Insulin/IGF signaling regulates the change in commitment in imaginal discs and primordia by overriding the effect of juvenile hormone

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A B S T R A C T

At the beginning of the final larval (fifth) instar of Manduca sexta, imaginal precursors including wing discs and eye primordia initiate metamorphic changes, such as pupal commitment, patterning, and cell proliferation. Juvenile hormone (JH) prevents these changes in earlier instars and in starved final instar larvae, but nutrient intake overcomes this effect of JH in the latter. In this study, we show that a molecular marker of pupal commitment, broad, is up-regulated in the wing discs by feeding on sucrose or by bovine insulin or Manduca bombyxin in starved final instar larvae. This effect of insulin could not be prevented by JH. In vitro insulin had no effect on broad expression but relieved the suppression of broad expression by JH. This effect of insulin was directly on the disc as shown by its reduction in the presence of insulin receptor dsRNA. In starved penultimate fourth instar larvae, broad expression in the wing disc was not up-regulated by insulin. The discs became responsive to this action of insulin during the molt to the fifth instar together with the ability to become pupally committed in response to 20-hydroxyecdysone. Thus, the Manduca bombyxin acts as a metamorphosis-initiating factor in the imaginal precursors.

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Introduction

During the last larval instar of holometabolous insects, imaginal discs and primordia initiate patterning and morphogenetic growth in preparation for metamorphosis. Prior to these metamorphic changes, the cells are subjected to a programmatic switch from larval to pupal, referred to as pupal commitment. In the general body epidermis, pupal commitment is orchestrated by the molting hormone, 20-hydroxyecdysone (20E), in the absence of juvenile hormone (JH) at the end of the feeding period (Riddiford, 1976, 1978), but commitment of the imaginal discs and primordia occurs earlier in response to both hormonal and unknown nutrient-dependent signals.

At the beginning of the final (fifth) larval instar of the tobacco hornworm, Manduca sexta, nutritional input is necessary to induce commitment of the imaginal eye and leg primordia and the wing discs (MacWhinnie et al., 2005; Truman et al., 2006). When larvae are starved, these imaginal precursor cells do not become committed. Similar diet-dependent induction of commitment is observed in the wing discs of the silkworm, Bombyx mori (Obara et al., 2002). When Manduca larvae are fed sugars, but not amino acids, a BTB/POZ type of transcription factor, broad, which is a molecular marker of pupal commitment (Zhou et al., 1998; Zhou and Riddiford, 2001), appears in the eye primordia (MacWhinnie et al., 2005). These primordia will subsequently begin their morphogenetic growth when given both sugars and amino acids. This indicates that final instar larvae are somehow able to sense sugar intake at least at the beginning of the instar and that nutrient-dependent signaling is involved in the change in commitment in the imaginal primordia and discs.

During the molt to the fifth instar of Manduca, the hemolymph JH titer gradually decreases to a low level, then abruptly increases to a very high level at ec dysis to the fifth instar (Fain and Riddiford, 1975). Once larvae are fed, the JH titer decreases to a low level; but when starved, the JH titer increases until they are fed (Cymborowski et al., 1982). Removal of the corpora allata, the source of JH (allatectomy) during the molt to the fifth instar allows pupal commitment and the onset of differentiation in the imaginal discs and primordia of starved larvae after ec dysis (Truman et al., 2006). Importantly, the application of a JH mimic (JHM) prevents this commitment in allatectomized larvae that are starved but not when they are fed. Similarly, JHM does not suppress these processes in the eye primordia in fed intact final instar larvae (Allee et al., 2006). Thus, a nutrient-dependent factor(s) overcomes the JH suppression of the imaginal primordia and discs in the final instar. This factor has been called metamorphosis-initiating factor (MIF) (MacWhinnie et al., 2005; Truman et al., 2006; Allee et al., 2006).

During the decline of ecdysteroid in the final larval molt, the wing discs of the silkworm, B. mori, gradually became competent to respond to 20E in vitro by becoming pupally committed (Koyama et al., 2004a). Initially, a low concentration of the JHM methoprene was sufficient to suppress this effect of 20E, but by 12 h after ec dysis even a...
high concentration had little effect. At this latter time, commitment occurred in vitro in the absence of any hormone although JH could partially suppress this commitment. By 24 h after ecdysis, neither 20E nor JH affected the progress of commitment. Therefore, imaginal disc cells become ‘competent’ to be committed before ecdysis to the final larval instar, but JH prevents the commitment until sometime after ecdysis.

