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N-glycoprofiling analysis in a simple glycoprotein model: A comparison between recombinant and pituitary glycosylated human prolactin

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ABSTRACT

Human prolactin (hPRL) is a polypeptide hormone occurring in the non-glycosylated (NG-hPRL) and glycosylated (G-hPRL) forms, with MM of approximately 23 and 25 kDa, respectively. It has a single, partially occupied N-glycosylation site located at Asn-31, which makes it a particularly simple and interesting model for glycosylation studies. The bioactivity of G-hPRL is lower than that of NG-hPRL (by ca. 4-fold) and its physiological function is not clear. However, carbohydrate moieties generally play important roles in the biosynthesis, secretion, biological activity, and plasma survival of glycohormones and can vary depending on the host cell. The main objective of this study was to determine the N-glycan structures present in native, pituitary G-hPRL and compare them with those present in the recombinant hormone. To obtain recombinant G-hPRL, genetically modified Chinese hamster ovary cells (CHO), adapted to growth in suspension, were treated with cycloheximide, thus increasing the glycosylation site occupancy from 5.5% to 38.3%, thereby facilitating G-hPRL purification. CHO cell-derived G-hPRL (CHO-G-hPRL) was compared to pituitary G-hPRL (pit-G-hPRL) especially with regard to N-glycoprofiling. Among the main differences found in the pituitary sample were an extremely low presence of sialylated (1.7%) and a high percentage of sulfated (74.0%) and of fucosylated (90.5%) glycans. A ~6-fold lower in vitro bioactivity and a higher clearance rate in mice were also found for pit-G-hPRL versus CHO-G-hPRL. N-Glycan profiling proved to be a useful and accurate methodology also for MM and carbohydrate content determination for the two G-hPRL preparations, in good agreement with the values obtained directly via MALDI-TOF-MS.

1. Introduction

Human prolactin (hPRL) is a pituitary-secreted polypeptide hormone whose essential role is on mammopoiesis. In addition, it exerts many other physiological actions on behavior and brain in general and on metabolism, immune responses and electrolyte balance (Bernichtein et al., 2010; Goffin et al., 2002). Several experimental studies have defined this hormone as the "regulator of maternal behavior", but recent studies also consider it to be a candidate "regulator of paternal behavior" (Gettler et al., 2012). The interest in hPRL stems from its clinical importance for women with lactation problems and infertility, as well as from the fact that elevated circulating levels or locally produced hPRL are associated with increased risks of breast and prostate cancer (Bernichtein et al., 2010; Fernandez et al., 2010; Suzuki et al., 2012; Tworoger et al., 2004).

Human prolactin (hPRL) is a 199 amino acid-polypeptide with a single potential N-glycosylation site located at Asn-31, which is partially (5–30%) occupied in the native pituitary form or in the recombinant form of the hormone (Heller et al., 2010; Price et al., 1995; Sinha, 1995; Soares et al., 2000, 2002). It thus exhibits the

Abbreviations: CDG, congenital disorders of glycosylation; CHO, Chinese hamster ovary; CHX, cycloheximide; hPRL, human prolactin; HPSEC, size-exclusion HPLC; MALDI-TOF-MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; MM, molecular mass; Mr, relative molecular mass; NHPP, National Hormone and Pituitary Program; PAGE, polyacrylamide-gel electrophoresis; SDS, sodium dodecyl sulfate; RP-HPLC, reverse-phase HPLC; t_R, retention time.

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simplest type of glycosylation macroheterogeneity: one protein population with and one without a single N-linked glycan (Heller et al., 2010; Shelikoff et al., 1994). This makes it particularly ideal for glycosylation studies, especially considering the complexity of glycan composition and the challenging task of the simultaneous accurate determination of: (i) protein concentration; (ii) oligosaccharide structures (i.e., the variation of the glycoform profile); and (iii) the glycosylation site occupancy of glycoproteins in general (Apweiler et al., 1999; Desaire, 2013; Lin et al., 2012; Pan et al., 2012; Petrescu et al., 2004).

Glycosylated hPRL (G-hPRL) has an approximately 4-fold lower potency compared to the non-glycosylated form (NG-hPRL), showing reduced lactotrophic and mitogenic activity (Heller et al., 2010; Price et al., 1995; Shelikoff et al., 1994; Sinha, 1995). Although it has been considered to be the major post-translational modification of NG-hPRL, with which it is co-secreted from childhood to the end of puberty, its physiological significance is still not yet well elucidated (Fideleff et al., 2012; Freeman et al., 2000).

Because it is such a simple monoglycosylation model, G-hPRL has been chosen to develop a methodology for determining oligosaccharide structures and glycosylation site occupancy via glycoprofiling analysis. After it has been validated, this methodology should then be applicable also to more complex polyglycosylated proteins. The importance of correctly knowing these parameters has been emphasized in the literature. Thus, comparative quantitative profiling of a glycoproteome and the accurate quantification of its glycosylation site occupancy have been related to folding, trafficking, initiation of inflammation, and host defense, as well as to disease states such as congenital disorders of glycosylation (CDG). These latter are a family of rare, inherited metabolic syndromes that affect the synthesis, transfer or processing of glycans and that cause motor and intellectual disability and variable multisystemic symptoms. In type-I CDG the early steps of glycosylation are impaired, resulting in unoccupied glycosylation sites; in type-II CDG the defects do not influence occupancy, but rather glycan processing (Barone et al., 2009; Fogli et al., 2012; Losfeld et al., 2012; Pan et al., 2012; Petrescu et al., 2004; Tian and Zhang, 2010).

