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Two-dimensional electrophoresis highlights haptoglobin beta chain as an additional biomarker of congenital disorders of glycosylation

Arnaud Bruneel[a,b,c,*], Florence Habarou[a], Tanya Stojkovic[d], Grégory Plouviez[a], Laure Bougas[a], Fanny Guillemet[a], Nadine Brient[a], Dominique Henry[a], Thierry Dupré[a], Sandrine Vuillaumier-Barrot[a], Nathalie Seta[a,e]

[a] AP-HP, Biochimie Métabolique et Cellulaire, Hôpital Bichat, Paris, France
[b] INSERM UMR-1193 "Mécanismes cellulaires et moléculaires de l'adaptation au stress et cancérogenèse", Université Paris-Sud, Châtenay-Malabry, France
[c] Université Paris-Sud, France
[d] AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Institut de Myologie, centre de référence des maladies neuromusculaires Paris Est, France.
[e] Université Paris Descartes, France

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ABSTRACT

Congenital disorders of glycosylation (CDGs) are rare inherited disorders affecting glycosylation of proteins and lipids and sharing very heterogeneous multivisceral symptoms. The biochemical screening of these diseases is currently limited to electrophoresis or HPLC separation/quantification of serum transferrin glycoforms and is relatively frequently hampered by genetic polymorphism. Further, it has been shown that transferrin glycosylation can be very poorly affected in confirmed CDGs. We developed a fast and simple two-dimensional (2-DE) Western-blot analysis applied to the simultaneous detection of various serum glycoproteins, i.e. haptoglobin, α1-anti-trypsin, transferrin and α1-acid glycoprotein, and applied it to a large cohort of CDGs and secondary glycosylation disorders. When separated using 2-DE, haptoglobin β glycoforms showed clear abnormalities in all interpretable CDG type I and CDG type II patterns. Although secondary glycosylation defects such as alcoholism, untreated fructosemia and bacterial neuraminidase remain to be excluded, we showed that 2-DE pattern of haptoglobin β glycoforms thus constitute a very reliable additional biomarker of all types of CDGs. Coupled with common screening techniques and glycans mass spectrometry, it can orientate and facilitate the way towards CDG molecular diagnostic.

1. Introduction

Glycosylation is a post-translational modification involving numerous molecular partners (nucleotide sugars, enzymes, transporters, lipid anchor, tethering factors, vesicular ATPases…) within the secretory pathway in endoplasmic reticulum (ER) and Golgi apparatus (GA) [1,2]. Depending on the oligosaccharide attachment site, i.e. amine of Asn or hydroxyl of Ser/Thr, glycosylation can be mainly subdivided into *N*- and *O*-glycosylation, respectively. Congenital disorders of glycosylation (CDG) are rare inherited diseases sharing heterogeneous symptoms with variable severity [3]. They are classically sub-grouped as type I (CDG-I) or type II (CDG-II). In CDG-I, the defect alters the lipid linked oligosaccharide (LLO) synthesis or its transfer to nascent proteins leading to unoccupied *N*-glycosylation sites with significant changes in both glycoproteins electric charge and molecular weight (Mw). In CDG-II, the defect alters the maturation of protein-linked oligosaccharide leading to incomplete or abnormal *N*-glycan structures with mainly significant charge modifications [4]. CDG screening is usually performed using isoelectric focusing (IEF) or capillary electrophoresis (CE) or HPLC of serum transferrin (Trf) [5–7] based on the separation of its *N*-glycoforms according to charge i.e., to the number of terminal sialic acids (SA). PAGE-SDS followed by Western-blot (PAGE-WB) of various serum glycoproteins has also been described as a valuable screening method for CDG-I since detecting associated Mw variations [8,9]. IEF,

Abbreviations: 2-DE, two-dimensional electrophoresis; AAT, α-anti-trypsin; AGP, α1-acid glycoprotein; ALG1, mannosyltransferase 1; ALG3, endoplasmic reticulum (ER) mannosyltransferase VI; ALG8, glucosyltransferase 2; ATP6V0A2, ATPase H + transporting V0 subunit a2; CDG, congenital disorder of glycosylation; CE, capillary electrophoresis; COG, conserved oligomeric golgi complex; DPGAT1, dolichol phosphate *N*-acetylglucosamine-1 phosphate transferase; DPM1, dolichol phosphate mannose synthase; DOLK, dolichol kinase; ER, endoplasmic reticulum; GA, Golgi apparatus; Hpt, haptoglobin; HUS, hemolytic uremic syndrom; IEF, isoelectric focusing; LLO, lipid linked oligosaccharide; MGAT2, *N*-acetylglucosaminyltransferase 2; PMI, phosphomannose isomerase; PMM2, phosphomannomutase 2; SA, sialic acid; SLC35A1, solute carrier family 35 member A1; Trf, transferrin

* Corresponding author at: AP-HP, Biochimie Métabolique et Cellulaire, Hôpital Bichat, Paris, France.
E-mail address: arnaud.bruneel@aphp.fr (A. Bruneel).

