Changes in glycosylation during Drosophila development. The influence of ecdysone on hemomucin isoforms

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Abstract

To explore a possible signal function of glycodeterminants and the tissue specificity of glycosylation in Drosophila melanogaster, hemomucin, a surface mucin previously isolated from cell lines was studied. It was shown to exist in two glycoforms with molecular masses of 100 and 105 kDa, respectively. The two forms differ by the presence of O-linked galactose, which was only detected in the larger glycoform using the β-galactose specific peanut agglutinin (PNA). The 105 form was found in cell lines after addition of the cell cycle inhibitor taxol and after induction with ecdysone. When whole animal tissues were analyzed using PNA, dramatic changes were observed during development. We were able to identify a number of proteins, which showed strong PNA-staining in stages with a high ecdysone titer, while virtually no staining was detected in adults. This pattern was specific for PNA and was not observed with any of the other lectins employed in this study. Surprisingly, in contrast to our observation in cell lines, PNA staining of hemomucin was not observed in late third larval and pupal stages, which are known to produce high ecdysone titers. The only organ, in which significant amounts of the 105 form were detected, were the ovaries, where hemomucin is produced in follicle cells during the late phase of oogenesis and subsequently incorporated into the chorion. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Glycodeterminants are covalently attached to proteins and lipids constituting an informational system, called glycocode, which regulates such diverse processes as fertilization, embryonic development and immune responses (Kaltner and Stierstorfer, 1998). The combination of various monosaccharides in different configurations ensures the specificity of the glycocode. In order for the glycocode to contain information about a particular cell type, glycodeterminants have to be synthesized in a cell- or stage-specific manner through the activity of glycosyltransferases (Mann and Waterman, 1998).

Compared to vertebrates, little is known about the glycosylation machinery in insects. It is generally believed that glycosylation in insects is less extensive, leading to different and less complex sugar determinants (Maerz et al., 1995). This holds true for both N- and O-linked glycosylation. Similar to vertebrates, mucin-type O-linked glycosylation in insects involves the addition of GalNAc to the protein backbone leading to the formation of the Tn determinant. Further addition of galactose, leads to the formation of the T determinant (or core 1, Maerz et al., 1995). There is little evidence in insects that the T-determinant is extended through further addition of sugars.

Recently, a number of studies have pinpointed the importance of glycosylation in developmental processes in Drosophila melanogaster, one of the most intensely analyzed experimental systems in developmental biology (Lin and Perrimon, 1999; Tsuda et al., 1999). A number of mutants have been shown to code for proteins with...
similarities to enzymes involved in carbohydrate metabolism (Yuan et al., 1997; Haecker et al., 1997; Sen et al., 1998). The substrates for these enzymes have not been identified in all cases. Analysis of developmental changes in glycosylation has so far mostly relied on the use of lectins with specificity for specific sugar configurations (Fredieu and Mahowald, 1994; Callaerts et al., 1995). The carbohydrate containing moieties, which include proteins and lipids, remained mostly unidentified. Given the underlying complexities, changes in the glycosylation pattern have not been studied extensively in whole animals, whereas Drosophila cell lines were successfully used for this purpose (Kramerov et al., 1983). One developmental aspect in particular has been addressed in cell lines; namely the influence of ecdysone on the regulation of glycosylation (Berger et al., 1978; Kramerov et al., 1983; Cherbas et al., 1989). In most cell lines, ecdysone has an anti-proliferative effect (Kramerov et al., 1983). In vivo, this effect is only observed in tissues, which are replaced during metamorphosis (Talbot et al., 1993). In developing tissues, ecdysone orchestrates the differentiation. The differential effects of ecdysone are mediated by different isoforms of the ecdysone receptor (Talbot et al., 1993).

Here we describe the differential glycosylation of hemomucin, a cell surface mucin, which we used as a model to study glycosylation in insects (Theopold et al., 1996). One rationale for using this protein is the highly glycosylated nature of mucins which facilitated our analysis. Another rationale is the fact that hemomucin is involved in insect immune reactions and that ecdysone has been shown to enhance immune reactivity in Drosophila by inducing a number of proteins involved in insect immunity (Dimarcq et al., 1997). Hemomucin, an inducer of insect immunity, was originally isolated from Drosophila hemocyte-like cell lines using Helix pomatia agglutinin (HPL), a lectin with specificity for GalNAc (Theopold et al., 1996). Here we present evidence for the stage- and tissue-specific appearance of two different hemomucin glycoforms in Drosophila.

