RAPID COMMUNICATION

Two-dimensional gel electrophoresis of apolipoprotein C-III and other serum glycoproteins for the combined screening of human congenital disorders of *O*- and *N*-glycosylation

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Congenital disorders of glycosylation (CDG) are inherited diseases that can affect not only the *N*-glycan (*e.g.* CDG type I and II) but also the *O*-glycan biosynthesis pathway. In the absence of specific clinical symptoms, there is a need for a reliable biological screening of these two groups of CDG. Using a few microlitres of human serum, 2-DE and immunoblotting were applied to the separation and simultaneous detection of the isoforms of the *O*-glycosylated protein apolipoprotein C-III (apoC-III) and of four *N*-glycosylated proteins, namely alpha-antitrypsin, alpha-1 acid glycoprotein, haptoglobin and transferrin. For the study of *O*-glycosylation, this technique allowed the reliable separation of the three fractions of apoC-III and the determination of normal percentage values in an adult population. Concerning *N*-glycosylation, the study of serum samples from patients with CDG type Ia revealed marked abnormalities systematically affecting the four 2-DE separated *N*-linked glycoproteins. 2-DE coupled to immunoblotting using a mixture of specific antibodies could be easily and reliably employed for the combined screening of both *N*- and *O*-glycosylation disorders in humans.

Keywords:
Apolipoprotein C-III / CDG / Glycoproteins / *O*-glycosylation / Two-dimensional gel electrophoresis

Congenital disorders of glycosylation (CDG) are a growing and ramified group of inherited diseases in which protein glycosylation is affected due to the presence of mutations in genes encoding enzymes or proteins involved in this post-translational process [1]. Biological screening of congenital disorders of *N*-glycosylation is currently based on IEF analysis of serum transferrin using either gel-based or fast CE techniques [2–4]; furthermore, IEF of the core 1 mucin-type *O*-glycosylated protein apolipoprotein C-III (apoC-III) has been recently proposed as a new test for the specific detection of congenital disorders of mucin-type *O*-glycosylation [5]. Once detected at the (glyco)protein level, CDG are further explored and possibly confirmed at the enzymatic and/or genetic levels. Clinically, the congenital disorders of *O*- and *N*-glycosylation are heterogeneous multisystem diseases often sharing common features such as mental retar-

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Abbreviations: AAT, alpha-antitrypsin; **AGP,** alpha-1 acid glycoprotein; **apoC-III,** apolipoprotein C-III; **CDG,** congenital disorders of glycosylation; ***Gal-GalNAc,*** galactose-*N*-acetylgalactosamine; **Hpt,** haptoglobin; **Trf,** transferrin

dation, neurological abnormalities and liver failure [1, 6]. Thus, there is a need for an efficient biological screening of these two types of CDG.

We carried out a 2-DE and Western-blotting procedure on human blood samples to separate and simultaneously detect not only the isoforms of apoC-III but also those of up to four *N*-glycosylated proteins, *i.e.* alpha-antitrypsin (AAT), alpha-1 acid glycoprotein (AGP), haptoglobin (Hpt) and transferrin (Trf). Proteins from 1 to 5 μL of serum/plasma were separated by 2-DE as previously described [7]; IEF was carried out using 180 mm linear pH 3–10 IPG strips and Protean® IEF Cell (40 kV·h; all products from BioRad, Marnes-la-Coquette, France). After IEF, the strips were incubated for 10 min in equilibration buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS, 5 mg/mL DTT and bromophenol blue in 50 mM Tris-HCl, pH 8.8) before being applied at the top of a 15% polyacrylamide SDS-PAGE gel for the second protein separation. After 2-DE, proteins were blotted (100 V for 50 min) onto an NC membrane (BioRad) and transfer efficiency was checked using Ponceau S red reversible staining (Sigma, L'Isle d'Abeau, France). Glycoproteins of interest were simultaneously detected with ECL reagents (Amersham Biosciences, Saclay, France) using a mixture of up to five primary rabbit antibodies (anti-apoC-III from Biodesign International, Saco, USA; anti-AAT, anti-AGP, anti-Hpt and anti-Trf from Dade Behring, Marburg, Germany; see figure legends for respective dilutions) and anti-rabbit IgG linked to horseradish peroxidase as the secondary antibody (Amersham Biosciences, 1/5000 v/v). Exposed films were developed on a Curix 60 processor (Agfa, Rueil-Malmaison, France). The identity of each detected protein spot could be assigned when comparing its experimental p*I* and molecular weight (p*I*/MW) values with the theoretical ones (http://expasy.ch). Finally, the films were scanned (GS-800 densitometer, BioRad) and the isoforms of apoC-III were quantified using the Image Master 2D Platinium software, Version 5.0 (Amersham Biosciences).