The present study is based on suspension-adapted prolactin-secreting CHO cells, cultivated in spinner flasks and incubated in the presence of cycloheximide in order to increase the proportion of G-hPRL in the total amount of prolactin and facilitate its purification. Purified G-hPRL, which is now a 100% occupied monoglycosylated protein, is then extensively characterized by SDS-PAGE, Western blotting, reverse-phase and size-exclusion HPLC, in vitro bioassay, pharmacokinetic analysis, MALDI-TOF-MS relative molecular mass (Mr) determination and, finally, N-glycan[1] profiling. Our recombinant preparation (CHO cell-derived) is compared to the native pituitary (lactotrophic cell-derived) preparation, using a well-known reference from the National Hormone and Pituitary Program (NHPP, USA). Accurate MALDI-TOF-MS molecular mass determination was employed to confirm that the glycoprofiling approach is indeed useful for correctly determining, besides the monosaccharide composition of each glycan, also the average glycan mass, the whole glycoprotein mass and, consequently, the percent molecular weight exclusively due to the carbohydrate moiety in the two preparations.

[1] Abbreviations for N-glycans were made by not considering the basic pentasaccharidic nucleus ("zero") and adding all other monosaccharides, as stated in the figures, in the following order: Man (**M**); GlcNAc (**N**); Gal (**G**); GalNAc (**Gn**); NeuAc/sialic acid (**S**); Fuc (**F**). So, for example, $NeuAc_1$ Gal_1 $GlcNAc_2$ $Fuc_1 + Man_3$ $GlcNAc_2$, becomes **N2G1S1F1**.

2. Materials and methods

2.1. Cell line

The clone expressing hPRL, obtained in our laboratory and utilized in this study, was derived from CHO dhfr-cells (DUKX-B11) that had been transfected with the vector pEDdc-hPRL (Soares et al., 2000) and adapted to suspension growth in serum-free medium (Arthuso et al., 2012).

2.2. G-hPRL production in spinners

For laboratory production, 250 ml spinner flasks containing 100 ml of CHO-S-SFM II medium, supplemented with antibiotics (50 unit ml^{-1} penicillin; 50 $\mu g\,ml^{-1}$ streptomycin, 40 $\mu g\,ml^{-1}$ gentamycin and 1.25 $\mu g\,ml^{-1}$ amphotericin B), were utilized. Cells were cultured at 37 °C under 5% CO_2, stirring at ~80 rpm. When a density of 1×10^6 cell ml^{-1} was obtained, the total volume of conditioned medium (100 ml) was centrifuged at 400 × *g* and substituted with fresh medium, with the initial cell concentration fixed at $\sim 1 \times 10^6$ cell ml^{-1}. In this phase, CHX (0–1 $\mu g\,ml^{-1}$, depending on the experiment) was added and the conditioned medium, collected daily by centrifugation at 400 × *g*, was replaced with a fresh one. This procedure was repeated for 10 days. The collected medium was stored at −40 °C.

2.3. Gel electrophoresis and Western blotting

Discontinuous SDS-PAGE, based on 15% polyacrylamide gels, was carried out under non-reducing conditions as described by Laemmli (1970). Coomassie Brilliant Blue G-250 (USB, Cleveland, OH) or silver nitrate was used for staining; the molecular mass markers were from GE Healthcare Bio-Sciences (Uppsala, Sweden).

For the Western blotting the proteins were transferred from the polyacrylamide gel to nitrocellulose membrane by the semi-dry transfer technique, probed with polyclonal anti-hPRL antiserum produced in rabbit (1:5000) and with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000). Visualization of proteins was performed with Luminata Forte (Millipore) on X-ray film.

2.4. HPSEC

A Shimadzu Model SCL-10A HPLC apparatus coupled to a SPD-10AV UV detector (Shimadzu, MD, USA) was used, employing the Class VP software, also from Shimadzu. For HPSEC, a TosoHaas (Montgomeryville, PA, USA) G2000 SW column (60 cm × 7.5 mm i.d., particle size of 10 μm and pore size of 125 Å) coupled to a 7.5 cm × 7.5 mm ID SW guard column was used. The mobile phase was 0.025 M ammonium bicarbonate, pH 7.0, with a flow-rate of 1.0 ml min^{-1} (Dalmora et al., 1997).

2.5. G-hPRL purification

The G-hPRL present in the conditioned culture medium was purified using a two-step purification method: SP-Sepharose Fast Flow followed by a C4 Grace-Vydac 214TP54 (25 cm × 4.6 mm ID, pore diameter of 300 Å and particle diameter of 5 μm) RP-HPLC column, used for preparative purposes. Briefly, conditioned medium (2000 ml) was adjusted to pH 5.0 using acetic acid. The material was then applied (5 ml min^{-1}) onto a 10 cm × 2.6 cm ID glass column packed with SP-Sepharose Fast Flow (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) that had been previously equilibrated in 50 mM sodium acetate (pH 5.0). UV absorbance was monitored at 280 nm. After washing with the same buffer, the column was eluted with 50 mM sodium acetate (pH 5.0), 90 mM NaCl. Human PRL (G-hPRL and NG-hPRL) was then eluted from the column with 25 mM

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