One candidate for MIF is insulin/insulin-like peptide. In Drosophila, there are seven insulin-like peptides, two of which are nutrition-dependent (Brogiole et al., 2001; Ikeya et al., 2002). Both loss and gain of function analyses of Drosophila insulin/insulin-like growth factor (IGF) signaling have shown that this signaling controls growth, size regulation and longevity, both at the cellular and the organismal levels (for reviews, Saudedo et al., 2003; Mirth and Riddiford, 2007). In the adults, mutations in the insulin/IGF signaling pathway alter JH synthesis in the corpora allata (Tatar et al., 2001; Tu et al., 2005). Recent studies have shown that the synthesis of 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase), a key enzyme in JH biosynthesis, in these glands is dependent on the insulin receptor (Belgacem and Martin, 2007).

In lepidopteran larvae, an insulin-like peptide, bombyxin, stimulates cell proliferation in the wing discs (Nijhout and Grunert, 2002; Nijhout et al., 2007). Interestingly, in Bombyx bombyxin is secreted from neurosecretory cells of the brain in response to sugar intake at the beginning of the fifth instar (Masumura et al., 2000).

In this study, we show that insulin induces broad expression in wing discs and eye primordia of final instar Manduca larvae in the absence of nutritional input. It does so by suppressing the JH repression of this commitment. This is a novel function of insulin/IGF signaling.

Materials and methods

Animals

Larvae of the tobacco hornworm, M. sexta, were individually reared in plastic cups containing artificial diet (Bell and Joachim, 1976) under a 12-h photophase: 12-h scotophase at 25.5 °C. To obtain timed fourth instar larvae, we used the onset of head capsule slippage (HCS), which is the separation of an old head capsule from the new head capsule, as a developmental marker. This onset was defined by the lack of withdrawal of the head in response to a mechanical stimulus. Larvae were observed every 30 min for this onset which was designated as HCS0h. Larvae eclosed to the fifth instar 28–29 h after the onset of HCS. To obtain timed fifth instar larvae, we selected newly molted fifth instar larvae every 30 min (5th 0 h). Larvae were raised on wet Kimwipes for starvation experiments.

Nutrient manipulation

To assess the effect of different nutrients, we fed 7.0% sucrose or 5.8% casein or a mixture of the two in non-nutrient agar (MacWhinnie et al., 2005). Sucrose and casein were used to investigate the effect of sugars and amino acids, respectively. Better survival was obtained with a base diet that contained vitamins, minerals, antibiotics, and preservatives: 15.65 g/l Wesson’s salt, 2.61 g/l sorbic acid, 13 g/l methyl paraaben, 6.52 g/l ascorbic acid, 0.26 g/l streptomycin, 0.07 g/l kanamycin, 0.11% formaldehyde, and 1.3% vitamin mixture (stock solution: 1.0 g/l nicotinic acid, 0.5 g/l riboflavin, 0.23 g/l thiamine, 0.23 g/l pyridoxine, 0.23 g/l folic acid, 0.02 g/l biotin), and 15.9 g/l non-nutrient Gelcarin PS 402 (FMC BioPolymer) for solidification.

Hormones and chemicals

A stock solution of 20E (SciTech Chemicals) was prepared in ethanol, and the concentration was measured spectrophotometrically at 240 nm ($\varepsilon_{240} = 12676$). A JHM, methoprene (Wellmark International), was dissolved in cyclohexane for tissue culture experiments or in acetone for topical application experiments. Stock solutions of 20E and JHM were stored at −20 °C until used. JHM-containing culture medium and containers for JHM-medium were prepared according to Riddiford et al. (1979). Rapamycin (Calbiochem) was dissolved in ethanol, wortmannin (Sigma) was dissolved in DMSO, and bovine insulin (Sigma) and partially purified Manduca bombyxin (gift of Dr. H.F. Nijhout, Duke University) were dissolved in 10 mM HCl.

Implantation assay of pupal commitment

The degree of the pupal commitment in the forewing discs was assessed by implantation into young fourth instar larvae and the type of cuticle on the implant determined after the host molted to the fifth instar as done for the epidermis by Riddiford (1976, 1978). The commitment score (0–5) was as in Obara et al. (2002). Briefly, score 0, entire disc is pale white and no tanned pupal cuticle; score 1, a few tanned spots appear; score 2, the tanned spots fuse and form a thin band; score 3, the band area expands approximately less than one-fourth of the surface area; score 4, the tanned area expands approximately less than half of the surface area; score 5, the tanned area covers more than half of the surface.

