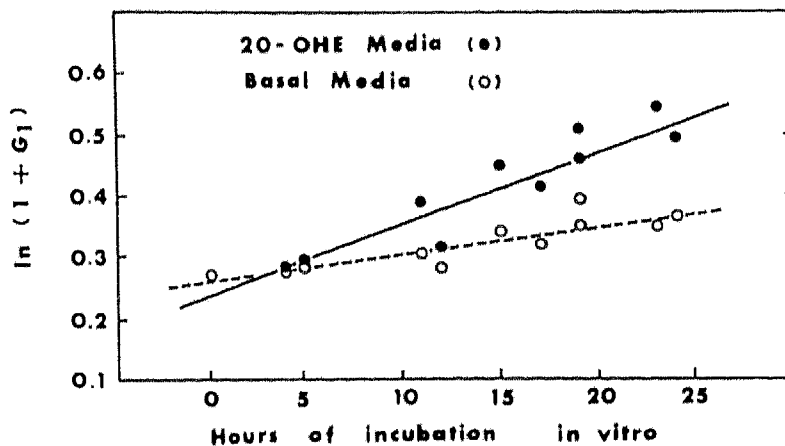presence of a control point 10-20 hours into G_2.

FIGURE 1. The rate at which cells from pupal BAGs
enter the G_1 compartment after addition of hydroxyurea (5
mM) in basal media and 20-hydroxyecdysone-containing
Landureau's S-20 media. Glands were dissected from 0 day
pupae and cultured at 25° C before nuclear isolation,
staining, and cytometry (6). Slopes of the regression
lines are 0.0045 (r=0.862) for basal media and 0.0114
(r=0.943) for hormone-containing media.

How long must the hormone be present to produce a
response? Exposures as short as 1 hour trigger a response
that persists for a full day after returning the glands to
basal media. In addition, 20-hydroxyecdysone did not
accelerate cell cycles in the presence of α-amanitin (5 x
10^{-5}M) or low doses of Actinomycin D (5 x 10^{-9}M). Our
data are consistent with a requirement for transcription
before ecdysteroid accelerates the flow into G_1.

A CHANGE IN COMPETENCE COINCIDES WITH THE ECDYSTEROID PEAK

At the start of the pupal stage, each BAG consists of a coat of presumptive muscle cells surrounding an epithelial monolayer that will be secretory in the mature adult. The patterns of Coomassie-stained protein bands on SDS-gels and the patterns of ^{3}H-leucine incorporation on fluorographs differ markedly between young pupal glands and adult ones (5). Many of the adult-specific spots appear to be suitable as markers for the onset of terminal differentiation. Two particularly useful spots were those designated #24 (apparent molecular weight 24 kd, pI 6.8) and #58 (apparent molecular weight 84 kd, pI 5.2) (5).

We suspected that the pupal ecdysteroid peak which affected cell cycling might be a prerequisite for subsequent synthesis of adult-specific proteins. To test this possibility, we transplanted young pupal glands, before and after exposure to high ecdysteroid in situ, into adult female hosts which have low titers of 20-hydroxyecdysone. Leucine incorporation and fluorography of two-dimensional gels was used to detect the synthesis of the adult specific proteins in cultured glands.

Glands were dissected from 0, 1, and 2 day pupae (before the pupal ecdysteroid peak) or from 4 and 5 day pupae (at the time of high ecdysteroid titers), aspirated into a transfer needle, and introduced into the abdomens of 0 day female pupae or adults. When the hosts had matured to become 9 day adults, the implants were recovered by dissection and incubated in vitro with ^{3}H-leucine. 0 Day pupal implants placed in 0 day pupal hosts grew and incorporated leucine into many adult-specific spots, most notably #24 and #58. When 0, 2, or 3 day pupal glands were implanted into 0 day adult hosts, no incorporation of leucine into adult-specific proteins was detected nine days later. But when older pupal BAGs (4 or 5 days) were placed in 0 day adults, spots #24 and #58 were visible on the x-ray film (Figure 2). These results indicate that a reprogramming occurs at the time of the pupal ecdysteroid peak. After reprogramming, the BAGs become competent to make adult proteins when placed in an adult environment. The data from the transplantation experiments and the stathmokinetic experiments indicate that the ecdysteroid peak which acts at 4-5 pupal days to accelerate cell cycles might also cause a change in commitment. All of

our morphological and biochemical data indicate that the
onset of terminal differentiation does not occur until 8
pupal days or older, well after ecdysteroid levels have
fallen. If we consider the time scale of the effect on
mitoses and the putative affect on differentiation, it
appears that ecdysteroids acting on the BAGs have two
short-term effects (on cell cycling and on competence)
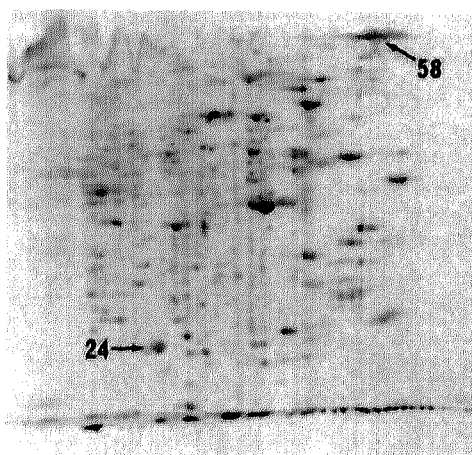which later lead to the long-term effects (terminal
differentiation).

