Identification and structural characterization of a mannose-6-phosphate containing oligomannosidic *N*-glycan from human erythropoietin secreted by recombinant BHK-21 cells

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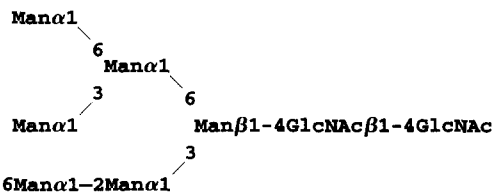
Abstract A sialidase resistant mono-charged *N*-glycan was isolated from glycosylation site I (Asn-24) of recombinant human erythropoietin expressed from baby hamster kidney (BHK-21) cells and constituted approximately 2–4% of the oligosaccharide material at this glycosylation site. Mass spectrometry and both 1- and 2-dimensional NMR techniques revealed a high mannose type structure (Man_6) with a phospho-diesterbridged N-acetylglucosamine as follows:



Key words: Recombinant human erythropoietin; Baby hamster kidney cells (BHK-21); Phosporylated oligomannosidic glycan; Mannose-6-phosphate, 1- and 2-D NMR spectroscopy; Lysosomal recognition signal

1. Introduction

Phosphomannosyl residues on glycoproteins serve as recognition markers for the translocation of lysosomal enzymes from the Golgi into their cellular compartment (for review see e.g. [1]). First, *N*-acetylglucosamine is transferred to a mannose residue by UDP-GlcNAc:*N*-acetylglucosaminylphosphotransferase. The phosphodiester is subsequently cleaved by a Golgi-associated *N*-acetylglucosaminidase generating the mannose-6-phosphate recognition signal on the glycoprotein, which is specifically targeted into the lysosome after binding to the mannose-6-phosphate receptor. The phosphotransferase reaction has been shown to be highly specific for lysosomal enzymes [2], while nonlysosomal glycoproteins are poor in vitro substrates at very high concentrations.

In the present report, we describe the structural characterization of a phosphorylated oligomannosidic oligosaccharide which was unexpectedly found at the first *N*-glycosylation site (Asn-24) of the obligate secretory human signal glycoprotein erythropoietin expressed from recombinant BHK-21 host cells.

2. Materials and methods

2.1. Isolation of the phosphorylated oligosaccharide

The *N*-glycans from 100 mg of a recombinant human EPO preparation [3] were enzymatically liberated by polypeptide:*N*-Glycosidase F (PNGase F). The *N*-glycans were desalted by Fast Protein Liquid Chromatography on a Sephadex G-25 Superfine Fast Desalting HR 10/10 column (Pharmacia, Uppsala, Sweden). Oligosaccharides were separated by charge on a Mono-Q column; the mono-charged fraction was re-chromatographed on aminobonded phase as described [3].

2.2. Enzymatic desialylation and analytical high-pH anion-exchange chromatography (HPAE-PAD) of asialo-oligosaccharides

Enzymatic removal of neuraminic acid and analytical high-pH anion-exchange chromatography of this desialylated oligosaccharide sample was performed as described [3].

2.3. Carbohydrate composition and methylation analysis

Monosaccharides were analyzed by GC on a Carlo Erba Mega Series instrument after methanolysis, reacetylation and trimethylsilylation as the corresponding methyl glycosides on a 30 m DB1 capillary column according to Chaplin [4].

The reduced oligosaccharide was methylated according to Hakomori [5]. The permethylated carbohydrate material was purified by chromatography on a Sephadex LH20 column, hydrolyzed, reduced and peracetylated. Analyses were performed on a Kratos MS 50 fast scan mass spectrometer connected to a Carlo Erba Mega Series gas chromatograph equipped with a 30 m DB1 column as described [6].

2.4. Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF MS)

2,5-Dihydroxbenzoic acid (DHB) was used as UV-absorbing matrix. 10 mg/ml DHB were dissolved in 10% ethanol in water. For analysis by MALDI/TOF MS the solution of the oligosaccharide was mixed with the same volume of matrix. 1 μl of the sample was spotted on a stainless steel probe tip and dried at room temperature. The concentration of the analyte was approximately 10 pmol/μl.