Tissue culture

Tissue culture was performed basically as described in Koyama et al. (2004a, b). Larvae were surface-sterilized in 70% ethanol followed by water anesthetization. Five to ten discs or primordia were cultured together in 1 ml of Grace's insect culture medium ( Gibco) at 25 °C under 95% O2/5% CO2. For eye primordium culture, Antibiotic-Antimycotic ( Gibco) was used.

RNA preparations and cloning of Manduca InR

Total RNA was isolated with TRIzol (Invitrogen). RNA concentrations were determined spectrophotometrically at 260 nm. Manduca Insulin Receptor (InR) was cloned by RT-PCR with degenerate primers that were designed based on an alignment of InR amino acid sequences of several insect species, B. mori, Drosophila melanogaster, Tribolium castaneum and Aedes aegypti. The partial sequence of Manduca InR obtained by RT-PCR was extended by the Rapid Amplification of cDNA End (RACE) method with the SMART RACE cDNA Amplification Kit (Clontech Laboratories). RACE-PCR reactions were performed according to the manufacturer's instructions.

In vitro RNA interference (RNAi)

For in vitro RNAi, wing discs were pre-cultured with double-stranded RNA (dsRNA) in Grace's media for 48 h, and then the discs were transferred into main culture media that included the same amount of dsRNA. The InR sequence was amplified with primers attached to the T7-promoter sequence, 5′-TACGGATATGGCCCTACTATC-TGG-3′ and 5′-TATCTCTGAGGATCGGAAATCCC-3′. The amplicin resistance gene (β-lactamase), which was amplified by primer pairs, 5′-ATGAGTATTCAATTTTCTGCCGAGC-3′ and 5′-ATCAATGTTTATGTCGATGAGC-3′, was used as a negative control. PCR products were purified and used as a template for RNA synthesis. RNA synthesis and annealing of RNA were performed according to Erezyilmaz et al. (2006).

Real-time reverse transcription-PCR analysis

 broad and InR mRNA levels were determined using real-time quantitative PCR. Real-time PCR was performed using a Chrono 4 System (Bio-Rad Laboratories) with a Brilliant SYBR Green QPCR Master Mix (Stratagene). Q-PCR reactions were performed according
to the manufacturer's instructions. Total RNA was extracted from 5 to 20 tissues, and three independent experiments were performed for reverse transcription. Expression levels of broad, InR and RpL3 were quantified based on standard curves, and those of both broad and InR mRNAs in one sample of total RNA were normalized with RpL3. Primers are: broad, 5′-TATAAGACGGACCTGATATACACCGCC-3′ and 5′-CTCTGAGATTTGAATCTCGGAG-3′; InR, 5′-GACTTACGGCAGAATCTGGAG-3′ and 5′-TACCCGCTTCTTCAAACTCCTG-3′; RpL3, 5′-GAGGATAAAGTAAAGTGGGCCAGGG-3′ and 5′-CACGCCGACACGCTTTCCAAAGTG-3′.

Immunohistochemistry

Eye primordia were fixed in 4% formaldehyde in phosphate-buffered saline [PBS: 0.283 g NaH2PO4 (H2O), 2.065 g Na2HPO4 (7H2O), 9 g NaCl] for 30 min at room temperature or for overnight at 4 °C. After washing with PBS containing 1% Triton-X 100 (PBS-TX), the tissues were blocked with 5% normal goat serum and 5% normal donkey serum, then incubated with 1:500 anti- Manduca Broad-core antiserum for 24 h at 4 °C (Zhou and Riddiford, 2001). After washing with PBS-TX, tissues were incubated with fluorescein-conjugated donkey anti-rabbit IgG (Jackson Immuno Research) for 24 h at 4 °C. Tissues were mounted in Vectashield (Vector Laboratories).