FIGURE   2   Two-dimensional   fluorograph   showing
^{3}H-leucine incorporation into proteins of BAGs, derived
from 5 day pupae and implanted for 9 days into female
adults.   Incorporation took place in vitro in Landureau's
S-20. Nine glands have been pooled for this gel.

ISOLATION AND CHARACTERIZATION OF A SPERMATOPHORIN

Two-dimensional   fluorography   after   leucine
incorporation is a cumbersome technique for scoring
differentiation.   To   obtain   better   probes   to   study
differentiation of the BAGs, our laboratory has prepared
monoclonal   antibodies   (12,13).   We   have   used   these
antibodies to detect antigens that are common to the
spermatophore and the BAG and that also have potential as
markers for differentiation.   We have recently isolated
one of the adult-specific antigens which promises to be a
suitable   marker   for   detecting   the   onset   of   terminal
differentiation.   This antigen is very similar (perhaps

identical) to a structural protein of the spermatophore,
a class which we term "spermatophorins".

Monoclonal antibodies were produced by immunizing
Balb-c mice with homogenates of the secretory plug of the
BAG and screening the hybridoma clones in an ELISA against
water-insoluble fractions of the spermatophore. One of
the resulting clones, designated PL21.1, recognized an
antigen of 23 kd, This antigen migrated on two dimensional
gels like spot #24 that we had previously used to detect
terminal differentiation. With western blots of organ
homogenates, we found that PL21.1 antigen is
organ-specific (found only in the BAGs and not in other
parts of the male tract or in the fat body) and is
differentiation specific (absent from 0 and 5 day pupae,
detected in 8 day pupae, and increasing rapidly
thereafter). Using immunocytochemistry, PL21.1 antigen
was localized in secretory granules of type 4 cells of the
BAGs and in a narrow zone within the multilayered wall of
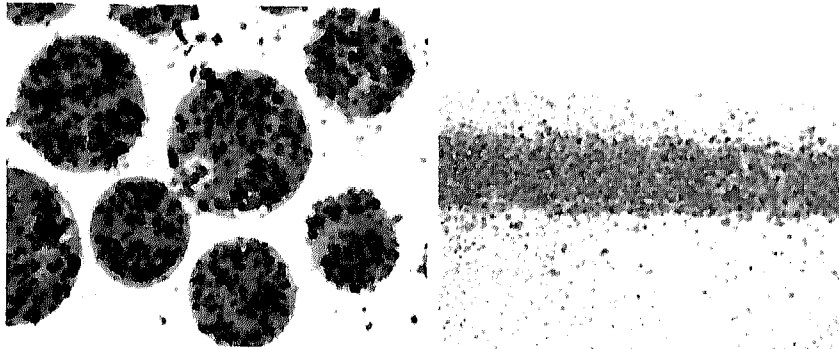the' spermatophore (Fig. 3).

FIGURE 3. Electron immunocytochemical localization
of PL21.1 after coupling with primary antibody,
biotinylated secondary antibody, and avidin-biotin-
peroxidase followed by diaminobenzidine (Vectastain). In
type 4 cells of the BAG, the secretion is confined to the
secretory granules (X34,000). In the spermatophore
(right), the staining is restricted to a discrete zone of
the outer wall (X18,000).

With the aid of PL 21.1 antibody, we isolated the
corresponding antigen. BAGs were homogenized and the
soluble fraction was applied to a Sephadex G-150 column.
Fractions containing antibody were identified by
dot-blotting, pooled and applied to an antibody affinity
column made by coupling PL21.1 to Affigel-10. After
washing thoroughly with binding buffer and high salt, a
peak was eluted with 0.2 N acetic acid. The antigen was
absent from the low salt and high salt washes and was
present in the acid-eluted peak. When this peak was run
on an SDS gel, there was a major band at 23 kd and also, a
minor one at lower molecular weight. Elution of the 23 kd
band from a preparative SDS gel gave a single band that
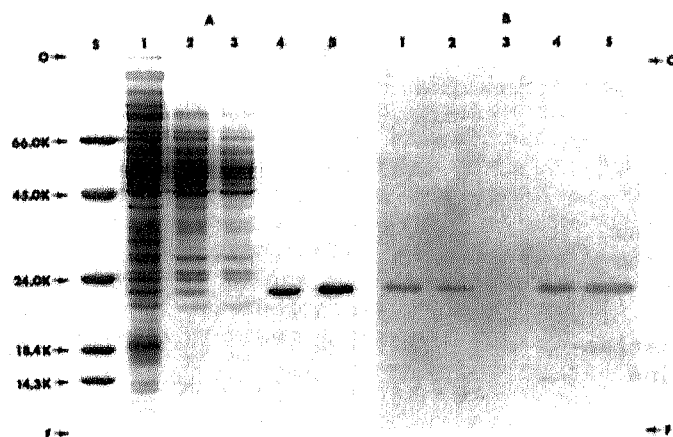was recognized by the PL21.1 antibody (Figure 4).