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HPLC and CE of Trf share the shortcoming to being restricted to one glycoprotein, commonly subjected to genetic polymorphism (0.5–2.0%) [10], and whose glycosylation is sometimes poorly affected in CDG. Indeed, several studies reported (*i*) screening difficulties related to Trf variants-associated charge heterogeneity [11–13] as well as (*ii*), cases of confirmed CDGs with normal or very discretely abnormal Trf profiles [14–16]. Regarding PAGE-WB, it is not subjected to protein variants interference but does not allow detecting the majority of CDG-II [8].

We previously showed that after separation of serum proteins according to charge and to Mw using two-dimensional electrophoresis (2-DE), it was possible, after electric transfer, to specifically detect on the same nitrocellulose sheet both glycoforms of serum haptoglobin β chain (Hpt), transferrin (Trf), α1-anti-trypsin (AAT), and α1-acid glycoprotein (AGP) [17]. When applied to CDG-I cases, we obtained encouraging results showing abnormal protein spots with decreased Mw (nonoccupancy of *N*-glycosylation sites) and acidic p*I* (loss of SA) by comparison with controls. In this work, we present a simplest and much faster 2-DE procedure and resume our two-years experience about its use besides routine CDG screening using CE of Trf. A large cohort of samples from confirmed CDG-I, CDG-II and PGM1-CDGs (described as 'mixed' CDGs) [18] were analyzed together with (*i*) samples with secondary glycosylation defects and (*ii*), samples with Trf polymorphism. In all CDG cases and studied secondary glycosylation defects, abnormalities affecting at least one glycoprotein were observed on 2-DE patterns. In Trf polymorphism cases, the patterns of others studied glycoproteins were systematically normal. Apart from one hemolyzed serum where undetectable, 2-DE profiles of haptoglobin β chain were interpretable and markedly altered in all CDGs, highlighting haptoglobin as a reliable and useful additional marker for these inherited diseases.

2. Material and methods

2.1. Samples

Tested samples i.e. serum, plasma or blood spotted on a Guthrie card, were sent to our lab for CDG screening or for chronic alcohol consumption-related 'carbohydrate deficient transferrin' (CDT) analysis.

They originated as follows: 14 non-CDG controls (negative Trf CE-based CDG screening). CDG-I samples: 5 PMM2-CDG; 3 PMI-CDG; 1 ALG3-CDG; 2 ALG8-CDG; 1 ALG1-CDG; 1 DPGAT1-CDG; 1 DPM1-CDG and 1 DOLK-CDG. CDG-II samples: 1 MGAT2-CDG; 5 COG-CDG (1 COG1, 3 COG5 and 1 COG7); 5 ATP6VOA2-CDG, 2 SLC35A1-CDG and 6 CDG-IIx (i.e. awaiting for molecular diagnosis; CDG-II Trf CE pattern). Previously described as 'mixed' CDG-I/CDG-II [18], 4 PGM1-CDG were also analyzed.

Samples with well-documented secondary glycosylation defects were analyzed: 1 untreated hereditary fructose intolerance, 3 *S. pneumoniae*-related hemolytic and uremic syndroms (HUS) [19] and 2 chronic alcohol abuses (ERNDIM CDG quality controls). Sixteen serum samples with transferrin polymorphism, as confirmed after neuraminidase treatment, were also analyzed.