Results

Insulin/IGF signaling is involved in pupal commitment of wing discs at the beginning of the fifth instar

Previous studies (MacWhinnie et al., 2005; Truman et al., 2006) have shown that nutrient input, specifically that of sugar, is necessary for the appearance of broad mRNA, a molecular marker for pupal commitment (Zhou et al., 1998; Zhou and Riddiford, 2001), in the eye and leg imaginal primordia. To confirm this nutrient dependency of induction of pupal commitment in the wing discs, we fed freshly molted fifth instar larvae on various special diets lacking certain components of the normal Manduca diet. Based on the implantation assay to assess the degree of pupal commitment (Riddiford, 1976; Obara et al., 2002) and quantification of broad mRNA, we confirmed that sugar intake was sufficient to initiate broad expression and pupal commitment, but not to maintain the expression level of broad or achieve the commitment level seen in discs of normally fed larvae unless either amino acids or vitamins and minerals (metabolic cofactors) were also in the diet (Fig. 1). No broad expression or pupal commitment occurred in discs of animals fed only amino acids, but both were seen when the metabolic cofactors were added to the amino acid diet (Fig. 1). These data show that induction of pupal commitment in the wing discs is dependent on nutrients, especially sugar, at the beginning of the fifth instar, and suggest that sugar-mediated signaling might be responsible.

Since sugar stimulates the release of the insulin-like peptide, bombyxin, from the neurosecretory cells in the brain of Bombyx at the beginning of the final instar (Masumura et al., 2000), we hypothesized that insulin/IGF signaling was involved in the change in commitment. To test our hypothesis, we first utilized wortmannin, a specific inhibitor of insulin/IGF signaling (Okada et al., 1994), and rapamycin, a specific Tor signaling inhibitor, which disrupts amino acid-specific signaling (Stanberg et al., 1997). In normally feeding fifth instar larvae, broad mRNA is seen in wing discs by 24 h (Figs. 2A, B). When 40 nmol of wortmannin was injected into freshly molted fifth instar larvae that were then fed normal diet for 24 h, no up-regulation of broad mRNA was observed in their wing discs (Figs. 2A, B). This suppressive effect of wortmannin on the wing discs was concentration-dependent (Fig. 2C). Wortmannin had a similar effect in the eye primordia on both broad mRNA (data not shown) and Broad protein (Fig. 3). In contrast, there was no effect of dietary rapamycin on the up-regulation of broad mRNA in these discs (Fig. 2A). Injected rapamycin also had no effect in 36 h [253.5±36.9 versus 291.0±32.9 for the 10% ethanol-injected control (average±S.E. for N=3) relative to broad mRNA as 100 at time of injection]. These data indicated that insulin/IGF signaling was necessary for the change in commitment in the imaginal tissues in response to nutrients.

To assess whether insulin or insulin-like peptides cause broad expression in the wing discs, we injected bovine insulin or Manduca bombyxin into freshly molted fifth instar larvae that were subsequently starved. Both bovine insulin and bombyxin caused the appearance of broad mRNA in the absence of nutrient input (Fig. 2D), and this effect of insulin on broad induction was concentration-dependent (Fig. 2E). When 50 μg of bovine insulin was injected into the newly molted fifth instar larvae that were then starved, broad expression was up-regulated in 24 h, and the commitment score was also increased concomitantly with the levels of broad mRNA (Fig. 2F).

In allatectomized (CAX) larvae, which lack the source of JH, the expression level of broad is up-regulated in a nutrient-independent manner (Truman et al., 2006). To determine whether or not insulin/IGF signaling alters the expression level of broad in the CAX larvae, we injected either wortmannin or bovine insulin shortly after ecysis to the fifth instar, then assessed broad expression in the wing discs after 36 h of starvation (Fig. 4). Neither wortmannin nor bovine insulin alters the expression level of broad (Fig. 4). These results suggested that insulin/IGF signaling modulated sensitivity to JH in the imaginal disc cells rather than directly up-regulating broad mRNA transcription.

Insulin/IGF signaling is needed to overcome the suppressive effect of JH on change in commitment at the beginning of the fifth instar

