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Glycan profiling discriminates between rEPO δ (Dynepo®, produced in human cells) and rEPO α/β (produced in CHO cells)

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Abstract

Current tests to differentiate between urinary endogenous (uEPO) and its recombinant analogues (rEPO, NESP) are based on differences in their iso-electric focussing (IEF) profiles. These differences are believed to stem from the cells/species in which they are expressed. The production of Dynepo[®] (rEPO δ) in human cells made it an interesting candidate to study the glycosylation patterns as compared to other recombinant products of non-human cells origin, (i.e. rEPO α/β). Comparison of both glycoproteins migration in SDS-PAGE suggested that N-glycan heterogeneity of rEPO δ must be lower than in rEPO α/β but the structural characterisation of the former had not been addressed. rEPO δ was found to contain mostly tri- and tetra-antennary structures, absence of di-antennary glycans and insignificant levels of *N*-acetyllactosamine repeats. Conversely, rEPO α/β presents a high proportion of di- and tri-antennary glycans and also *N*-acetyllactosamine repeats. Glycans from rEPO δ do not seem to contain charges other than sialic acids, so the slightly more acidic IEF profile must be due to a higher degree of sialylation. Analysis of O-glycans showed significant differences in sialylation. While in rEPO δ almost 100% of O-glycans were di-sialylated, in rEPO α/β similar proportion of mono- (~56%) and di-sialylated (~44%) glycoforms were found. At the sialic acid level, while rEPO α/β was found to contain small amounts (~1.3%) of the non-human *N*-glycolylneuraminic acid, it was not detected in rEPO δ under the conditions used. As only complexity differences and non additional negative charges have been found in rEPO δ, current studies are focused at revealing the structural differences of recombinant preparations (independently of cell type origin) and endogenous EPO. Thus, the sugar profiling could be useful for unambiguous anti-doping analysis or pathology diagnosis. Note: Part of this work is published in Analytical Biochemistry.

Introduction

Recombinant erythropoietin (rEPO) has become the standard therapy for treatment of the anaemia of chronic kidney disease (CKD), cancer patients on chemotherapy and surgical patients to avoid red blood cell transfusion. In addition, the abuse of this hormone and its analogues in sports has also become common practice. The development of second and third-generation products have improved their pharmacokinetic properties and/or minimised immunogeneicity. Thus, the doping control laboratories face different erythropoiesis-stimulating biopharmaceuticals, and it is very important to know the structural composition in order to differentiate the exogenous EPO analogues [1]. Recently, a novel rEPO named epoetin delta (rEPO δ; Dynepo®) from Shire Pharmaceuticals was launched for the treatment of CKD. It is homologously expressed by gene-activation in a human fibrosarcoma cell-line (HT-1080) [2]. With respect to the sequence of their 165 aminoacids, the rEPOs are identical with the naturally occurring human forms. However, from a biochemical viewpoint, there is an interesting diversity in their post-translational modifications, most probably in the glycosylation. Like endogenous EPO, epoetins contain one O-linked (Ser-126) and three N-linked (Asn-24, 38, 83) oligosaccharides and these account for ca.40% of the total molecular weight (~ 30 kDa). The biosynthesis of glycans is species-, tissue-, and cell-type dependent but also the culture conditions may contribute to the so-called microheterogeneity resulting in a large diversity of glycan structures. Chinese Hamster Ovary (CHO) and Baby Hamster Kidney (BHK) cells are the hosts used for the production of rEPO pharmaceuticals. The enzymatic endowment of these host cells is similar to human cells. However, some human tissue-specific terminal carbohydrate motifs are not synthesised by BHK and CHO cells since they lack the proper sugar-transferring enzymes; for example α2-6 sialyltransferase, α1-3/4 fucosyltransferase, bisecting *N*-acetylglucosamine transferase, etc. [3]. Contrary, these cells contain the enzyme responsible for *N*-glycolylneuraminic acid synthesis (CMAH) that is absent in humans. The IEF analysis of rEPO displays a profile in which more acidic isoforms can be observed with respect to CHO-derived marketed recombinant EPOs, albeit not as acidic as present in endogenous erythropoietin (urinary or plasmatic) [4, 5]. These different IEF profiles suggest that the glycosylation could be different. Even though several papers have been published on the gene expression of rEPO δ and its use in medicine, details on the glycosylation affecting the biological activity, have not yet been reported. In this study we describe the glycosylation of rEPO δ as compared to CHO cell-derived epoetins.

