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Glucuronic acid can extend *O*-linked core 1 glycans, but it contributes only weakly to the negative surface charge of *Drosophila melanogaster* Schneider-2 cells☆

Isabelle Breloy[a], Tilo Schwientek[a,1], Stefan Lehr[b], Franz-Georg Hanisch[a,c,*]

[a] *Institute of Biochemistry II, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, 50931 Köln, Germany*
[b] *German Diabetes Center, Heinrich-Heine-University, Düsseldorf, Germany*
[c] *Center for Molecular Medicine Cologne, University of Cologne, Köln, Germany*

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Abstract Previous studies of the mucin-type *O*-glycome of the fruit fly *Drosophila melanogaster* have revealed a restricted pattern of neutral core-type glycans corresponding to the Tn-(GalNAcα) and the T-antigen (Galβ1-3GalNAcα). In particular, no extension of the core 1 glycan with acidic sugars, like sialic acid, was detected. Here we report on the identification of an acidic *O*-linked trisaccharide expressed on secreted endogenous and recombinant glycoproteins of the embryonal hemocyte-like *Drosophila* Schneider-2 (S2) cell line. The glycan is composed of glucuronic acid, galactose and *N*-acetylgalactosamine and its structure was determined as GlcA1-3Gal1-3GalNAc. The *O*-linked trisaccharide resembles the peripheral structures of acidic *D. melanogaster* glycosphingolipids. Glucuronic acid may substitute for sialic acid in this organism, however its expression on the S2 cell surface may only marginally contribute to the negative surface charge as revealed by free-flow cell electrophoresis prior to and after β-glucuronidase treatment of the cells.

Keywords: Glucuronic acid; Glycoprotein; *O*-glycosylation; *Drosophila melanogaster*

1. Introduction

Carbohydrate chains of glycoproteins and glycolipids carry a wealth of information concealed in a highly diverse set of linear and branched polymer structures. The information stored in the glycan chains controls a multitude of processes such as cell recognition and cell–cell interactions in development and disease. Comprehensive structural analyses provide keys to decipher the code of the glycan moiety and glycomics – systematic analyses of the complete set of glycan structures expressed by a cell, tissue or organism – therefore has become a major trend in postgenomic research [1]. Glycomic analyses of the fruit fly *Drosophila melanogaster* revealed restricted patterns of *N*-linked sugar chains on proteins and simple mucin-type *O*-glycans dominated by the T-antigen (Galβ1-3GalNAcα) were found in embryonal tissues and cultivated cells [2–4]. Glycosylation is a non-template driven synthetic process directed by an orchestra of glycosyltransferases whose individual cellular expression, topology and combined specificities control the individual pattern of structures produced [5]. Acidic sugars are known to play vital roles in a variety of glycan environments and acidic *Drosophila* glycolipids were identified, nevertheless the presence of charged *N*- or *O*-linked glycans has yet to be confirmed [6]. After completion of the genome indirect evidence for the existence of more complex structures on glycoproteins was obtained by identification of orthologous *Drosophila* glycosyltransferase gene families known to be involved in mammalian complex glycan formation [7]. A functional sialyltransferase of *Drosophila* suggests the potential capability to synthesize sialylated glycans, but their presence in vivo is still controversial and the functions of the sialyltransferase therefore remains elusive [8]. Three β1-3glucuronyltransferases with discrete functions in glycosaminoglycan and glycolipid synthesis were identified and two isoforms were shown to efficiently use the T-antigen as acceptor substrate [9].

Here we report the first example of an acidic mucin-type *O*-glycan on secreted endogenous and recombinant glycoproteins of *Drosophila* Schneider-2 (S2) cells. Schneider-2 is an embryonic hemocyte-like cell line that has emerged as a model system to study hemocyte function [10]. In Drosophila these specialized blood cells phagocytose microbes and conduct various other functions related to innate immunity. Schneider-2 cells do not express sialic acids on *O*-linked glycans [11], and other acidic sugars are likely to substitute these, for example by contributing to the negative surface charge. The acidic glycan characterized in this study is composed of glucuronic acid (GlcA), galactose and *N*-acetylgalactosamine in equimolar proportions and its structure was determined as GlcA1-3Gal1-3GalNAc thus resembling the peripheral structures of acidic glycosphingolipids identified in *Drosophila* embryos [3].

