Biochimica et Biophysica Acta 1834 (2013) 1474–1483

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Mass spectrometry investigation of glycosylation on the NXS/T sites in recombinant glycoproteins

Izabela Sokolowska, Armand G. Ngounou Wetie, Urmi Roy, Alisa G. Woods, Costel C. Darie *

Biochemistry & Proteomics Group, Department of Chemistry & Biomolecular Science, Clarkson University, 8 Clarkson Avenue, Potsdam, NY 13699-5810, USA

ARTICLE INFO

Article history:
Received 24 February 2013
Received in revised form 15 April 2013
Accepted 22 April 2013
Available online 28 April 2013

Keywords:
Recombinant protein
Post-translational modification
Glycosylation
Mass spectrometry
Targeted proteomics

ABSTRACT

We used a targeted proteomics approach to investigate whether introduction of new N-linked glycosylation sites in a chimeric protein influence the glycosylation of the existing glycosylation sites. To accomplish our goals, we over-expressed and purified a chimeric construct that contained the Fc region of the IgG fused to the exons 7 & 8 of mouse ZP3 (IgG-Fc-ZP3E7 protein). Immunoglobulin heavy chain (IgG-HC protein) was used as control. We then analyzed the IgG-HC and IgG-Fc-ZP3E7 proteins by liquid chromatography-tandem mass spectrometry (LC–MS/MS) and by Western blotting (WB). We concluded that in control experiments, the glycosylation site was occupied as expected. However, in the IgG-Fc-ZP3E7 protein, we concluded that only one out of three NXS/T glycosylation sites is occupied by N-linked oligosaccharides. We also concluded that in the IgG-Fc-ZP3E7 protein, upon introduction of additional potential NXS/T glycosylation sites within its sequence, the original NST/S glycosylation site from the Fc region of the IgG-Fc-ZP3E7 protein is no longer glycosylated. The biomedical significance of our findings is discussed.

1. Introduction

To regulate the solubility of recombinant therapeutic proteins or of immunoglobulin (IgG)-based recombinant chimeric proteins used as therapeutics, artificial glycosylation such as modification of proteins by polyethylene glycol (PEG) or PEG-ylation is a standard procedure. However, the reproducibility of batches of recombinant proteins or chimeric proteins produced for therapeutic use is critical for their commercialization. Therefore, intense efforts have been made by protein chemists, biochemists and mass spectrometrists, focused on the full structural characterization of these recombinant chimeric proteins. The methods of choice are usually biochemical and whenever possible, mass spectrometry [1–9].

One important feature of the IgG-based chimeric proteins is the formation of the correct disulfide bridges within the chimeric protein. For a protein, failure to have correct disulfide connectivities may lead to changes in the three dimensional structure, as well as formation of disulfide-linked multimeric proteins [4,6,8,10–13]. As such, changes in disulfide linkages can lead to changes in the solubility, half-life and renal clearance, and hence the therapeutic effectiveness of the chimeric protein.

Another important feature of the IgG-based chimeric proteins is the NXS/T N-linked glycosylation site from the IgG part of the protein, which gives the recombinant chimeric protein almost a mandatory glycosylation of the asparagine residues from the NXS/T site, which also increases the chimeric protein's solubility. Therefore, full characterization of the chimeric protein and its NXS/T glycosylation site is a mandatory step in characterization of this protein. A fair assumption is that the NXS/T glycosylation site is occupied by an oligosaccharide and that one must determine the structure of the oligosaccharide. However, this is not always the case and one may also expect that the same glycosylation site is not occupied. In addition, if one has within the chimeric protein sequence more than one NXS/T glycosylation site, then one should consider that within the three dimensional structure of the chimeric protein, the glycosylation sites that are expected to be glycosylated may not necessarily be glycosylated anymore.

Here, we performed a targeted MS analysis of the IgG-Fc-ZP3E7 protein to investigate the glycosylation sites for N-linked oligosaccharides. IgG-Fc-ZP3E7 is a fusion between the IgG Fc region and the exon 7 of the zona pellucida protein 3; zona pellucida 3 is the protein responsible for egg–sperm interaction and fertilization [2,10,11,14–16]. We over-expressed the chimeric construct IgG-Fc-ZP3E7 and purified the protein product, and then analyzed it by LC–MS/MS using data dependent analysis (DDA) and information dependent analysis (DDA with inclusion list or IDA). We also analyzed the control IgG-HC by the same approach.