2. Experimental procedures

2.1. Flies

Flies carrying the w 1118 mutation were kept on cornmeal/yeast food at 25°C with a 10/14 h light/dark cycle.

2.2. RNA slot blots

RNA blots were hybridized according to conventional protocols (Sambrook et al., 1989).

2.3. Preparation of antisera

The hemomucin specific antiserum was prepared as described (Theopold et al., 1996). For the production of an antiserum against recombinant hemomucin, a PCR amplified fragment covering amino acids 178–299 (Theopold et al., 1996) was expressed in the expression vector pQE32 (Quiagen). The resulting fusion-protein was purified according to the instructions of the manufacturer and excised from a preparative polyacrylamide gel. Rabbits were immunized according to Harlow and Lane (1988) with approximately 10 µg protein used for each immunization.

2.4. Purification of glycoproteins using lectin columns

Purification on lectin columns was performed essentially as described (Theopold et al., 1996). The sugar used to elute mucin from a PNA column was 125 mM lactose. For smaller scale precipitations of mucin from cell- or tissue-lysates, 50 µl of column material containing 50% beads was incubated with the lysate for 10 min at 4°C, washed six times in the buffer used for binding and eluted by boiling with SDS-PAGE loading buffer.

2.5. Electrophoretic techniques

SDS–polyacrylamide gel electrophoresis on a Mini-Protean II electrophoresis unit (Bio Rad) was essentially performed according to Laemmli (1970). Molecular weights were determined using prestained SeeBlue™ or Mark12™ molecular weight markers (Novex). The proteins were blotted onto a nitrocellulose membrane (Amersham) as described by Theopold and Schmidt (1997). The amount of protein loaded was ca. 5 µg/lane in Western blots or as indicated in the figure legends. The blotting efficiency was determined by staining the blot with Ponceau S. In Western blots, peroxidase-conjugated HPL (Sigma), Bandeira (Griffonia) simplicifolia lectin (BSL) and Vicia villosa B4 lectin (VVL) were used at a concentration of 0.1 µg/ml. Peroxidase-conjugated PNA was used at a concentration of 1 µg/ml. For detection of signals by enhanced chemiluminescence, the BM Chemiluminescence reagents from Boehringer (Mannheim) were used according to the instructions of the supplier. Detection by antibody on the immoblot and by lectin-mediated chemiluminescence on an autoradiograph allowed us to superimpose the two patterns and to identify signals that stained with both the specific antibody and the lectin.

2.6. Treatment of cells prior to Western blot analysis

Schneider cells (SL2 cells, a cell line which has been used as a model system to study ecdysone induction and immune reactions Kramerov et al., 1983; Theopold and
Schmidt, 1997) were diluted 10-fold and grown for another day to obtain an exponentially growing cell sample. For induction, 20-hydroxyecdysone (Sigma) was added to a concentration of 5 μg/ml for the duration indicated in the figure legends. Taxol (Sigma) was added at the concentrations indicated and the cells were left to grow for another five days.

2.7. Treatment of hemomucin with O-glycosidase

Approximately 1 μg of hemomucin eluted from a PNA column was incubated for 20 h after addition of 5 mU of O-glycosidase (Boehringer) in 50 μl reaction buffer (50 mM Tris/HCl, pH 7.5, 0.5% Nonidet P40). After that time, an aliquot of 20 μl was taken for analysis. The remainder was heat-denatured for 10 min at 65°C and incubated with the same amount of freshly added O-glycosidase for another two days. Only the 105 form of hemomucin was digested by O-glycosidase when incubated alone or in a mixture with the 100 kDa form, which remained undigested (data not shown).

2.8. Staining of ovaries and cells

Freshly emerged Drosophila females were transferred into a new culture vessel and incubated for another two to four days. Ovaries were dissected and fixed in 4% paraformaldehyde in PBS overnight. After four washes (5 min each) in PBS, FITC-conjugated PNA was added to a concentration of 50 μg/ml in PBS containing 0.5% NP40. After 1 h, the sample was washed twice to remove excess PNA and analyzed using fluorescence microscopy. Other ovaries were analyzed in Western blots using the hemomucin specific antiserum.