To assess whether insulin/IGF signaling directly stimulates broad expression, the wing discs from freshly molted fifth instar larvae were
broad quantitation at 0 h of the fourth instar larvae to assess commitment (open diamonds). Expression level of and on commitment in the wing discs. Wing discs were dissected, and total RNA was diet as in (A) and (B). (D) Effects of injection of purified Manduca bombyxin or bovine insulin into freshly molted fifth instar larvae on broad expression in the wing discs. Freshly molted fifth instar larvae were injected either with 40 nmol of wortmannin in 10% DMSO (open circles) or with 10% DMSO (closed circles), then normal Manduca diet was fed. Rapamycin was fed as 40 nmol of rapamycin in 4 g of normal diet. After 24 h of feeding, the larvae were dissected and total RNA was extracted to assess the broad mRNA level. (B) Time course of the effects of 40 nmol wortmannin after injection into freshly molted fifth instar larvae on broad expression in the wing discs. Freshly molted fifth instar larvae were injected either with 40 nmol of wortmannin in 10% DMSO (open circles) or with 10% DMSO (closed circles), then normal Manduca diet was fed. (C) Concentration-dependence of wortmannin inhibition of broad expression in the wing discs 36 h after injection into freshly molted fifth instar larvae that were fed normal diet as in (A) and (B). (D) Effects of injection of purified Manduca bombyxin or bovine insulin into freshly molted fifth instar larvae on broad expression after 36 h of starvation. Larvae were given 10 or 50 μg of bovine insulin or 2.5 brain equivalents of Manduca bombyxin. (E) Concentration-dependence of bovine insulin induction of broad expression 36 h after injection into freshly molted fifth instar larvae that were subsequently starved. (F) Time course of the effects of 50 μg bovine insulin given to freshly molted fifth instar larvae that were subsequently starved on broad expression and on commitment in the wing discs. Wing discs were dissected, and total RNA was extracted to assess broad expression (closed circles), or they were implanted into young fourth instar larvae to assess commitment (open diamonds). Expression level of broad at 0 h of the fifth instar was designated as 100. Each point is the average ± S.E. N = 3 for broad quantification and N = 11–13 for implantation assay.

Fig. 3. The effect of wortmannin on Broad appearance in the eye primordia as detected by the Manduca Broad-core antibody. (A) DMSO-injected control. (B) Wortmannin-treated. Forty nmol of wortmannin were injected into freshly molted fifth instar larvae, which were then returned onto normal diet for 48 h. Dashed lines indicate Broad-positive cells in (A) and corresponding area in (B). Inset in (A) shows magnification of indicated area. Anterior is to the left. Bar=50 μm.

Fig. 4. Effects of injection of wortmannin or bovine insulin into freshly molted allatectomized fifth instar larvae on broad expression after 36 h of starvation. Allatectomy was performed several hours before HCS of the fourth instar as described by Truman et al. (2006). Closed bars, allatectomized (CAX) larvae; open bars, sham-operated larvae. Ins, bovine insulin; Wor, wortmannin; Fed, fed normal diet; St, starved on wet Kimwipe. Expression level of broad at 0 h of the fifth instar was designated as 100. Each point is the average ± S.E. N = 3.

Fig. 5. Insulin/IGF signaling overcomes the suppressive effect of JHM on broad expression in vitro. (A) Effects of purified Manduca bombyxin and bovine insulin on broad expression in wing discs in vitro in the presence or absence of JHM. Wing discs dissected from freshly molted fifth instar larvae were cultured with various hormones and chemicals for 24 h. Concentrations of hormones and chemicals are: methoprene (JHM), 1 μg/ml; wortmannin (Wor), 1 μM; bombyxin (BBX), 2.5 brain equivalents/ml; bovine insulin (Ins), 10 μg/ml. (B) Concentration-dependence of the effect of bovine insulin on overcoming the JHM suppression of broad expression in wing discs in vitro. Wing discs of freshly molted fifth instar larvae were cultured for 24 h with 1 μg/ml of methoprene and various concentrations of bovine insulin. Open circle, level of broad mRNA in the hormone-free condition; closed circles, levels of broad mRNA in JHM and bovine insulin. The level of broad mRNA in the hormone-free condition at 24 h was designated as 100. Each point is the average ± S.E. N = 3.
expression by the JHM. This effect was dependent on the degree of insulin could no longer block the suppression of the suppression of (dsAmp) gene (dsAmp) was used as a negative control dsRNA and reduced to 46% and 44% of no dsRNA control levels at 24 h and 48 h, and 50

in the imaginal disc cells can overcome the suppressive effect of JH on the appearance of broad mRNA, but that insulin/IGF signaling cannot directly stimulate broad mRNA expression.

Suppression of the insulin receptor expression by RNAi shows that insulin/IGF signaling is necessary for the change in commitment

To confirm the effect of insulin/IGF signaling in the wing discs, we cloned and partially sequenced Manduca insulin receptor (InR) cDNA, then used InR dsRNA to suppress insulin/IGF signaling by RNA interference (RNAi). Since InR is a single copy gene in all insect species so far examined, we isolated Manduca InR using an RT-PCR method with degenerate primers, which were designed based on amino acid sequences of several insect InRs, and a rapid amplification of cDNA end (RACE) method. This sequence has been deposited in GenBank, Accession No. FJ169464. The cloned partial Manduca InR sequence (3911 bp, 1064 aa) showed 75% identity with B. mori, 46% with T. castaneum and 37% with D. melanogaster InR at the amino acid level (data not shown).