Materials and Methods

Recombinant human EPOs were obtained from European Pharmacopoeia Commission (rEPO α/β) or kindly provided by P. Hemmersbach and C. Reichel (rEPO δ). Sialic acids were released from the carbohydrate chains in 2M AcOH at 80°C for 3h, derivatised with DMB and analysed by two methods: 1) reverse phase HPLC-FLD (Zorbax SB-C$_{18}$ 150 x 0.3mm) using ACN:H$_2$O (20:80) as eluent at 4 µl/min. Analyses were performed on an Agilent 1100 capillary instrument equipped with a Jasco21FP fluorescence detector (λ_{ex}=373 and λ_{em}=448 nm). 2) UPLC ESI-TOF MS using an Acquity^{TM} instrument from Waters coupled to an LCT premier XE ESI TOF instrument from Micromass. LC analyses were performed at a flow-rate of 0.2 ml/min using an BEH C$_{18}$ column (100 x 2.1mm, 1.7µm) and MeOH:ACN:H$_2$O (7:25:68) as eluent. MS analyses of O-glycoproteins were performed using an Acquity^{TM} UPLC from Waters coupled to a LCT premier XE ESI-TOF instrument (Micromass). Samples were analysed in flow-injection configuration at a flow-rate of 0.05 ml/min using a mobile phase of H$_2$O:ACN:FA (95:5:0.1). O-glycopeptides were separated using an Acquity UPLC BEH C$_{18}$ using Waters instrument and under conditions reported by Guan *et al* [6]. N-glycans were released from rEPOs with PNGaseF, purified through a graphitised carbon column and derivatised with 2-AB. WAX HPLC profiling were carried out using an Agilent 1090 HPLC equipped with a fluorescence detector (λ_{ex}=330 and λ_{em}=420 nm). The column was a VYDAC 301 VHP (7.5 x 50 mm) with the following gradient: solvent A was 20% ACN in water; solvent B was 20% ACN in 500 mM ammonium acetate pH 4.4. Initial conditions were 100% A at a flow rate of 0.4 ml/min. Following injection, samples were eluted by a linear gradient of 100-0% B over 5 min, followed by a linear gradient of 0-100% B over the next 35 min, returning to the start conditions over the next 15 min. Normal-phase HPLC analyses were performed on an Agilent 1100 capillary instrument equipped with a Jasco21FP fluorescence detector (λ_{ex}=330 and λ_{em}=420 nm). NP profiling was carried out on a TSK gel Amide-80 column (0.5 x 150 mm), using the following gradient conditions: solvent A was 10% 50 mM ammonium formate (pH 4.4) in 90% ACN, solvent B was 90% 50 mM ammonium formate (pH 4.4) in 10% ACN, and the flow rate was 15 µl/min. Following injection, samples were eluted by a linear gradient of 20-55% B over 100 min, followed by a linear gradient of 55-100% B over the next 5 min. The column was eluted with 100% B for 2 min, and re-equilibrated in 20% B before injection of the next sample. MALDI-TOF MS experiments were carried out on a Voyager-DE^{TM} STR (Applied Biosystems) [7].

Results and Discussion

Given that the two rEPOs compared in this study share their polypeptide chain, molecular weight (Mw) determination was used to infer the microheterogeneity of both glycoproteins. The average molecular weight was calculated from MALDI-TOF MS data. Despite that peaks were wide and badly resolved, the mean value 29.75 kDa for rEPO δ resulted to be slightly higher than the reference rEPO α/β (Mw 29.39 kDa). This result, reported previously by Kohler *et al.* [8] contrasted with the Mw determined by SDS-PAGE in which rEPO δ migrates faster and as a narrower band indicating lower hydrodynamic value and possibly lower glycan heterogeneity. Glycan profiling of both glycoproteins were performed for evaluating the effect of human cell system in rEPO production.