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*Corresponding author. Address: Institute of Biochemistry II, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, 50931 Köln, Germany. Fax: +49 221 478 7788.
E-mail address: franz.hanisch@uni-koeln.de (F.-G. Hanisch).

[1]This author contributed equally to the study as the first author.

Abbreviations: EI, electron impact; ESI-MS, electrospray ionization-mass spectrometry; FFE, free-flow electrophoresis; IMAC, immobilized metal chelate affinity chromatography; GC/MS, gas chromatography–mass spectrometry; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; Man, mannose; RP-HPLC, reversed phase high-pressure liquid chromatography; DG, dystroglycan; S2, Schneider-2

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2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich and were of the highest grade available. Exceptions are indicated in the text.

2.2. Cell culture

Serum-free culture of *Drosophila* S2 cells was performed as described previously [11].

2.3. Expression of recombinant glycosylation probes

The expression vectors encoding the fusion proteins Sgs1VH and MUC1VH were generated as described in [11]. The ectodomain of *Drosophila* dystroglycan (DG) was amplified by PCRusing the EST clone LD 33152 (Invitrogen, Karlsruhe, Germany) with the sense primer 5′-CTAGCGGCCGCATGAGATTCCAGTGGTTC-3′ and the anti-sense primer 5′-CATGCGGCCGCGCCGAAAGAGGACTTA-TGT-3′ with NotI restriction sites. The PCR product was cloned into the NotI site of the expression vector pAc5.1V5-His (Invitrogen). Transfection and expression of the constructs in S2 cells was performed as described previously [11].

2.4. Isolation of recombinant proteins from cell culture supernatant

The recombinant fusion proteins were purified by immobilized metal chelate affinity chromatography (IMAC) as described [12]. Further purification of Muc1VH and Sgs1VH was performed by reversed phase high-pressure liquid chromatography (RP-HPLC) on C8 columns essentially as described [12]. Purification of DGVH was achieved by gel permeation chromatography on a Superdex 200 HR10/30 column using an Äkta-FPLC system from Amersham Biosciences (Freiburg, Germany). The sample was run in 50 mM ammonium hydrogen carbonate with a flow rate of 0.5 ml/min. Eluting protein was detected spectrophotometrically at 280 nm.

2.5. Isolation of endogenous proteins

Endogenous mucin-type *O*-glycoproteins from the cell culture supernatant of serum-free growing S2-cells were isolated by serial lectin affinity chromatography as described in [11].

2.6. Monosaccharide composition analysis

Monosaccharide compositions were determined by GC/MS of the trimethylsilylated (TMS) 1-*O*-methylglycosides [13] on a Fison MD800 GC/MS (Thermo Fisher, Dreieich, Germany), equipped with a 15 m RTX5-SILMS column (Restek, Bad Homburg, Germany). After an isothermal phase at the initial temperature (100 °C, 1 min), a gradient of 6 °C/min up to 260 °C was applied. MS spectra were registered by electron impact (EI) ionization at 70 eV. Relative masses between *m/z* 100 and 700 were scanned every second at 400 V.

2.7. Isolation of oligosaccharides and permethylation of glycan alditols

To identify the core sugar and the oligosaccharide structures, the glycan chains were released from the protein by reductive β-elimination as described [11]. Permethylation of glycan chains was performed by sequential incubations of the dry samples with finely powdered NaOH in DMSO and methyliodide as described [11].

2.8. Glycan analysis by collision-induced dissociation-electrospray (QTOF) mass spectrometry

Electrospray ionization (ESI)-mass spectrometry (MS) data were acquired according to a previously published protocol by static nanospray application of the samples and analysis of the protonated or sodiated molecular ions on a QTOF2 instrument (Waters, Eschborn, Germany) as described previously [14].