Measurements were performed on a Bruker Reflex MALD/TOF mass spectrometer using a N_2 laser (337 nm) with 3 ns pulse width and 10^7 to 10^8 $W \cdot cm^{-2}$ irradiance at the surface (0.2 mm^2 spot). Both positive and negative ion spectra were recorded at an acceleration voltage of 20 kV using the reflectron for enhanced resolution.

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Abbreviations: BHK, baby hamster kidney; CHO, Chinese hamster ovary; COSY, correlation spectroscopy; EPO, erythropoietin; GC, gas chromatography; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPAE-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; HPLC, high performance liquid chromatography; MALDI/TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Man, mannose; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement and exchange spectroscopy; PNGase F, polypeptide:N^4-(*N*-acetyl-glucosaminyl)asp aragine-amidase F; TOCSY, total correlation spectroscopy.

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2.5. 600 MHz ^1H NMR spectroscopy

Prior to ^1H NMR spectroscopic analysis, the oligosaccharide was repeatedly treated with ^2H_2O (Fluka, >99.95 atom % ^2H) at p^{2}H 7 at ambient temperature. Resolution enhanced 600 MHz ^1H NMR spectra were recorded using a Bruker AM 600 NMR spectrometer interfaced with an Aspect-3000 computer at a probe temperature of 27°C. A water elimination Fourier-transform pulse sequence was applied for suppression of the residual HO^{2}H resonance. Chemical shifts of the 'structural reporter groups' [7] are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to free acetate (δ = 1.908 in ^2H_2O at p^{2}H 6–8 and 27°C), with an accuracy of 0.002 ppm. ^1H 2D TOCSY [8] and NOESY spectra [9] were performed on the same instrument with mixing times of 80 and 500 ms, respectively. All 1D and 2D spectra were recorded using the standard Bruker software package and data manipulation of the 2D spectra were performed on a Bruker Aspect X32 data station.

3. Results

3.1. Isolation of the phosphorylated oligosaccharide

The N-linked oligosaccharides from recombinant erythropoietin were liberated by digestion with polypeptide:*N*-glycosidase F (PNGase F). After precipitation of the de-*N*-glycosylated EPO-protein, oligosaccharides were desalted and were separated according to their charge by ion-exchange chromatography on Mono-Q as described [3]. Preliminary characterization by HPAE-PAD of the four enzymatically desialylated fractions yielded the anticipated oligosaccharides for the di-, tri- and tetra-sialylated MonoQ fractions (not shown, cf. [3]). However, an unexpected oligosaccharide peak (Fig. 1, panel B, peak h) with a retention time similar to monosialylated *N*-glycans was observed for the desialylated mono-charged Mono-Q fraction. In order to isolate this unusual compound, the respective fraction was further separated by chromatography on a NH_2-bonded phase (see Fig. 1, panel A). The sialidase resistant charged oligosaccharide was detected solely in fraction 1 (Fig. 1, panels A and C). A total of 240 μg of this oligosaccharide material was obtained from 100 mg of recombinant EPO protein. Proteolytic peptide-mapping and HPAE-PAD analysis of N-linked oligosaccharides from individual glycosylation sites showed that this glycan was exclusively attached to Asn-24 (data not shown).

The oligosaccharide material from the HPLC fraction 1 (Fig. 1A) was further subjected to carbohydrate compositional analysis, methylation analysis, MALDI/TOF MS and NMR studies as described below.

3.2. Structural characterization of the phosphorylated oligomannosidic N-glycan

Mannose and *N*-acetylglucosamine were found in a ratio of approximately 2:1 after carbohydrate compositonal analysis. Since no neuraminic acid was detected, a phosphorylated (probably containing a phosphodiester bridge, since under the alkaline conditions of HPAE-PAD only one negative charge could be observed) or sulfated structure must be assumed. Negative ion MALDI/TOF MS (Fig. 2A) of the underivatized sample yielded a molecular ion at 1678 Da (calculated for $C_{60}H_{101}O_{49}N_3P$: 1678.5 Da) compatible with $[Hex_6\ HexNAc_3\ H_2PO_4]^-$, which can be explained by a phosphorylated high mannose type compound bearing an additional *N*-acetylglucosamine residue. In the positive mode (Fig. 2B) three signals at 1724, 1740 and 1756 Da were detected, indicating the addition of 2 Na^+, 1 Na^+ and 1 K^+ or 2 K^+ ions. Methylation analysis confirmed the presence of a high mannose type structure and indicated the presence of an additional terminal GlcNAc residue.