InR mRNA expression in the wing discs was high at the beginning of the fourth instar, then declined to a plateau level during the feeding phase (Fig. S2). The fifth instar discs showed the same pattern, high at ecysis with a decline to a plateau level during the feeding phase. We also analyzed InR expression in the abdominal epidermis as a control. Interestingly, InR mRNA expression in the general larval epidermis was similar to the wing discs in the fourth instar, but in the fifth instar differed as it was low until the final day of feeding when this epidermis is becoming pupally committed (Riddiford, 1978).

To suppress InR mRNA expression by in vitro RNAi, we pre-cultured wing discs obtained from freshly molted fifth instar larvae with double-stranded InR dsRNA and 1 μg/ml methoprene in a culture medium for 48 h followed by a culture with dsInR RNA, methoprene and 50 μg/ml bovine insulin for either 24 or 48 h. When 20 μg/ml of dsInR RNA was used in this culture-regimen, InR expression level was reduced to 46% and 44% of no dsRNA control levels at 24 h and 48 h, respectively (Fig. 6). The double-stranded ampicillin resistance (β-lactamase) gene (dsAmp) was used as a negative control dsRNA and had no effect on InR expression. When InR expression was suppressed, insulin could no longer block the suppression of broad mRNA expression by the JHM. This effect was dependent on the degree of suppression of InR.

Ecdysteroids are not necessary to induce pupal commitment by insulin in the starved larvae

Our CAX and in vitro culture results showed that insulin did not induce broad expression in the absence of JH or JHM, but prevented the suppressive effect of JH or JHM on broad expression (Figs. 4, 5). Recently, one of the insulin-like peptides of the mosquito, A. aegypti, was found to stimulate ecdysioderoid production in the ovaries (Brown et al., 2008). To examine whether insulin/IGF signaling stimulates ecdysteroid production in the prothoracic glands (PG) that then causes the change in commitment, we injected 50 μg of bovine insulin into decapitated (neck-ligated, NL), mesothorax–metathorax intersegment-ligated (T2–T3 ligation, TL) or non-ligated (NoL) freshly molted fifth instar larvae and assessed the expression level of broad in the hindwing discs 24 h later (Fig. 7). TL larvae lack both corpora allata (CA), source of JH, and PG, source of ecdysteroid, and NL larvae lack only CA. Hindwing discs of non-injected NL and TL larvae showed greater than 3 times higher level of broad expression than that of non-injected starved NoL larvae (Fig. 7). Apparently, the absence of JH due to the removal of the CA enhanced the elevation of broad expression, which was not dependent on insulin. To avoid this problem, we applied 1 μg of methoprene immediately after ligature and injection of insulin, and assessed broad expression under the same conditions (Fig. 7). In all 3 cases, insulin increased broad expression over the basal level with JHM. The hindwing disc of insulin-injected NL larvae showed a slightly higher level of broad expression than that of TL or NoL larvae, but this difference was not significant. Since a low concentration of 20E strongly induced broad expression during this period (see Fig. S1), ecdysteroids are not involved in the insulin-mediated change in commitment in the imaginal discs at least during the first 24-h period after insulin injection.

Sensitivity to insulin is acquired during the molt to the fifth instar

In order to examine whether insulin/IGF signaling can suppress JH effects only at the beginning of the fifth instar, we injected bovine insulin during the molts to the fourth and to the fifth instar, and assessed broad mRNA expression. The scheme of the experiment is described in Fig. 8A. Bovine insulin was injected into larvae (i) immediately after the ecysis to the fourth instar, (ii) several hours before third instar HCS and immediately after the ecysis to the fourth instar and (iii) immediately after the ecysis to the fifth instar, and
then injected larvae were starved for 48 h after ecysis. At the beginning of the fourth instar, insulin did not induce broad mRNA even if larvae were injected twice with insulin (Fig. 8B). These results showed that this stimulatory effect of insulin on broad induction in the presence of JH was specifically seen after the molt to the fifth instar, i.e., the imaginal discs acquired sensitivity to insulin sometime during the fourth instar.