Abbreviations: ZP, zona pellucida; SP, signal peptide; FLCS, furin-like cleavage site; TM, transmembrane; IgG-HC protein, immunoglobulin heavy chain protein; IgG-Fc-ZP3E7 protein, the protein product of the Fc region of the IgG fused to the exons 7 & 8 of mouse ZP3; ZP3E7, polypeptide that corresponds to exon 7 from ZP3; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WB, Western blotting; ECL, enhanced chemiluminescence; MS, mass spectrometry, LC–MS/MS, liquid chromatography tandem mass spectrometry; m/z, mass/charge; CID, collision-induced dissociation, DDA, data dependent analysis; IDA, information dependent analysis (DDA with inclusion list); TIC, total ion chromatogram; XIC, extracted ion chromatogram

* Corresponding author. Tel.: +1 315 268 7763; fax: +1 315 268 6610.
E-mail address: cdarie@clarkson.edu (C.C. Darie).

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http://dx.doi.org/10.1016/j.bbapap.2013.04.022

This experimental approach allowed us to identify the N-linked glycosylation sites in both IgG-HC and IgG-Fc-ZP3E7 proteins. We concluded that in IgG-HC, the only one predicted NST glycosylation site is occupied by a N-linked oligosaccharide residue. We also concluded that in IgG-Fc-ZP3E7 protein, one out of three NXS/T glycosylation sites is occupied by an N-linked oligosaccharide. These studies also allowed us to conclude that addition of a new potential NXS/T glycosylation sites within the sequence of IgG-Fc-ZP3E7 protein will prevent the glycosylation of the original NST/S glycosylation site from the Fc region of the IgG-Fc-ZP3E7 protein. The significance of this finding for biotechnology and for pharmaceuticals is discussed.

2. Materials and methods

2.1. Reagents

Reagents were obtained from the following commercial sources. Cell culture reagents were from Invitrogen (Carlsbad, CA). AspN, goat anti-mouse IgG-HRP, formic acid, and acetonitrile from Sigma-Aldrich (St. Louis, MO), trypsin from Roche Applied Science (Indianapolis, IN), peptide-N-glycosidase (PNGase F) from New England Biolabs (Beverly, MA), SDS-PAGE gels from Bio-Rad (Hercules, CA), M_r markers from Bio-Rad, Invitrogen and New England Biolabs, nitrocellulose membranes and enhanced chemiluminescence (ECL) kits from Amersham Pharmacia Biotech (Piscataway, NJ).

2.2. Construction of IgG-Fc-ZP3E7 chimeric construct

A chimeric construct was designed in which the gene responsible for the Fc portion of human IgG1 heavy chain was joined to the exons 7 and 8 of the mouse ZP3 (*mZP3*). The chimeric construct was named *IgG-Fc-ZP3E7* [Note: Polypeptide encoded by exon 8 is removed by excision at the FLCS and is not found in the mature, secreted ZP3 or in IgG-Fc-ZP3 protein product, so for simplicity, we named the chimeric construct *IgG-Fc-ZP3E7* not *IgG-Fc-ZP3E78* (although it contains exon 8)]. An IgG fusion strategy that facilitates purification and characterization of the fusion proteins has been used successfully by other investigators [17]. The extensive procedure for building the *IgG-Fc-ZP3E7* chimeric construct was described elsewhere [18]. For stable transfection, the *IgG-Fc-ZP3E7* construct was linearized with *Hind*III/*Eco*RI and then introduced into EC cells by electroporation and a stably transfected EC cell line was obtained.

2.3. Secretion, purification, and characterization of IgG-Fc-ZP3E7

EC cells, stably transfected with *IgG-Fc-ZP3E7*, were cultured under standard conditions and the serum-free supernatant was collected and purified over an IgG-affinity column. The full procedure is described elsewhere [18]. Briefly, supernatant containing recombinant protein was collected after culturing EC cells in serum-free culture medium for 20–24 h. Supernatant was screened for *IgG-Fc-ZP3E7* by purification over an ImmunoPure Immobilized Protein G (on agarose) column (Pierce) as described by the manufacturer; 50 ml supernatant was centrifuged at low speed at room temperature and filtered (Millipore), and then 50 ml ImmunoPure (A/G) IgG binding buffer (Pierce) was added, and then run over a column packed with 0.5 ml of settled Protein G (~1 ml of gel slurry). The flow-through was collected, applied once more to the Protein G column and then discarded. To remove unbound protein, the column was washed with ImmunoPure (A/G) IgG binding buffer and bound proteins were eluted with ImmunoPure IgG elution buffer (Pierce). Neutralizing buffer (1 M Tris, pH 9) was added (100 μl/ml) immediately upon elution to adjust eluted fractions to physiological pH. Eluted fractions were pooled, dialyzed extensively against H2O at 4oC, lyophilized, and stored frozen. Aliquots of bound IgG protein were analyzed by SDS-PAGE and Western immunoblotting to determine whether EC cells synthesized and secreted IgG-Fc-ZP3E7 protein. Here we will use the term IgG-Fc-ZP3E7 protein for the protein product of the *IgG-Fc-ZP3E7* construct. The heavy chain of the IgG1 (IgG-HC) used as a control was purchased from Sigma.