3. Results

3.1. Two glycoforms of hemomucin

To examine whether hemomucin, like other proteins involved in insect immunity, is induced by ecdysone, we analyzed RNA from SL2 cells after ecdysone induction for different time periods (Fig. 1A). Induction of hemomucin transcription was observed after two days. To address possible changes in O-linked glycosylation, we also analyzed samples from cells treated in the same way using lectins with specificity for the first two most common steps of O-linked glycosylation. GalNAc-specific HPL recognized a band of the size of hemomucin in all lysates (although there seemed to be a slight difference in their migration behavior, Fig. 1B), whereas galactose-specific PNA detected a slightly slower migrating band in lysates from induced cells (Fig. 1C). To find out if the two proteins detected by HPL and PNA are both hemomucin, a lysate from ecdysone induced cells was
enriched for the fractions that bind the lectins using affinity purification on lectin columns. The fractions from the two columns were then analyzed with two hemomucin-specific antisera and the two lectins used for purification. Similar to whole-cell lysates, the two forms differed slightly in molecular weight and reacted differentially with PNA (Fig. 2A, 4), whereas HPL and antibodies recognized both forms (Fig. 2A, 1–3). These results confirm that the two bands are different glycoforms of hemomucin. Since ecdysone is known to act as a cell-cycle inhibitor, we also analyzed cells, which had been treated with the cell-cycle inhibitor taxol and were indeed able to detect the larger glycoform in growth-arrested cells (Fig. 2B). Similarly, cells grown to high density, which did not divide further, showed the larger glycoform of hemomucin (Fig. 2C). When an aliquot of these cells was diluted into medium and observed on a glass slide using FITC-conjugated PNA, membrane particles (microparticles) were released as described before (Theopold and Schmidt, 1997). The microparticles showed strong PNA binding (Fig. 2D, E).

To confirm that the observed galactosylation happens on O-linked sugars, the PNA purified fraction was treated with O-glycosidase, which cleaves the bond

Fig. 2. (A) Enrichment of two hemomucin glycoforms: Hemomucin was purified from edysone induced SL2 cells on a PNA column (Pp). The flow-through was subsequently purified on an HPL column (Hp). The two fraction were analyzed on an immunoblot using an antiserum against recombinant hemomucin (1), an antiserum against hemomucin purified from cells (2), HPL (3) and PNA (4). (B) Galactosylation of hemomucin in taxol-treated cells; control cells (c) and cells treated with taxol (0.1 μM: t1; and 2μM: t2) were analyzed using peroxidase-conjugated PNA in a Western blot. (C) Galactosylation of hemomucin in cells in stationary phase; Cell lysates from cells grown to the stationary phase (high density, hd), exponentially growing cells (low density, ld) and cells grown at low density after induction with ecdysone for three days (ecd), were analyzed using peroxidase-conjugated HPL and PNA. (D, E) PNA stains microparticles; cells from a high density culture were diluted into fresh medium and allowed to attach to a glass slide, followed by staining with FITC-conjugated PNA (D) (E shows the same section in phase contrast).
between serine or threonine and the disaccharide Gal-GalNAc in the T determinant. This treatment led to a decrease in PNA binding of hemomucin and a second smaller protein (Fig. 3, left). At the same time, HPL-labeling increased for both proteins after glycosidase treatment, most likely due to better accessibility of GalNAc after removal of the T determinant (Fig. 3, right). This indicates that two forms of hemomucin can be enriched from cultured cells, which differ, by the presence or absence of the T determinant. The molecular mass of the two forms was 100 kDa (with the Tn determinant) and 105 kDa (with the T determinant), respectively. In summary, our in vitro data indicated that ecdysone influences glycosylation in SL2 cells leading to the exposure of galactose on hemomucin. This effect could be due to ecdysone directly or, more likely to the antiproliferative effect of ecdysone, since it was also observed in non-dividing cells.

3.2. Expression of galactosylated proteins in different developmental stages

Having identified two hemomucin glycoforms in *Drosophila* cell lines, our next goal was to analyze expression of hemomucin glycoforms in vivo in order to identify any differences in glycosylation during stages that are characterized by high ecdysone titers. Our expectation was that during the stages with high ecdysone, strong PNA binding occurs in contrast to adult males, where the ecdysone titer is low. Based on our in vitro findings, we analyzed different developmental stages using lectins with specificity for the Tn (GalNAc) and the T (galactose in β-anomeric configuration) determinant. We also included a lectin with specificity for α-galactose (BSL). All samples obtained from whole animals or tissues showed a complex lectin-staining pattern compared to the cell lines. GalNAc-specific lectins showed very similar patterns, one of which is shown in Fig. 4A.