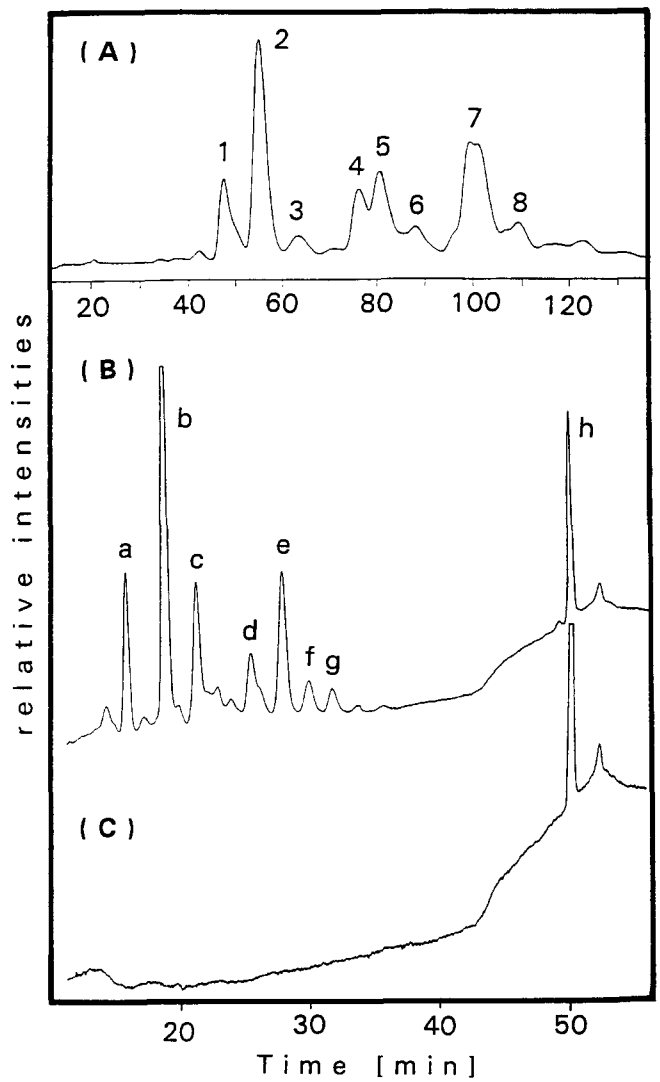

Fig. 1. (A) Separation on amino-bonded phase HPLC of the mono-charged Mono-Q oligosaccharide fraction detected at 202 nm. Compositional analysis of peak 1 revealed mannose and GlcNAc in a ratio of axproximately 6:3 and was further structurally analyzed. (B) HPAE-PAD mapping of the enzymatically desialylated total oligosaccharides from the monocharged Mono-Q fraction. Based on their comparison with reference oligosaccharides (see [3]) peaks were identified as follows: a = hybrid type, b = NeuAc, c,d,e = di-, tri- (both isomers) and tetra-antennary oligosaccharides, respectively, f = triantennary (2,6-branched) plus 1 lactosamine repeat, g = tetraantennary plus 1 lactosamine repeat); peak h = sialidase resistant monocharged oligosaccharides. (C) HPAE-PAD elution profile of the sialidase resistant NH_2-HPLC fraction 1 from panel (A).