Sialic acids analysis:

Analysis by reverse phase HPLC of the fluorecently tagged sialic acids released by mild acid hydrolysis allowed to determine the percentage of *N*-glycolylneuraminic acid (Neu5Gc), *N*-acetylneuraminic acid (Neu5Ac), and other variants. Identification was carried out by comparing retention times with reference compounds (figure 1A). Neu5Ac resulted to be the major constituent in both recombinant preparations accounting for 98.1 mol% in rEPO δ and 87.8 mol% in rEPO α/β. In addition, O-acetyl modifications could be also identified although far less abundant in rEPO δ; $Neu5,9Ac_2$ accounted for 1.9 mol% in rEPO δ while it was up to 7.9 mol% in rEPO α/β. Also $Neu5,7Ac_2$ (1.2 mol%) and the $Neu5,7(8),9Ac_3$ (1.7 mol%) could be detected, but in rEPO α/β only. Finally, an interesting feature in sialic acid speciation is the potential presence of Neu5Gc. As expected, rEPO δ produced in human cells did not contain any Neu5Gc (LOD: 6 fmol, i.e. 0.3 mol% in these experiments) while rEPO α/β had a ca. 1.3 mol% Neu5Gc. In order to corroborate these results, sialic acids were simultaneously analysed by UPLC-ESI-TOF (figure 1B). Confirming what was seen by fluorimetric detection, rEPO δ showed only a detectable peak at the trace corresponding to *m/z* 424.14 (Neu5Ac) but none at *m/z* 440.13, characteristic for Neu5Gc. On the contrary, the analyses of rEPO α/β showed a clear peak at the trace *m/z* 440.13 confirming the presence of Neu5Gc. Although this particular sialic acid occurs frequently in animal cells and is absent in humans due to the absence of CMP-Neu5Ac hydroxylase, a potential Neu5Gc uptake from the culture medium and incorporation to endogenous glycoproteins has been reported. The successive injections products containing Neu5Gc have been suggested to produce allergy-like technology, so the monitoring of this residue is important for quality control of biopharmaceuticals. Furthermore, besides the most abundant peak of Neu5Ac (*m/z* 424.14), in

rEPO α/β other species with acetyl groups at different positions were also found; Neu5,7(9)Ac$_2$ (*m/z* 466.14) and Neu5,7,9Ac$_3$ (*m/z* 508.15).
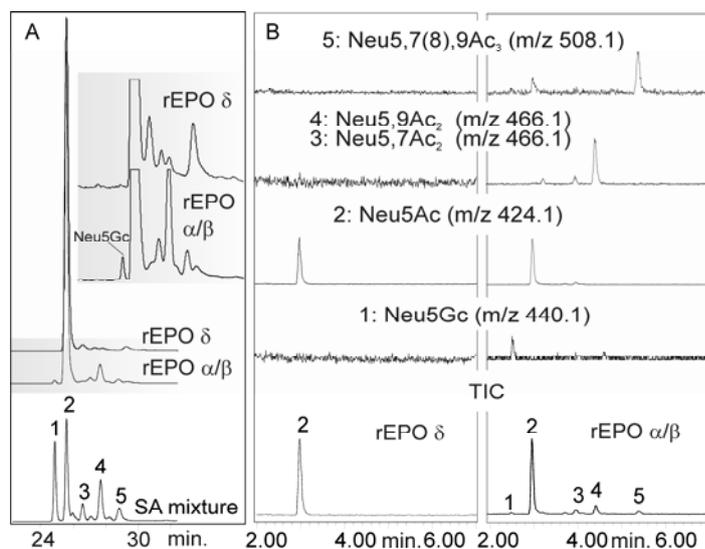

Figure 1. (A) HPLC-FLD pattern of fluorescent DMB derivatives of sialic acids from rEPO δ and rEPO α/β in comparison to the standard sialic acid mixture included in the lower panel. The identification of the numbered species is given in panel B. The insert is a zoom of the chromatograms where the presence of *N*-glycolylneuraminic acid can be appreciated in the rEPO α/β only. (B) Extracted ion chromatograms of LC-MS analysis of the DMB-derivatised sialic acid residues derived from rEPO δ (left) and rEPO α/β (right). The total ion chromatogram (TIC) is included at the bottom.