Hemomucin is labeled in SL2 cell lysates and a band of the same size is detectable in all developmental stages. Although the size difference between both mucin glycoforms is visible in SL2 cells, no such difference was apparent among any of the lysates from different stages. A similar result was obtained using BSL (Fig. 4B). In accordance with that, no signal in the size range of hemomucin could be detected in any of the stages analyzed using PNA (Fig. 4C). The only PNA-reactive signal in the size range of hemomucin in the early pupa (Fig. 4C asterisk) is due to a different glycoprotein, for two reasons: (1) affinity purification of lysates from pupal stages did not lead to an enrichment of binding of hemomucin specific antibodies and (2) the antibody signal and the lectin-signal did not co-localize in blots where they were analyzed side by side (data not shown).

Our results indicate that in contrast to the situation in cell lines, hemomucin is not likely to be galactosylated in vivo, even during stages where the ecdysone titer is high. However, a strong correlation between the appearance of a number of other PNA-reactive proteins and the increase in ecdysone titer could be detected. A group of bands in the 200 and 150 kDa range were strongly labeled in all stages that have a high ecdysone titer and were virtually absent in lysates from adult male flies (Fig. 4C). In addition, a PNA-reactive band of 33 kDa was visible in lysates from pupal stages and not in third larval and adult tissues (Fig. 4D). The dramatic changes in PNA staining are thus unique and differ from the patterns we obtained with other lectins where staining was of similar overall intensity in all developmental stages.

3.3. Tissue specificity of hemomucin glycoforms

In addition to its stage specific expression, ecdysone is active in a tissue-specific manner showing high titers in ovaries. We therefore decided to analyze ovarian tissue lysates for the presence of the two glycoforms. As shown in Fig. 5A and C, a hemomucin band, which showed both the size difference and the differential labeling with PNA observed in SL2 cells, could be detected in ovarian tissues. In lectin-mediated precipitation experiments we could confirm that the band observed in ovaries was indeed hemomucin (Fig. 5B).

While analyzing different tissues from third larval instar the 105 glycoform was not observed as expected. Instead, an additional band of approximately 103 kDa
Fig. 4. Hemomucin is not galactosylated in larvae and pupae stages. Lysates from feeding 3rd instar larvae (fl), wandering 3rd instar (wl), early pupae (ep), late pupae (lp) and adult males (ad), as well as lysates from ecdysone induced (S+) and non-induced cells (S−) were analyzed on a Western blot using peroxidase-conjugated lectins and a hemomucin specific antiserum (Ab). The amount loaded is equivalent to 10% of a whole fly lysate, except for adult and third larval stage lysate, of which 25 and 20%, respectively, were loaded to compensate for the difference in hemomucin expression. The band marked by an asterisk in (C) is not identical with hemomucin (see text). Samples were analyzed using *Vicia villosa* lectin (A), *Bandeira simplicifolia* lectin (BSL) and PNA. PNA staining was repeated on a 15% gel in (D).

was detectable in the salivary glands (Fig. 5C). Though having a slightly higher molecular weight than the 100 kDa form, this form showed no labeling with PNA and thus seems to contain no additional β-galactose.

In whole-mount preparations of ovaries labeled with FITC-conjugated PNA, we observed that the staining was restricted to the latest stages of oogenesis, when the chorion is deposited onto the egg surface (Fig. 6A and B). Strong PNA binding was also found on freshly laid eggs, confirming that hemomucin is likely to form part of the chorion (Fig. 6C). When whole lysates from flies younger than two-days- and four-days-old flies were analyzed, only the older flies showed the 105 form of hemomucin, underlining the role of this glycoform as a marker for mature follicle cells. Additional higher molecular bands were also only observed in the lysates from older ovaries indicating a possible crosslinking of hemomucin during the hardening of the chorion.