To examine when the discs become sensitive to insulin, we injected 50 μg of insulin at various times during the molt to the final larval instar and assessed the expression level of broad in the wing discs 48 h later (Fig. 9A). The larvae were not fed after ecysis. When insulin was injected before or at HCS, we saw no broad induction 48 h later (Fig. 9A). At HCS+12 h, insulin induced some broad expression (Fig. 9A). The greatest response to insulin was seen in discs from larvae that were treated with (i) 1 μg of methoprene or 1 μl acetone was applied as in (i), but the larvae were starved for 48 h after ecysis before dissection. (iii) 1 μg of methoprene or 1 μl acetone was applied immediately before HCS and either bovine insulin or solvent was injected immediately after ecysis to the fifth instar, then the larvae starved 48 h before dissection. (D) Level of broad mRNA in the wing discs. (E) Scheme of the experiments for (F). One μg methoprene was applied 24 h, 12 h or shortly before HCS in the molt to the fifth instar. After ecysis the treated larvae were then either fed or given 50 μg bovine insulin immediately and starved before dissection at 48 h. (F) Level of broad mRNA in the wing discs and commitment score. Closed circles, broad expression in the wing discs of insulin-injected larvae; open circles, broad expression in the wing discs of fed larvae; closed bars, commitment score in the wing discs of insulin-injected larvae; open bars, commitment score in the wing discs of fed larvae; hatched bars, commitment score in the wing discs of starved larvae. The level of broad mRNA in the wing discs of insulin-treated larvae at 48 h of the fifth instar was designated as 100. Each point is the average ± S.E. N= 3 for broad quantification and N= 12–15 for implantation assay.

During the molt to the fifth instar, the hemolymph JH titer of Manduca larvae declines (Fain and Riddiford, 1975). This JH drop seems to play a role in the acquisition of competence of the wing discs to become pupally committed in response to 20E (Fig. 9C) as seen in the wing discs of Bombyx (Koyama et al., 2004a). In Manduca wing discs, cells begin to respond to low concentrations of 20E (0.1 μg/ml) in vitro shortly after HCS, but JH can prevent this response up to the time of ecysis (Fig. 9C). At ecysis JH no longer prevented the response to 20E, but prevented the pupal commitment that occurred in hormone-free medium. To examine whether the drop of JH titer and/or the onset of the commitment process (acquisition of sensitivity to 20E) in the imaginal discs is essential for the effect of insulin, we topicaly applied 1 μg of methoprene at the onset of HCS, which was 28–29 h before ecysis to the fifth instar, then injected insulin immediately after the molt to the fifth instar (see Fig. 8C for details). The broad response to insulin was not significantly affected in the discs from the JHM-treated larvae (Fig. 8D). When JHM was applied at earlier times (12 or 24 h before HCS), broad expression was slightly reduced in both insulin-treated and fed larvae, but these differences were not significant (Figs. 8E, F). The commitment score in these conditions was also slightly suppressed, but these scores were significantly higher than in the starved controls (Fig. 8F). In addition, larvae that were given 1 or 10 μg of methoprene 24 h before or at HCS pupated with a few days delay, and all these pupae showed slightly larger body size with complete appendages (data not shown). Thus, the responsiveness to insulin at ecysis is not dependent on the decline of JH during the molt.

Discussion

Pupal commitment of larval tissues, accompanied by the appearance of the transcription factor broad (Zhou and Riddiford, 2001), is the first cellular step to metamorphosis. It is caused by endocrine signaling but the timing and the hormonal cues differ between the general epidermis and the imaginal discs and primordia. In the general epidermis, it occurs late in the feeding period in response to 20E in the absence of JH (Riddiford, 1978) which also causes the cessation of feeding and the onset of metamorphosis. The imaginal discs and primordia, in contrast, become committed during the first
Insulin counteracts the effects of starvation on pupal commitment of wing discs

Previous studies with the eye and leg primordia of Manduca have shown that pupal commitment as defined by the appearance of broad mRNA occurs early in the final instar after the larvae feed (MacWhinnie et al., 2005; Truman et al., 2006). None appeared in starved larvae, but feeding on a sucrose diet was sufficient to induce broad in the eye primordia whereas feeding on amino acids was not (MacWhinnie et al., 2005). We have confirmed these findings for the wing discs and further shown that the appearance of broad coincides with the onset of pupal commitment as assayed by the production of pupal cuticle by an implanted disc during the larval molt of the host. When provided as single nutrients, feeding on sucrose caused progressive commitment of the discs whereas feeding on an amino acid source did not. When the diet was supplemented with vitamins and minerals, the protein diet became as effective as the sucrose diet, presumably because the cofactors facilitated the conversion of amino acids into sugar. The effects of these diets on broad expression were similar. Therefore, we conclude that sugar is the primary cue to initiate broad expression and pupal commitment. We however cannot explain why the broad response was transient under the sucrose-only conditions. Supplementation of the sucrose diet with either amino acids or the cofactors allowed continued broad expression, but the underlying metabolic basis is unclear.