O-glycan analysis:

The heterogeneity associated to the single O-glycan was evaluated by means of LC-ESI-TOF MS of proteins after de-N-glycosylation, as well as analyses of O-glycopeptide after trypsinolysis [9]. Quantitative differences in sialylation of the O-glycans were found when rEPO δ was compared with rEPO α/β. With regard to the O-glycoprotein the di-sialylated peak was the only one detected in rEPO δ. For rEPO α/β both di- and monosialylated O-glycan, could be clearly detected accounting for 56.36% and 43.64% respectively (figure 2). Data obtained from O-glycopeptide (fragment 117-131) corroborated the absence of mono-sialylated O-glycoform in rEPO δ but also showed a small proportion of non-sialylated and non-O-glycosylated peptide. In case of rEPO α/β, again the di- and mono-sialylated O-glycoforms were predominant in similar amounts. However, the non-O-glycosylated peptide, the O-glycopeptide containing and GalNAc and the O-glycopeptide containing Gal-GalNAc were also present in lower proportion (figure 2).
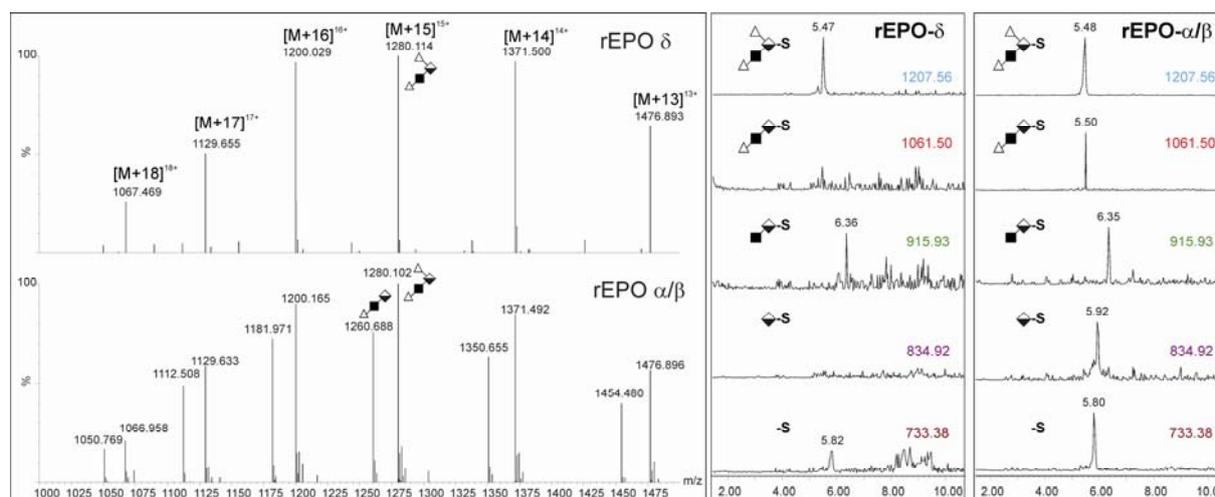
Figure 2. Comparison of the O-glycoforms from rEPO δ and rEPO α/β by ESI-TOF MS of de-N-glycosylated protein (left) and O-glycopeptide (right). Symbols: (⬧) *N*-acetylgalactosamine, (■) galactose, (Δ) sialic acid.

N-glycan analysis:

Weak anion exchange (WAX) carbohydrate profiling allowed the analysis of N-glycans charge content (figure 3). For rEPO δ three major peaks were observed showing a much less heterogeneous profile than rEPO α/β. The single peak of tetra-charged structures of rEPO δ accounted for 44.28%, tri-charged for 37.69% and di-charged 16.81%. Although it was supposed to contain low proportion of mono-charged entities, they could not be quantified because of the 2-AB excess. Neutral structures were almost absent (~1.2%). For rEPO α/β, once the areas of the two peaks accounting for tetra-sialylated N-glycans were summed, they yielded 47.85%. The five different peaks of tri-charged species contributed with a 36.85% and another four peaks accounting for 12.63% of di-charged N-glycans. Mono-charged and neutral N-glycans were minority accounting for 0.76% and 1.90% respectively.
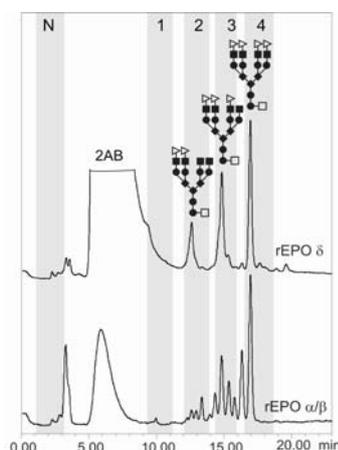