4. Discussion

We present evidence for the existence of different glycoforms of *Drosophila* hemomucin—our model glycoprotein for the analysis of developmental and tissue-specific regulation of O-linked glycosylation. We were able to identify two glycoforms of hemomucin in a *Drosophila* cell line, which differ by the presence or absence of β-galactose leading to a molecular mass of 105 and 100 kDa, respectively. Both the T- and Tn-antigen have also been observed on proteins from three lepidopteran cell-lines (Lopez et al., 1999), where the Tn-antigen was more abundant, similar to our results (Fig. 1B, C). Interestingly, the signals in both studies were in a similar molecular weight range as hemomucin (Lopez et al., 1999). Galactose and N-acetylgalactosamine, the terminal sugars in the two glycoforms have previously been implicated in regulating developmental processes and
inflammatory reactions in mammals (Weigel, 1994). Our observation that the 105 form seems to predominate in non-dividing cells led to the expectation that this form of hemomucin would also be present in larval tissues that stop dividing and undergo histolysis during metamorphosis, mediated by specific isoforms of the ecdysone receptor. Surprisingly, we were not able to isolate the 105 glycoform in any of the stages, which show high ecdysone titers. Instead, the only organ where we could identify the 105 glycoform in significant amounts was the ovary, where it is produced by the follicle cells and probably deposited onto the egg surface as part of the chorion. The formation of microparticles that we observed in this study (Fig. 2D, E) and before (Theopold and Schmidt, 1997) may be a paradigm for the deposition of membrane proteins like hemomucin onto extracellular surfaces, including the egg surface.

The regulation of hemomucin glycosylation thus reflects some, but not all the effects that ecdysone has on different tissues. These differences may be due to the presence of different isoforms of the ecdysone receptor (Talbot et al., 1993) or to the activity of different ecdysteroids compared to 20-hydroxyecdysone used for cell induction (Hiruma et al., 1997).

Sugar modifying enzymes, which could be responsible for differential galactosylation, include galactosyltransferases and galactosidases, some of which are known to be regulated by ecdysone (Best-Belpomme et al., 1978).

Our results also indicate that other proteins, which contain the T determinant, are highly expressed in both late third larval and pupal stages. This difference to hemomucin may be the result of the induction of proteins with a high content of the T determinant or of the induction of enzymes that modify existing carbohydrate determinants leading to the exposure of the T determinant. An example for the first case are the larval glue proteins, which lead to the signal in the 150 kDa range we observed in larval and pupal lysates (Kress, 1982 and our own unpublished results). An example for the latter case may be the differential galactosylation of laminin which, quite similar to hemomucin, has been shown to exist in two isoforms only one of which reacts with PNA (Callaerts et al., 1995). The signals in the 200 kDa range observed in larval and pupal stages (Fig. 4C) might correspond to some of the laminin subunits.
Fig. 6. PNA staining of ovarioles. Whole-mounts of ovarioles (A and B) and freshly laid eggs (C) were stained with FITC-conjugated PNA and analyzed under the fluorescence microscope. A and B show the same section of ovaries from four-day-old flies under phase contrast (A) and fluorescence (B). C shows a freshly deposited egg after staining with FITC-conjugated PNA. (D) The 105 form of hemomucin is restricted to mature ovaries; Ovary lysates derived from animals two days (d2) and four days (d4) after hatching, were analyzed on an immunoblot using the hemomucin-specific antiserum. The amount equivalent to ovaries from a whole animal (1) or 50 (1/2) and 25% (1/4) fractions, respectively, were loaded. Asterisks indicate higher molecular weight bands that stain with the antiserum.

Using the information available through the EST/BDGP sequencing project it will be possible to identify candidates for galactosyltransferases and glycosidases, which might be responsible for the differences described for laminin (Callaerts et al., 1995) and for hemomucin, and study their regulation by ecdysone. The discovery of multigene families in Drosophila coding for both glycosyltransferases and carbohydrate binding proteins (Theopold et al., 1999) suggests the existence of a complex coding-decoding system based on carbohydrate determinants that may be used during development and for the discrimination between self and non-self. It is interesting to note that amongst Drosophila gene products that have been molecularly characterized, there are a number of proteins with possible specificity for galactose. They include the only member of the C-type lectin family that has to date been purified and was shown to bind galactose (Haq et al., 1996) and the brainiac gene product, which shows sequence homology to galactosyltransferases (Yuan et al., 1997) and is expressed amongst other tissues in the ovaries (Goode et al., 1996). Our future work will concentrate on the analysis of hemomucin glycoforms in mutants of these and other genes involved in glycosylation.

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