2.9. Linkage analysis of glycans

Partially methylated alditol acetates were prepared from permethylated samples as described [14]. GC/MS analysis was performed on a Fison MD800 GC/MS (Thermon Fisher), which was equipped with a 15 m RTX5-SILMS column (Restek). The initial temperature was 60 °C. The gradient was from 60 °C to 100 °C with 40 °C/min followed by 10 °C/min up to 280 °C. Masses were scanned between 100 and 450 Da.

2.10. Colorimetric detection of glucuronic acid on the surface of S2 cells

6×10^8 S2-cells were harvested at $300 \times g$ for 5 min, washed, and diluted in 0.5 ml PBS. After addition of 15 µl (2 U) β-glucuronidase (Roche) cells were incubated for a maximum of 75 min at 37 °C under continuous agitation. Cells were pelleted at $300 \times g$ and the released glucuronic acid was detected in 250 µl of the supernatant by application of the carbazol assay and measurement at 525 nm [15]. The assay was calibrated by standard dilutions of GlcA ranging from 0 to 20 µg.

2.11. Free-flow cell electrophoresis

FFE experiments were performed on a TECAN PRO TEAM FFE apparatus in zonal electrophoresis mode. The chamber was equipped with a 0.5 mm spacer and 0.8 mm filter paper and coated with 0.4% HPMC prior to the experiment. All FFE media were used exactly as described previously [16]. Separation of the cells was performed at 278 V and 272 mA at a temperature of 8 °C. Media were pumped vertically with 254 ml/h.

Cells were analysed untreated and pretreated with β-glucuronidase as described above. Prior to FFE, 1×10^8 cells were washed, pelleted at $300 \times g$ and diluted in 1 ml separation medium. The sample was injected at 0.74 ml/h via the middle inlet. Electrophoresed cells were collected in a standard 96 well plate for 3.5 min and pelleted by centrifugation at $500 \times g$ for 5 min.The supernatant was discarded and cell collection repeated for 4 times. Cells were detected by $OD_{580\ nm}$ -measurement in a standard ELISA-Reader.

3. Results

This study was undertaken to characterize the *O*-glycosylation on *Drosophila* proteins expressed in the hemocytic cell line S2. In particular, we were interested in the detection of glycans representing extensions of the core 1 disaccharide, which is the only reported complex *O*-linked glycan in *Drosophila*. For this purpose, we analysed secreted glycoproteins from the culture supernatant of S2 cells (referred to as endogenous glycoproteins) and recombinant fusion proteins based on the tandem repeat domain of human MUC1 (hMUC1VH), on the tandem repeat domain of the *Drosophila* mucin Sgs1 (dSgs1VH) and on the ectodomain of the *Drosophila* dystroglycan (dDGVH).

3.1. Purification of the fusion proteins

The V5- and His_8 immunoreactive fusion protein dDGVH corresponding to the ectodomain of dystroglycan from *D. melanogaster* was enriched from serum-free culture supernatant of S2 cells by affinity chromatography on nickel chelate. V5-immunoreactive dDGVH was further purified by gel permeation chromatography on Superdex 200 HR10/30 (not shown). The eluate was analysed by gel electrophoresis of proteins in each fraction revealing a silver-stained band at the apparent molecular mass of approximately 200 kDa and absence of contaminating proteins in fractions A8 and A9. dDGVH in the pooled fractions was identified in a western-blot as V5-reactive protein with an apparent molecular mass of approximately 200 kDa and was demonstrated by DIG glycan detection to be free of contaminating glycoproteins (not shown).

Purification of endogenous S2-glycoproteins by serial lectin affinity chromatography and of the recombinant probes dSgs1VH and hMUC1VH by nickel chelate affinity chromatography was described previously [11].

3.2. Analysis of O-glycosylation

3.2.1. O-glycan composition analysis of dDGVH. The composition of glycans on dDGVH was analysed after methanolysis and formation of the 1-*O*-methylglycosides by GC/MS