Figure 3. Weak anion exchange HPLC profiles of 2-AB-labeled glycans from rEPO δ (upper panel), rEPO α/β (lower panel).). The regions corresponding to neutral (N), mono-, di-, tri-, and tetra-charged glycans (1,2,3,4) are indicated with grey boxes. 2-AB indicates the excess of derivatisation reagent. Structures were tentatively assigned based on mass spectrometry data (*vide infra*). Short-hand notation: (□) fucose, (●) N-acetylglucosamine, (◆) mannose, (■) galactose, (Δ)

Percentages of N-glycans with different degrees of sialylation together with percentages of each sialylated O-glycoform (obtained from O-glycoprotein MS analyses) allowed us to calculate the average number of sialic acids. Values obtained for

rEPO δ were 11.72 sialic acids while for rEPO α/β the value was 11.39 sialic acids. Further structural information on the N-glycan composition was obtained from MALDI-TOF MS analyses of the 2-AB-labelled structures (figure 4).
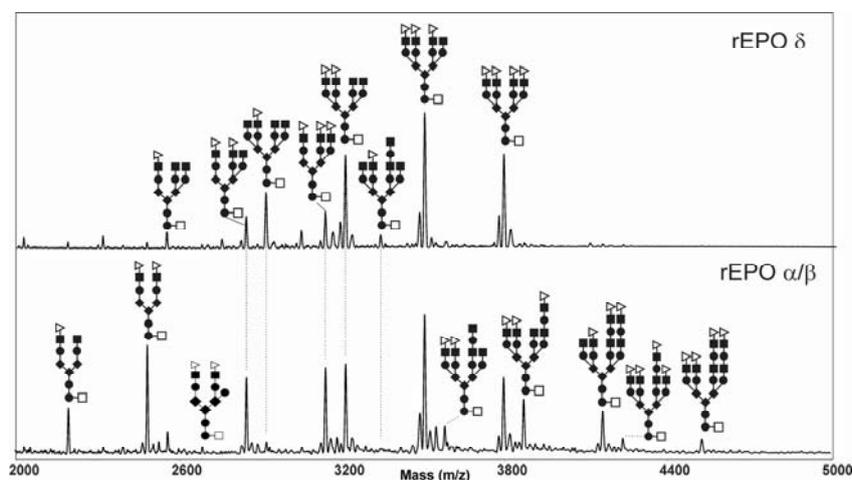

Figure 4. Negative-ion mode MALDI mass spectra of 2-AB-labeled glycans from rEPO δ (top), rEPO α/β (bottom). The depicted structures indicate all possible isomers. Short-hand notation: (□) fucose, (●) N-acetylglucosamine, (◆) mannose, (■) galactose, (Δ) sialic acid. The structural assignments for rEPO α/β are based on NMR data. The structural assignments for rEPO δ have been done by analogy to those of rEPO α/β and are indicative only.

Profiles obtained for rEPO δ displayed mainly core-fucosylated tetra-antennary complex type N-glycans containing up to 4 sialic acid residues, some tri-antennary N-glycans containing up-to 3 sialic acids and no di-antennary N-glycans (figure 4). These structures are also present in rEPO α/β but in this case also di-antennary N-glycans were present. Furthermore, in the latter a relatively higher content of tri-antennary N-glycans and other structures with up to 2 LacNAc repeats was observed. Normal phase HPLC profiling of these structures was conducted, following the observations of the different charge profiles. The individual chromatograms represent the structural heterogeneity present in the N-glycans of each sample. From a comparison between both recombinant pharmaceuticals, it becomes evident that rEPO δ is a more homogeneous preparation. rEPO δ profile showed 5 peaks in 10-12 GU retention time interval. Conversely, rEPO α/β yielded a much greater heterogeneity with at least 13 distinctive structures that elute at GU values comprised between 8 and 14. Structures at GU values higher than 12 are those potentially indicating the presence of glycans larger than standard tetra-antennary structures (*i.e.* presence of LacNAc repeats). These structures, as seen by MS, were minor in rEPO δ while abundant in rEPO α/β. In order to corroborate the initial structural assignments, reagent array digestions (RAAM) were conducted in conjunction with both, HPLC and MALDI-TOF MS analyses. The HPLC profile for rEPO δ after de-sialylation basically showed 3 structures; core-fucosylated tetra-antennary N-glycan (81.07%), core-fucosylated tri-antennary N-glycan (12.81%), core-fucosylated tetra-antennary N-glycan containing a single LacNAc repeat (6.12%). The equivalent treatment for rEPO α/β yielded a profile where apart from those structures, also core-fucosylated di-antennary N-