In larvae of the silkworm B. mori, intake of sugar causes the release of bombyxin, the insect ortholog of insulin (Masumura et al., 2000). We found that either bombyxin or bovine insulin injected into freshly ecdysed Manduca larvae that were subsequently starved caused the appearance of broad and concomitant pupal commitment of the wing discs. Conversely, wortmannin, an insulin-signaling inhibitor (Okada et al., 1994), prevented the appearance of broad in both the wing discs and the eye primordia of normally fed larvae. These findings together strongly suggest that insulin is the nutrient-dependent signal that leads to the appearance of broad mRNA and pupal commitment in the wing discs and the other imaginal primordia (Fig. 10).

Paradoxically, insulin did not have a similar effect in the early fourth instar larva, even though the InR mRNA is present in the wing discs at similar levels to that in the fifth instar. Presumably during the penultimate and likely earlier instars, the discs and other tissues are sensitive to insulin in order to coordinate their growth with their dietary intake as seen in Drosophila (Britton et al., 2002). Then in the final instar, insulin appears to have a morphogenetic function, initiating pupal commitment and subsequent morphogenetic growth of the discs and primordia. Manduca fourth instar wing discs also are not competent to respond to 20E in vitro by becoming pupally committed (Fig. 9C), just as Obara et al. (2002) and Koyama et al. (2004a,b) found for Bombyx wing discs. In both cases, this competency is gained gradually during the molt to the final instar and is completed within about 16–18 h after ecdysis in fed animals. During this period, the discs also lose their sensitivity to JH to suppress the commitment response to 20E. The switch in insulin function is associated with the gain in competence to respond to 20E as it is fully manifest at ecdysis (Figs. 9A, B). Yet the insulin effect does not depend on endogenous...
ecdy steroids since it occurred in hindwing discs of T2–T3-ligated larvae in the absence of the source of the ecdy steroids. Importantly, neither insulin nor bombyxin was able to stimulate broad expression in the wing discs in vitro beyond the increase seen in hormone-free medium. In addition, neither wortmannin nor bovine insulin had any effect on the broad expression that normally occurs in starved CAX larvae (Fig. 4). Therefore, although insulin is directly acting on the wing discs via the InR, it is not directly up-regulating broad, but rather regulating its response to JH.

Antagonistic interaction of JH and insulin

Our studies have shown that insulin can overcome the suppressive effects of JH on the induction of broad and pupal commitment in the wing discs in starved final instar larvae, even when exogenous JH is given during the molt to the final instar. Moreover, this effect of insulin appears to be directly on the discs since our in vitro studies show that insulin suppresses the ability of JH to inhibit the increase in broad mRNA that occurred when early fifth instar discs were incubated in hormone-free conditions. Either wortmannin or InR dsRNA can repress this action of insulin. Thus, insulin is acting as a MIF. How insulin overcomes the effect of JH is not yet understood.

Our simplified model for the interaction of JH, insulin and 20E in the commitment of the wing discs and imaginal primordia is seen in Fig. 10. In early larval instars, JH allows isomorphic growth of the wing discs and imaginal primordia in response to nutrient intake, but prevents the appearance of broad, pupal commitment and subsequent morphogenetic growth of these structures. Insulin is likely involved in this isomorphic growth linked to nutrient input, but in the penultimate instar it cannot cause the appearance of broad mRNA and pupal commitment. 20E also cannot cause commitment in these discs unless JH and/or its covert effects are allowed to decay in a hormone-free environment before the 20E is added (Koyama et al., 2004a). Then during the molt to the final instar, the discs become competent to respond to either insulin or 20E in the presence of JH by expressing broad and pupal commitment. Insulin appears to be repressing the suppressive effect of JH since it cannot stimulate broad expression in vitro (Fig. 5). In contrast, 20E has a direct effect on the disc in vitro to induce broad expression and pupal commitment that cannot be overcome by JH (Figs. S1, 9C). The molecular changes that bring about competence as well as the hormonal changes that cause them are still to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ydbio.2008.09.017.

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