2.2. Two-dimensional electrophoresis (2-DE) and Western-blot of glycoproteins

The 'new' 2-DE procedure we used is detailed in Supplementary File 1. For serum/plasma, 2 μL of 10-fold diluted sample in deionised water were analyzed; for blood spot on Guthrie card, one circular punched spot was eluted in 100 μL of deionised water and 10 μL of the eluate was used. 2-DE was carried out as described by the manufacturer (Life Technologies) using ZOOM Strip pH 4–7 for the first dimension and 4–12% NuPAGE Bis-Tris gels for the second dimension. After the first dimension, IEF gels can be stored at − 80 °C before subsequent steps. Proteins were transferred to nitrocellulose (100 V, 1 h) and glycoforms of Trf, AAT, Hpt and AGP were both revealed on the same sheet using a mix of rabbit primary antibodies and HRP-linked anti-rabbit IgG secondary antibody. More precisely, antibodies dilutions (v/v) in TTBS were as follows: anti Trf (Siemens): 1/4000; anti-Hpt (Dako): 1/5000; anti-AAT (Siemens): 1/10000; anti-AGP (Dako): 1/2000 and secondary anti-rabbit IgG: (GE healthcare): 1/5000. Lastly, 2-DE profiles were acquired using Chemidoc XRS camera system from Bio-Rad.

3. Results

3.1. The 'new' 2-DE procedure accelerates serum proteins glycosylation analysis

By greatly accelerating IEF gel rehydration and migration steps, the presented 2-DE/Western-blot method allowed the fast separation and accurate identification of various protein glycoforms from less than 1 μL of pure serum/plasma. Indeed, separation and reliable antibody-based detection of Hpt β chain, AAT, Trf and AGP glycoforms could be achieved on the same nitrocellulose sheet (6 sheets per run) in less than 12 h with minimal manipulations compared to around 48 h for the previous 'tricky' technique (Supplementary File 1).

3.2. 2-DE profile of control samples

As illustrated Fig. 1A, typical 2-DE control pattern showed several glycoforms of the four investigated *N*-glycoproteins i.e. Hpt, AAT, Trf and AGP. All 4 glycoproteins could be localized at their expected calculated p*I*/Mw values and up to 14 glycoforms could be individualized for Hpt, up to 6 for AAT and up to 5 for Trf. Concerning Trf, because of p*I* values close to the upper limit of the used pH gradient (i.e.

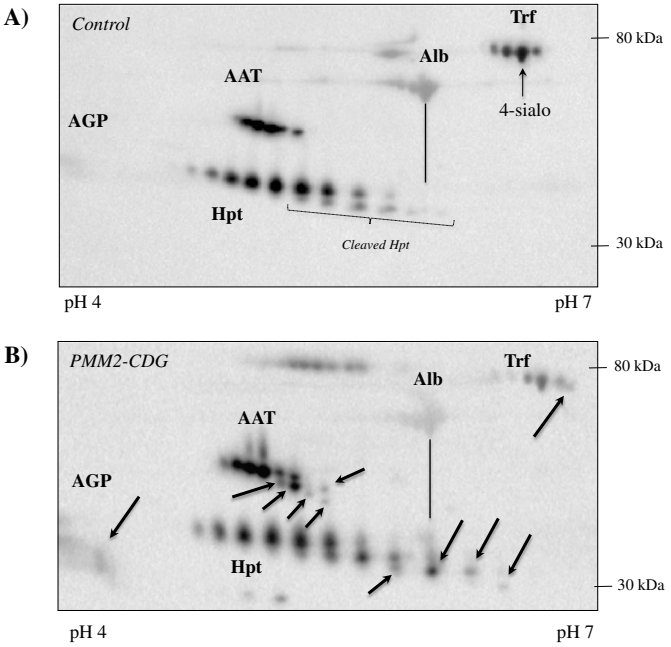

Fig. 1. Two-dimensional Western-blot profiles of control and CDG type I (PMM2-CDG).p*I* range: 4.0 to 7.0; Mw range: 20 kDa to 100 kDa. AGP: glycoforms of α1-acid glycoprotein; AAT: glycoforms of α-anti-trypsin; Hpt: glycoforms of haptoglobin β chain; Trf: glycoforms of transferrin; Alb: isoforms of non-glycosylated albumin. In control (A), AGP glycoforms are non-detectable or very poorly resolved because of out-of-range p*I* values. Hpt β chain can be subdivided into entire protein glycoforms (upper train of spots) and cleaved protein glycoforms (lower train of spots). For Trf, the major tetra-sialo glycoform (vertical arrow) can be systematically detected. No entire Hpt spot can be detected just under the major Alb spot. In PMM2-CDG (B), various additional abnormal glycoforms (plain arrows) can be detected showing modifications in Mw (underglycosylation) coupled to cathodical shift (loss of sialic acid residues).