The 600 MHz 1D ^1H NMR spectrum (Fig. 3A; Table 1) showed three resonances in the region of the *N*-acetyl protons of aminosugars, corroborating the compositional and MALDI data. In the anomeric proton region, resonances of 5 α-linked mannose residues were observed. As expected, the signal of the β-linked inner common core mannose was obscured by residual HOD. The H-1 signals of the chitobiose unit of the common

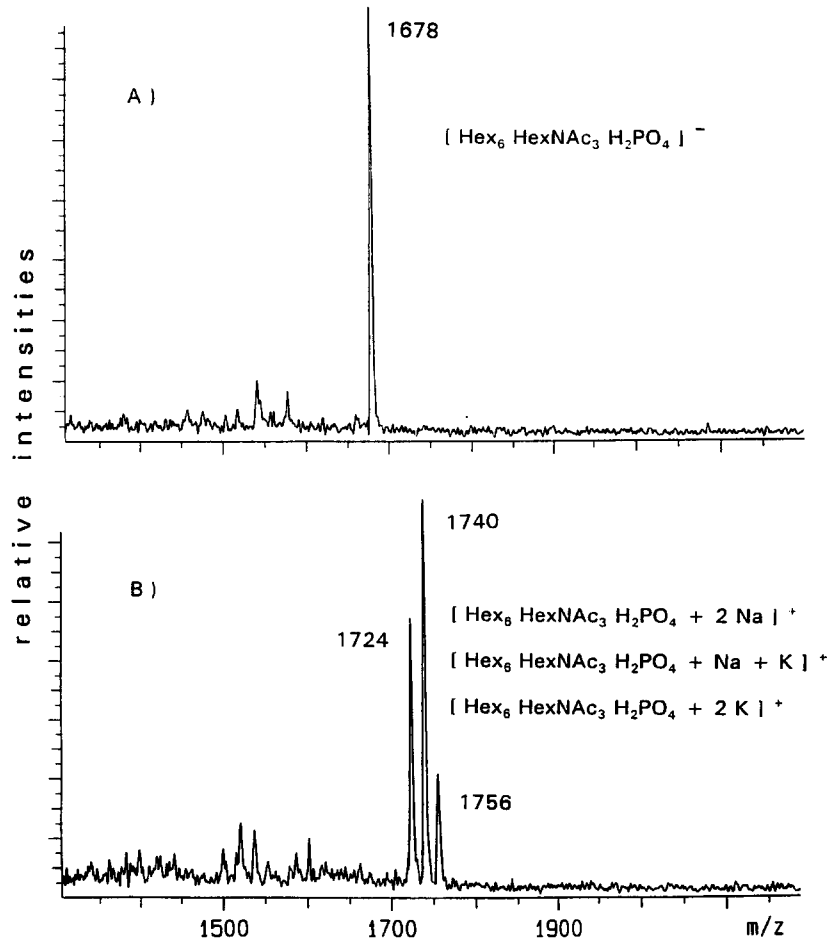

Fig. 2. Negative ion (A) and positive ion (B) MALDI mass spectra of the phosphodiester-bridged oligosaccharide isolated from recombinant human EPO expressed from BHK-21 cells.

core showed no irregularities nor did the H-1 and H-2 resonances of Man-**4,6,7**, when compared to the standard oligosaccharide Man_6 $GlcNAc_2$, whereas Man-**5** showed a downfield shift of approximately 0.02 ppm and Man-**8** a highfield shift of 0.03 ppm similar to the variations in chemical shifts observed by de Waard et al. for the analoguous mannose residues of a phosphorylated mannan compared to the unphosphorylated derivative [10]. For the H-1 resonance of the third *N*-acetylglucosamine at 5.479 ppm, not only the characteristic coupling of the anomeric proton of an α-linked *N*-acetylglucopyranose to its H-2 proton of approximately 3.5 Hz was observed, but an additional coupling of approximately 7 Hz, which is expected for a phosphodiester bridged compound ($J_{1H\text{-}31P}$ ca. 6–8 Hz). An inverse ^1H-detected ^1H-^{31}P correlated 1D NMR experiment showed upon irradiation of the phosphorus atom spin transfer to the H-1 and H-2 protons of the neighbouring GlcNAc residue and the H-6a and H-6b protons of a mannose residue (Fig. 3B). The linkage of phosphoric acid to position 6 of mannose was confirmed by detection of the respective derivatized compound after methanolysis, trimethylsilylation of the sugar hydroxyl groups and methylation of the phosphoric acid as described by Ferguson et al. [11].