glycan, and core-fucosylated tetra-antennary N-glycan containing 2 LacNAc repeats were seen. Mass spectra obtained for these samples corroborated the presence of the structures assigned to the NP-HPLC peaks. With the aim of determining the linkage type present in the sialic acid residues, de-sialylation was performed also with an α2-3 sialidase. The mass spectra after both, this treatment and that from a complete desialylation yielded the same results suggesting that all sialic acids are α2-3 linked. Next in RAAM analysis, the β-1,4-galactosidase digestion, evidenced the presence of LacNAc repeats; a very small proportion of tri- and tetra-antennary glycans containing one LacNAc in rEPO δ but more abundant peaks corresponding to tri- and tetra-antennary glycans containing one and two LacNAc repeats in rEPO α/β. Finally, after the complete digestion the only detectable structure was the common core-fucosylated pentasaccharide.
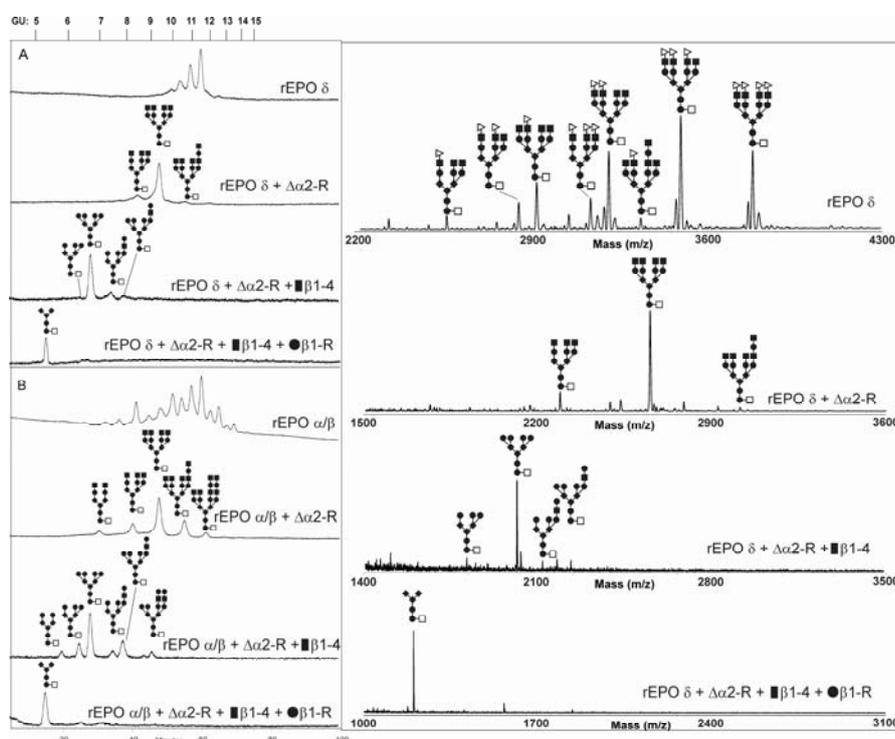

Figure 5. On the left, the normal-phase HPLC profiles of 2-AB-labeled glycans from rEPO δ (A), rEPO α/β (B). The top profile shows undigested pool of glycans, followed by a series of exoglycosidase digestions in the lower panels. GU: glucose units. On the right, the MALDI mass spectra of rEPO δ before (upper panel, negative ion mode) and after exoglycosidase digestions (lower panels, positive ion mode). The structural assignments for rEPO δ follow those of rEPO α/β that are based on NMR data but should be considered as indicative only.