In the field of *O*-glycosylation, 2-DE patterns developed after saturating exposure time (Fig. 1A) showed that apoC-III was most often separated into three distinct protein fractions *i.e.* two major spots with p*I* 4.3–4.5 (spot 2) and 4.5–4.7 (spot 1) and one vertical cluster of minor spots with p*I* 4.8–5.0 (spots 0). More precisely, spot 2 (9.4 kDa) and spot 1 (9.1 kDa) respectively matched with the already described disialyl (apoC-III_2) and monosialyl (apoC-III_1) glycoforms of apoC-III [8]. Concerning the apoC-III_0 fraction, it could be separated into up to four spots (0a, 0b, 0c and 0d) sharing identical p*I* but distinct MW values; while spots 0a and 0b respectively matched with apoC-III substituted with *N*-acetylgalactosamine (GalNAc) and with galactose-*N*-acetylgalactosamine (Gal-GalNAc), spots 0c (9.3 kDa) and 0d (9.6 kDa) could not be evidently assigned to other known asialylated isoforms of the protein. Incubation of plasma with neuraminidase (Sigma, cat. no. N-8271, ratio 1:3 for 1 h at 37°C) induced the nearly complete disappearance of

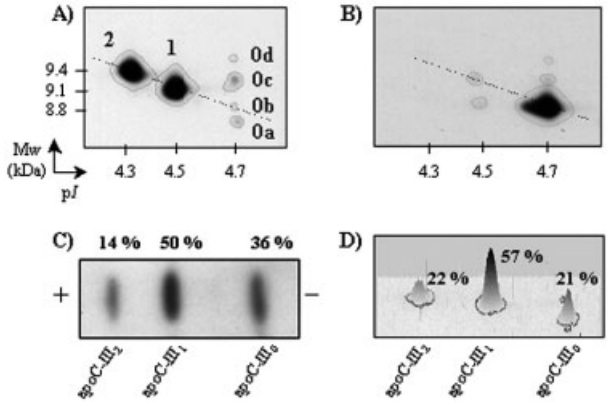

Figure 1. (A) Typical 2-DE pattern of apoC-III isoforms separated from 5 μL of human serum and (B) the corresponding 2-DE pattern obtained after 1 h of neuraminidase treatment. (C) Abnormal IEF profile of apoC-III and (D) the corresponding 2-DE data provided by Image Master 2D Platinium software.

spots 2 and 1 confirming their identifications as apoC-III_2 and apoC-III_1, respectively; concomitantly, neuraminidase treatment induced the marked increase of spot 0b in accordance with its identification as apoC-III_0 substituted with Gal-GalNAc (Fig. 1B). Under these conditions and after nonsaturating exposure time (5–40 s), the mean (SD) percentage (%) values of apoC-III isoforms determined from the analysis of 24 plasma samples from healthy adult donors were as following: apoC-III_0, 1.5 (2.0)%, apoC-III_1, 52.8 (9.0)% and apoC-III_2, 45.7 (9.6)%. These data differed from those obtained by Wopereis *et al.* [5] with IEF only, in particular when considering the mean % value of apoC-III_2 (45.7% *vs.* 36.0% using IEF) and of the apoC-III_0 fraction (1.5 *vs.* 8.5%), which could notably be related to differences in anti-apoC-III antibodies and/or protein blotting procedures. Lastly, the determination of the mean (SD) percentage (%) values of apoC-III isoforms separated from the same control plasma sample on six different days (between-run reproducibility) gave the following results: apoC-III_0, 0.5 (0.1)%, apoC-III_1, 54.2 (1.2)% and apoC-III_2, 45.3 (1.3)%. We also analysed one plasma sample with known increased level of apoC-III_0 isoform as determined by IEF only (Fig. 1C; apoC-$III_{0/1/2}$: 36/50/14%) and confirmed this abnormality, once again, with lower apoC-III_0% value and higher apoC-III_2% value (Fig. 1D; apoC-$III_{0/1/2}$: 21/57/22%).