The complete primary structure of the oligosaccharide was resolved by 2D NMR spectrometry. Fig. 4A shows the 2D TOCSY spectrum of the oligosaccharide, which in combination with the 2D COSY spectrum allowed the complete or partial assignment of the spin systems of all monosaccharide residues. The interresidual crosspeaks of the 2D NOESY spectrum were then used for determination of the sequence of the individual monosaccharide units. Thus, the linkage of Man-**8** to Man-**5** could be unequivocally determined. The H-6a and H-6b protons of Man-**8** did not show a crosspeak in the respective H-1 trace of the TOCSY spectrum nor did the H-2 and H-1 protons of this residue in the H-6a trace. However, after reprocessing of the TOCSY data (Fig. 4B), the complete spin system of Man-**8** could be observed on its H-**2** trace, confirming unequivocally the linkage of phosphoric acid to this residue.

4. Discussion

The carbohydrate structures of recombinant human erythropoietin expressed from Chinese hamster ovary (CHO) and baby hamster kidney (BHK-21) host cells have been thoroughly investigated by several groups (Sasaki [12], Takeuchi [13], Nimtz [3] and Tsuda [14]). From the three *N*-glycosylation sites present in this polypeptide, Asn-24 of the first tripeptide recognition

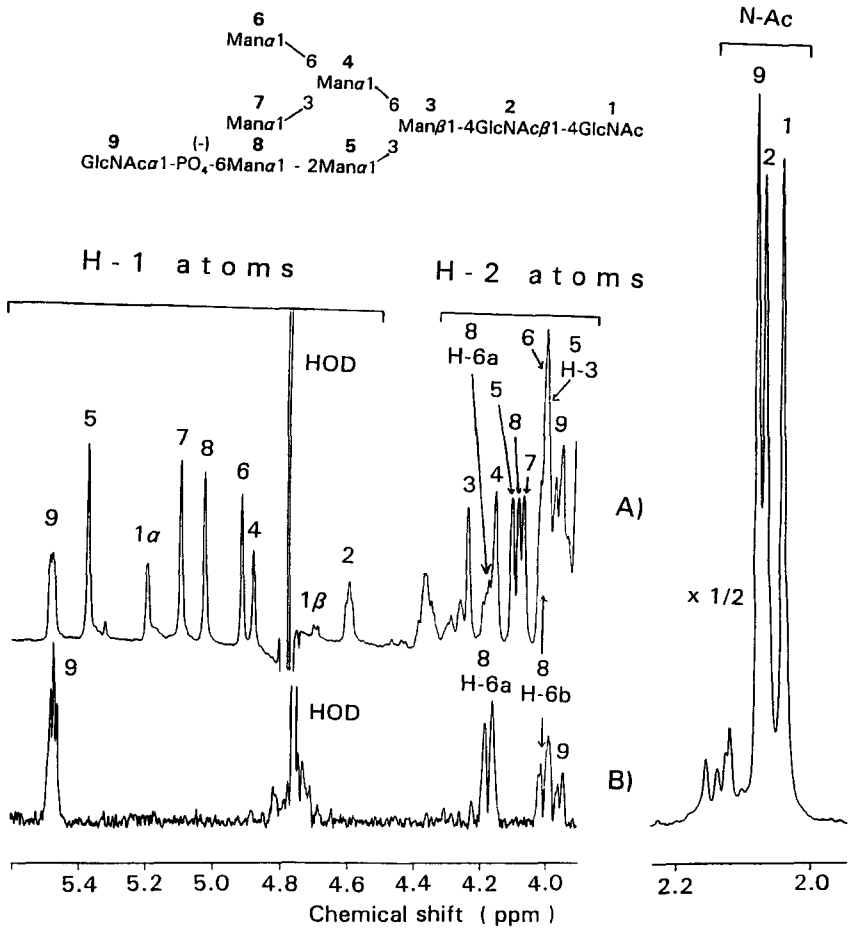

Fig. 3. (A) 600 MHz ^{1}H NMR spectrum of the phosphorylated oligosaccharide (structural reporter group signals) isolated from BHK-EPO. (B) ^{1}H-^{31}P correlation via heteronuclear zero and double quantum coherence using ^{1}H-inverse detection (optimized for long-range couplings).