Gel-separated glycoforms

Upon separation by IEF, according to the isoelectric point, rEPO δ was resolved in up to 9 glycoforms. Most of the isoforms are also present in rEPO α/β, but rEPO δ extends its profile towards more acidic pH values, with 2 isoforms being exclusively present in rEPO δ. In order to characterise individual rEPO δ glycoforms, with particular interest in the two more acidic bands, two-dimensional electrophoresis was combined with MALDI-TOF MS analysis of the glycans obtained from the excised bands. Bands were numbered according to their acidity; *i.e.*

rEPO α/β (from 1 to 7 in order of increasing pI) and rEPO δ (the same series from 1 to 7 plus additional more acidic bands α and β following nomenclature of WADA). Purified N-glycans, labelled with 2-AB and analysed by MALDI-TOF MS as described above allowed the structural identification of the glycans contained in individual bands. Mass spectra of purified N-glycans from the least abundant bands (7 and β for rEPO δ) could not be obtained. However, the results clearly show the trend towards a higher degree of sialylation following the decreasing pI values of the IEF band. As rEPO δ possesses much less heterogeneity, this variation resides mainly on the sialylation degree of tetra-antennary chains showing a progressive increase in tetra-charged structures and a progressive decrease of mono-charged species with increasing acidity of the band. While rEPO α/β also showed this trend, the larger structural heterogeneity in this product (presence of di- and tri-antennary structures and LacNAc repeats) rendered a less pronounced phenomenon in individual structures. Overall, these results confirmed that sialic acid residues are the sole charges present in the glycans. The presence of other charged residues such as sulphates described for other human glycoproteins were not observed despite the fact that rEPO δ is produced in human cells.
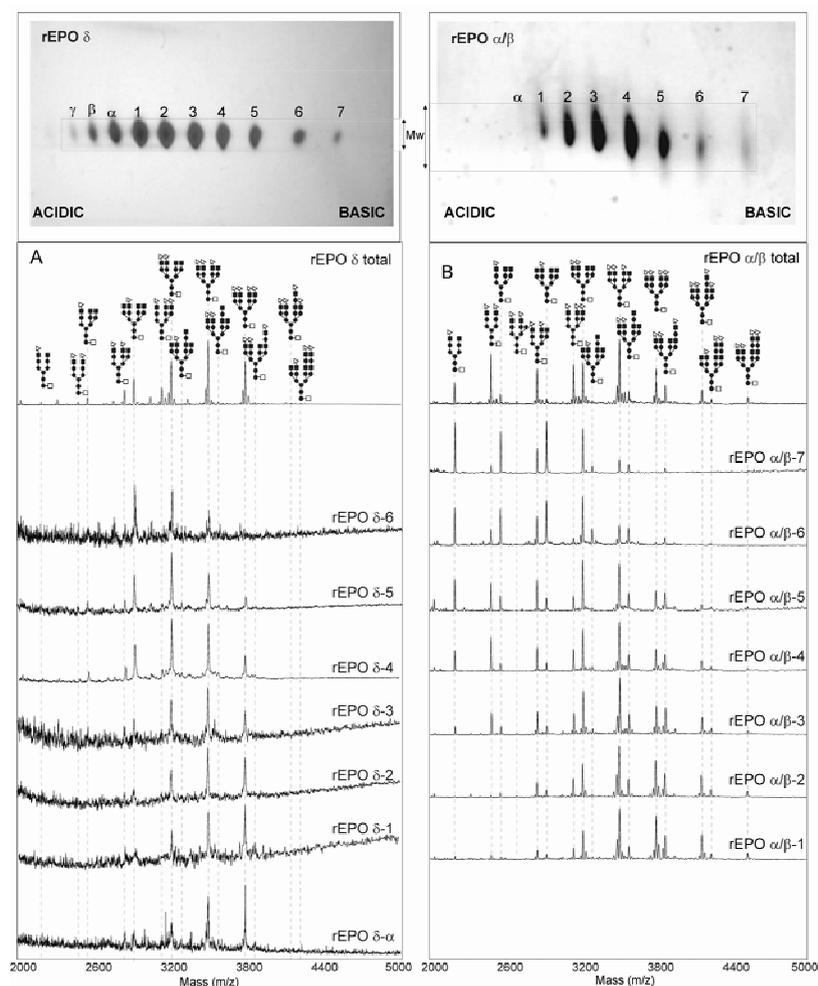