Beside the apoC-III isoforms, we were also able to specifically detect on the same gel and at the same time the isoforms of up to four major circulating *N*-linked glycoproteins *i.e.* AAT, AGP, Hpt (β chain) and Trf (Fig. 2A). With the exception of very acidic AGP isoforms which were difficult to efficiently separate under our IEF conditions, the other isoforms were all correctly separated, with reproducible experimental p*I* values between samples ($\Delta pI \leq 0.2$ pH-units), as previously emphasized for the IPG focusing technology that we used [7–9]. Additionally, in case of insufficient or saturated ECL labelling of some glycoforms at a

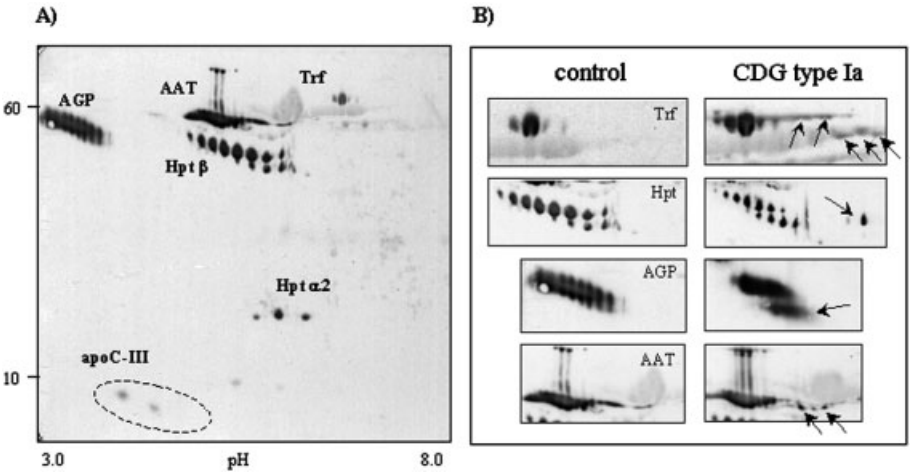

Figure 2. (A) Typical 2-DE patterns obtained after simultaneous detection of apoC-III, AGP, AAT, Hpt (antibodies dilution: 1/5000 v/v) and Trf (1/2500 v/v). (B) Enlarged areas showing marked *N*-glycan abnormalities in one plasma sample from a patient with CDG-Ia (right; arrows) in comparison with control (left).

given exposure time (*e.g.* in Fig. 2A, concerning apoC-III isoforms), this time could be easily adjusted in order to ameliorate their specific detection and/or quantification. Thus, the 2-DE pattern of plasma *N*-glycosylated proteins from one patient with CDG type Ia (CDG-Ia; deficiency in phosphomannomutase), *i.e.* sharing an enzymatic defect in the assembly of oligosaccharide to the asparagine residue of protein, revealed unequivocal p*I*/MW differences in comparison with controls (Fig. 2B); in particular, while CDG-Ia-associated abnormal cathodal isoforms of Hpt appeared relatively faint and isolated, those of Trf, AGP and AAT were typically strongly marked and numerous. Preliminary results in five plasma samples from patients with CDG-Ia confirmed these types of *N*-glycan abnormalities systematically affecting all the four tested *N*-linked glycoproteins; further, 2-DE patterns of apoC-III isoforms were all normal among these samples (not shown), in accordance with the absence of an oligosaccharide to protein transfer step in the *O*-glycosylation pathway [6].