site has been shown to be glycosylated heterogeneously with hybrid-type, bi-, tri- and tetra-antennary chains, whereas the two *N*-glycosylation sites Asn-38 and Asn-83 are occupied exclusively by tetra-antennary chains with or without 1–3 lactosamine repeats [3]. In accordance with this oligosaccharide distribution pattern the unexpected GlcNAc-P-Man$_6$GlcNAc$_2$ oligosaccharide structure characterized in this study was found to be attached solely at Asn-24 of the recombinant EPO polypeptide and was calculated to be present in about 2–4% of the total molecules. To our knowledge this report describes, for the first time, the complete primary structural analysis of a phosphorylated oligomannosidic oligosaccharide synthesized on an obligate secretory glycoprotein from a mammalian cell line.

So far no structural primary sequence or three-dimensional motif in lysosomal polypeptides has yet been identified that explains the specificity of the *N*-acetylglucosaminyl 1-phosphotransferase for lysosomal enzymes with oligomannosidic *N*-glycans [1,15]. Recently, Dustin et al. [16] have presented a model which ascribes the interaction of distinct peptide regions of lysosomal enzymes a role in the recognition by the phosphotransferase. However, it is known that a small fraction of lysosomal hydrolases, containing phosphorylated mannose, is transiently delivered to the extracellular milieu and is subsequently targeted into the lysosome after binding to cell surface mannose-6-phosphate receptors and internalization via clathrin coated vesicles [17]. This recapture mechanism involves the presence of the phosphomonoester form of the oligomannosidic glycan attached to the polypeptide. Such a structure would be expected to elute in the region of the doubly charged oligosaccharide fraction in anion-exchange chromatography on Mono-Q. However, upon inspection of the disialylated glycan fraction from recombinant erythropoietin preparations from BHK-21 cells no sialidase-resistant oligosaccharide material was detected in our studies. Therefore, either the *N*-acetylglucosaminidase, if expressed in the BHK cells, is inactive with the phosphodiester intermediate of EPO, or all of the mannose-6-phosphate glycoforms resulting from this enzyme activity are targeted directly into the lysosomal compartment via the intracellular Man-6-phosphate receptor pathway.

The detection of the GlcNAc-P-Man$_6$GlcNAc$_2$ oligosaccharide structure may be explained by the high level of expression of the protein in BHK-21 host cells. Thus, overloading of the intracellular glycoprotein processing and transport machinery could lead to the observed aberrant modification of a small proportion of the molecules. However, it should be emphasized that the high concentration of glutamine, which is a major energy source of cultured cells [18], in the culture medium used in biotechnological production processes is known to result in