FIGURE 4   Polyacrylamide gel electrophoresis with
Coomassie staining of the gel (left) and electroblotting
and visualization with PL21.1 antibody, secondary antibody
conjugated with peroxidase, and o-diansidine (right). S,
standard. Lane 1, crude homogenate; Lane 2, positive
fractions from Sephadex G-150; Lane 3, non-binding
fractions from PL21.1 affinity column; Lane 4, acid eluant
from PL21.1 affinity column; Lane 5, the 23 kd band from
another gel (acid eluant of affinity column) which has
been eluted and rerun.

Electrophoresis of homogenates on non-denaturing
pore-limiting (4% - 20%) gels indicated a molecular weight
for this antigen of 370 kd, suggesting that there are 16
monomers in a native protein molecule.

The amino acid composition of the isolated protein was determined after acid hydrolysis (Table 1). The antigen corresponding to PL21.1 is distinguished by its high glutamic acid/glutamine content (15.4%) and even higher proline content (25%). Methionine was absent. Preliminary sequence analysis (23 of 30 N-terminal amino acids) shows the presence of six prolines but does not suggest their involvement in an obvious repeat such as is found in collagen.

TABLE 1

AMINO ACID COMPOSITION OF PL21.1 ANTIGEN

AMINO ACID	Mol per cent*
Aspartic acid/asparagine	6.9
Threonine	3.9
Serine	3.5
Glutamic acid/glutamine	15.4
Proline	25.2
Glycine	5.7
Alanine	5.9
Valine	4.5
Methionine	0
Isoleucine	5.4
Leucine	4.0
Tyrosine	4.0
Phenyalanine	3.2
Histidine	1.7
Lysine	3.2
Arginine	3.5
Cysteic acid**	4.1

* Data from duplicate samples hydrolyzed for 24 hr in 6 N HCL _in vacuo_, 110° C.
** Duplicate samples oxidized with performic acid before hydrolysis.

PL21.1 antigen from the BAG is an unusual protein by virtue of its extraordinarily high proline content (25%). High proline content suggests a relatively rigid structure which has a low α-helical content. This characteristic may be shared by many other spermatophorins of _Tenebrio_, since it was earlier shown that hydrolysates of the wall of the spermatophore of _Tenebrio_ are high in proline (14).

In its high proline content, PL21.1 antigen may bear some
resemblance to the proteins of the vitelline membrane of
Drosophila [18.3% proline (15)] and some cuticle proteins
of cecropia [17.9% proline (16)]. Like many cuticle
proteins and unlike chorion ones, methionine is absent
from Sp23. Unlike cuticle proteins and like some chorion
proteins, there is a relatively high content of cysteine
in PL21.1 (16, 17).

Spermatophores, the most elaborate products of male
accessory glands, are utilized for sperm transfer in
diverse insect species, including many agricultural pests
and disease vectors. Spermatophorins assemble in
extracellular space to form the layers of the wall and the
core of the spermatophore of Tenebrio. At least in broad
outline, the assembly pattern we have seen for Tenebrio
seems to be representative of other species.

According to our evidence from gel electrophoresis
and immunochemistry, PL21.1 antigen from the BAGs is
indistinguishable from a similar antigen in the wall of
the spermatophore. Since the PL21.1 antigen from the
spermatophore is a spermatophorin, this 23 kd protein
should be designated Sp23. The antigen from the gland
appears to be a precursor to Sp23, in the sense that
vitellogenins are precursors to vitellins. Further
research is required before we can judge whether Sp23 is
representative of spermatophorins of Tenebrio or the
analagous proteins of other species.

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The accessory glands of Tenebrio have proved to be
convenient materials with which to demonstrate the
short-term transcription-dependent effects of ecdysteroids
on cell cycles. At the same time that high levels of
ecdysteroids accelerate cell cycles, the BAGs become
competent to mature further and thus to make
adult-specific proteins. The reprogramming is expressed
days later in the long-term effects - increased expression
of genes for spermatophorins. With antibodies as probes,
we plan to isolate the cloned cDNAs corresponding to
spermatophorin messenger RNAs and to begin to study the
genes for these proteins. With the aid of molecular
biology, we hope to understand better the differences
between the short-term and long-term effects of
ecdysteroids and the linkages among them.

ACKNOWLEDGEMENTS

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