In contrast to methods associating 2-DE with nonspecific protein staining procedures such as silver nitrate or colloidal blue [10, 11], the presented technique allowed us to specifically and easily visualize several glycoforms of interest with formal identification and with near-absence of any interfering 'protein background' on developed films. As clearly shown in Fig. 2A, our patterns were very slightly disturbed by albumin towards some isoforms of AAT and by probable heavy chains of IgM (deduced from www.expasy.ch) towards isoforms of Trf. For the study of core 1 mucin type *O*-glycosylation, this technique was successfully applied to the separation and relative quantification of apoC-III isoforms, showing good reproducibility and appreciable sensitivity since it was applicable to a few microlitres of the serum. In contrast to IEF only, it allowed the efficient separation of apoC-III$_0$ with Gal-GalNAc and also revealed an 'unexpected' heterogeneity in the asialylated apoC-III$_0$ fraction whose accurate characterization will be investigated using MS/MS and/or specific glycosidases. Concerning *N*-glycosylation, the use of a mixture of specific antibodies allowed us to detect the 2-DE separated isoforms of AAT, AGP, Hpt and Trf, further displaying typical *N*-glycan abnormalities related to CDG type Ia. In addition during detection the modulation of film exposure time allowed us to adjust the intensity of the spots, irrespective of the potential differences of serum-specific protein concentrations between samples, avoiding overexposition and saturation of the film leading to a nonlinear response.

This method which improves the resolution of the protein separation over IEF or SDS-PAGE alone, could be dedicated to different purposes depending on the antibodies used. It can be routinely applied with only anti-apoC-III antibody for the specific screening of disorders of *O*-glycosylation. With the five antibodies tested, it will also be employed as a secondary technique to get an accurate overview on *N*- and/or *O*-glycan abnormalities in patients suggestive of CDG on the basis of abnormal routine screening test, *i.e.* Trf IEF or SDS-PAGE [12]. Lastly, since this method is easily adaptable to minigel electrophoresis equipment offering relatively 'high throughput' capabilities (*e.g.* up to 12 gels *per* run), we may speculate that it could be shortly applied to the overall biological screening of both *O*- and *N*-glycosylation congenital disorders.

References

[1] Grunewald, S., Matthijs, G., Jaeken, J., *Pediatr. Res.* 2002, *52*, 618–624.

[2] Jaeken, J., van Eijk, H. G., van der Heul, C., Corbeel, L. *et al.*, *Clin. Chim. Acta* 1984, *144*, 245–247.

[3] Iourin, O., Mattu, T. S., Mian, N., Keir, G. *et al.*, *Glycoconj. J.* 1996, *13*, 1031–1042.

[4] Carchon, H. A., Chevigné, R., Falmagne, J., Jaeken, J., *Clin. Chem.* 2004, *50*, 101–111.

[5] Wopereis, S., Grunewald, S., Morava, E., Penzien, J. M. *et al.*, *Clin. Chem.* 2003, *49*, 1839–1845.

[6] Wopereis, S., Lefeber, D. J., Morava, E., Wevers, R. A., *Clin. Chem.* 2006, *52*, 574–600.

[7] Gorg, A., Weiss, W., Dunn, M. J., *Proteomics* 2004, *4*, 3665–3685.

[8] Vaith, P., Assmann, G., Uhlenbruck, G., *Biochim. Biophys. Acta* 1978, *541*, 234–240.

[9] Corbett, J. M., Dunn, M. J., Posch, A., Gorg, A., *Electrophoresis* 1994, *15*, 1205–1211.

[10] Harrison, H. H., Miller, K. L., Harbison, M. D., Slonim, A. E., *Clin. Chem.* 1992, *38*, 1390–1392.

[11] Henry, H., Tissot, J. D., Messerli, B., Markert, M. *et al.*, *J. Lab. Clin. Med.* 1997, *129*, 412–421.

[12] Seta, N., Barnier, A., Hochedez, F., Besnard, M. A. *et al.*, *Clin. Chim. Acta* 1996, *254*, 131–140.