Figure 6. Silver stained 2-DE gels (upper panel) and negative-ion mode MALDI mass spectra of 2-AB-labeled N-glycans excised from 2-DE bands in the lower pannels. On the left the analyses for rEPO δ and on the right the analyses for rEPO α/β. Identification of bands in the 2-DE gels is according to the criteria employed by doping-control laboratories; (γ for more acidic, to 7 for more basic band). Dashed lines drawn in the gel indicate molecular weight dispersion of bands. In the mass spectra the numbering corresponds to the band annotation in the 2-DE gels. Short-hand notation: (□) fucose, (●) N-acetylglucosamine, (◆) mannose, (■) galactose, (Δ) sialic acid. The structural assignments for rEPO δ follow those of rEPO α/β that are based on NMR data but should be considered as indicative only.

In 2-DE gels, as well as in 1D SDS-PAGE, rEPO α/β migrates slower than rEPO δ (figure 6). This phenomenon, accompanied by Mw differences between contiguous glycoforms was analysed in depth. Glycoforms from rEPO α/β were observed as a "train" of bands which not only differ in pI but also in apparent MW ("diagonality"). The latter effect was much less pronounced in rEPO δ and absent in urinary EPO. With the obtained knowledge of the structural differences between the two recombinant homologues, the diagonality in a 2-DE and band broadness in SDS-PAGE can be attributed predominantly to the presence of LacNAc repeats, whereas sialic acids play a much less prominent role than assumed thusfar. In order to validate this assumption, the average Mw conferred to a glycoform through the 3 N-glycans in a single band, both with and without sialic acids were calculated. Results showed a clearly different slope when the total glycans were considered, which is fairly consistent with the diagonal trends observed in the 2-DE of the intact glycoproteins. Without sialic acids, rEPO δ yielded a near-horizontal mass trend, as expected since it contains predominantly tetra-antennary N-glycans, while rEPO α/β still shows a diagonal trend albeit with a lower slope (figure 7).
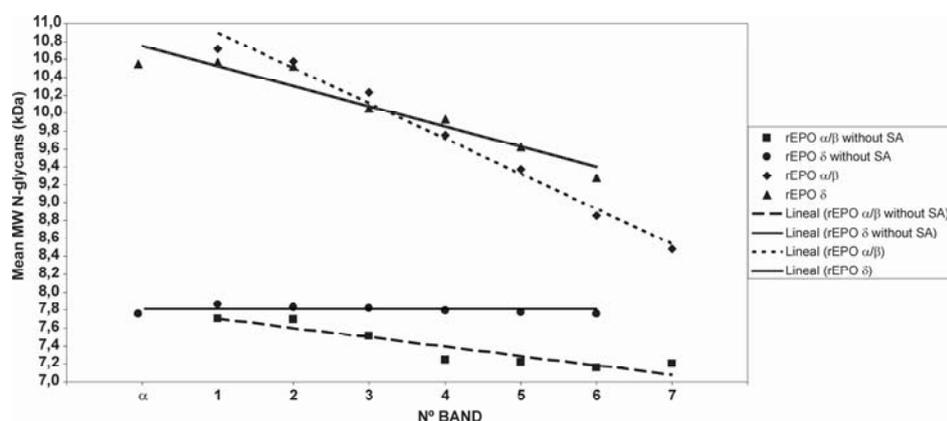

Figure 7. Trend-lines of the cumulative mass of the N-glycans from the individual bands in the 2-DE gels based on mass spectrometry data. A clear difference in slope can be visualised for the intact glycans from rEPO δ (lower slope) and rEPO α/β (higher slope) indicating a narrower mass distribution over the glycans for the former than for the latter. This slope difference is further exemplified after desialylation where a near horizontal line is observed for rEPO δ whereas for rEPO α/β still preserves diagonality.

To sum up, can be concluded that the glycosylation of both rEPOs (α/β and δ) contains the same structural elements but being rEPO δ a more homogeneous preparation (mainly tetra-antennary N-glycans with different sialylation). At sialic acids level rEPO δ presents higher degree of sialylation in the O-glycan and similar levels in N-glycans when is compared to rEPO α/β. Importantly, the unique structural feature of rEPO from non human cell lines (Neu5Gc) is absent in rEPO δ.

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