2.4. N-glycanase digestion

Aliquots of IgG-Fc-ZP3E7 (100–200 ng) and IgG-HC (500 ng) were dissolved in denaturing buffer containing 5% SDS and 10% β-mercaptoethanol (final concentrations of 0.5% SDS and 1% β-mercaptoethanol), the sample was boiled for 10 min, cooled, and one-tenth volume of 0.5 M sodium phosphate, pH 7.5, one-tenth volume of 50 mM Chaps, and 500 U of Peptide:N-Glycosidase F (New England BioLabs) was added (total volume ~15 μl). The sample was incubated at 37 °C for 24 h, then placed at −20 °C, and stored frozen prior to analyis by SDS-PAGE and WB.

2.5. SDS-PAGE and WB

The glycosylated and de-glycosylated proteins were separated by SDS-PAGE and then stained by Coomassie, according to published procedures. The protein gel bands of interest were excised and subjected to enzymatic digestion. SDS-PAGE gels were also electroblotted on PVDF membrane (Millipore, Bedford, MA) and probed with antibodies against human IgG (Sigma) or polypeptide that corresponds to exon 7 of mouse ZP3 (ZP3E7) [18] or ConA-HRP (ConA, Sigma). The immune reaction was visualized by ECL reaction kit (Pierce, Rockford, IL).

2.6. Enzymatic digestion of proteins for MS-based analysis

Digestion of gel pieces containing individual proteins with proteases was carried out using published protocols [3,5,19–21]. Gel pieces containing 0.05–1 μg of protein were excised from the gel and incubated with 60% (v/v) acetonitrile for 20 min, dried completely in a SpeedVac evaporator, and rehydrated for 10 min with digestion buffer (25 mM ammonium bicarbonate, pH 8.0). This procedure was repeated three times. After drying, gel pieces were again rehydrated in digestion buffer containing 10 mM DTT and incubated for 1 h at 56 °C. Reduced cysteine residues in the proteins were blocked by replacing the DTT solution with 100 mM iodoacetamide in 25 mM ammonium bicarbonate, pH 8.0, for 45 min, in the dark, at room temperature, with occasional vortexing. Gel pieces were then dehydrated, dried and subjected to digestion for 16–18 h at 37 °C in digestion buffer containing 15 ng/μl of trypsin (cleaves at the C-termini of R and K residues). The gel pieces were then dehydrated, dried and subjected to a second digestion for 16–18 h at 37 °C in digestion buffer containing 15 ng/μl of AspN (cleaves at the N-termini of D and E residues). Following digestion, peptides were extracted twice from gel pieces by addition of 200 μl of 60% acetonitrile/5% formic acid in 25 mM ammonium bicarbonate, pH 8.0, and shaking for 40–60 min at room temperature. Solutions containing peptide mixtures were then dried in SpeedVac concentrator, solubilized in 20 μl of 0.1% formic acid/2% acetonitrile and cleaned with a P10 ZipTip μ-C18 (Millipore Corporation, Billerica, MA). For LC–MS/MS analysis, the samples were resuspended in 10 μl of 0.1% formic acid in 2% acetonitrile.

2.7. MS, protein identification and data analysis

The resulting peptide mixture was analyzed by reverse phase liquid chromatography (LC) and MS (LC–MS/MS) using a NanoAquity UPLC coupled directly to a Q-Tof Premier MS or Q-Tof Micro MS (Waters, Milford, MA). The procedure used was previously described [3,7,22,23]. Briefly, the peptides were loaded onto a 100 μm × 10 mm nanoAquity BEH130 C18 1.7 μm UPLC column (Waters, Milford, MA) and eluted over a 120 minute gradient of 10–85% acetonitrile in 0.1% formic acid at a flow rate of 250 nl/min. The column was coupled to a Picotip