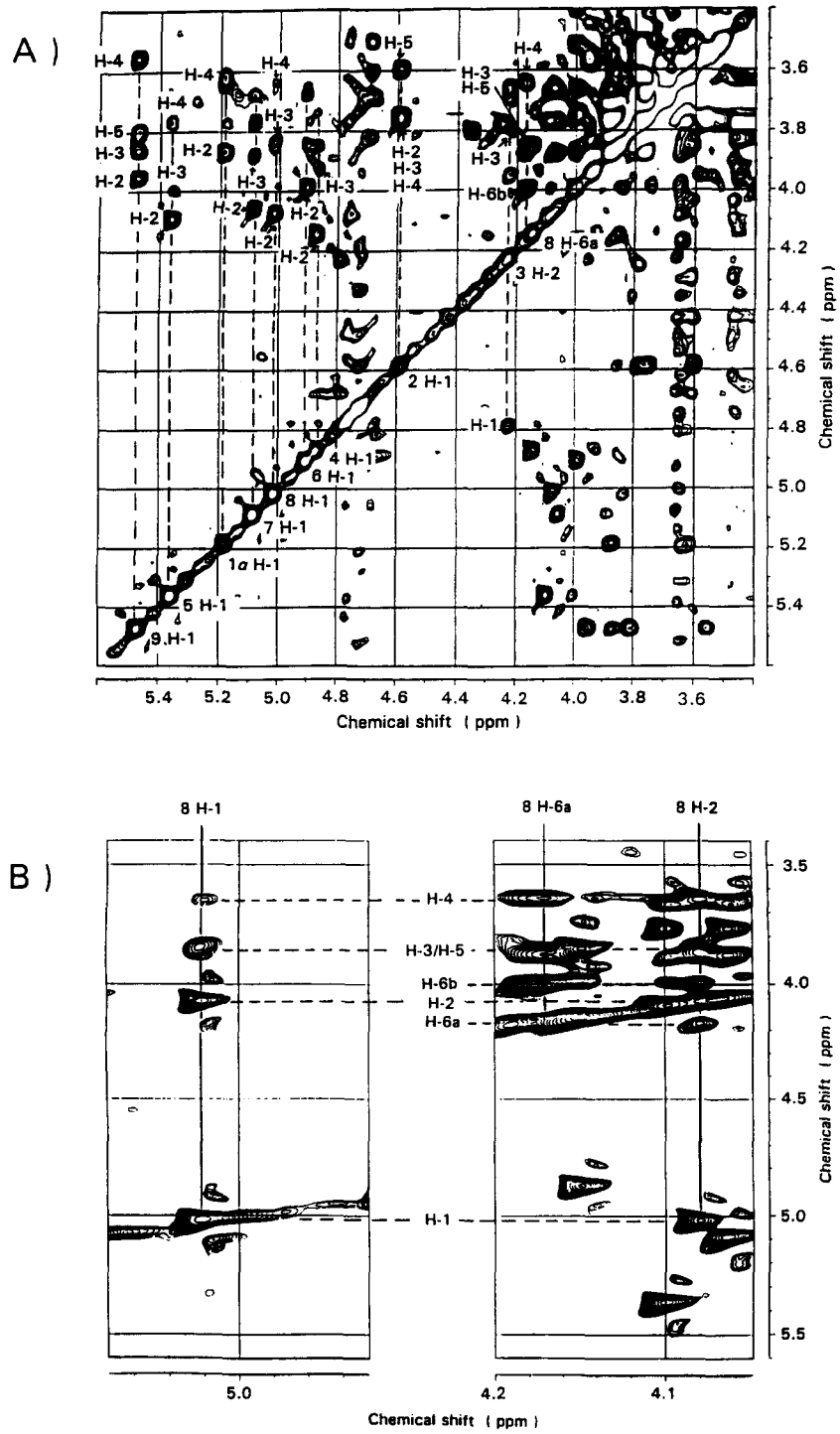

Fig. 4. (A) 600 MHz ^{1}H NMR TOCSY contour plot of the phosphorylated oligosaccharide. (B) Part of the TOCSY spectrum, 5.9–3.0 ppm, which was reprocessed, in both dimensions with 1024 real points in F2 and 512 real points in F1. QSINE window functions with SSB2 = 2.5 and SSB1 = 1 were used. The position of the H-1, H-6a and H-2 traces of the mannose residue 8 are marked at the top of the figure and their respective correlated signals in the centre.

high NH_4-concentrations (2–4 mM) [19] in the medium due to thermal degradation of the amino acid. The addition of exogeneous NH_4Cl to the culture medium of transfected BHK-21 cells has been shown to profoundly affect the final glycosylation pattern of recombinant glycoproteins [20]. Furthermore, Hasilek et al. [21] and Horst et al. [22] have reported that the mannose-6-phosphate dependent targeting can be inhibited and a portion of the precursor can be directed into the normal secretory pathway when cells are grown in the presence of 10 mM NH_4Cl.

This phenomenon deserves special attention in cases where the biotechnological production of recombinant phar-

Table 1
600 MHz ^{1}H NMR chemical shifts of fraction 1 (Fig. 1A)

Protons	Chem. shift (ppm)	Protons	Chem. shift (ppm)
H-1		H-4	
GlcNAc-1	5.190 (α)	GlcNAc-1	3.63 [N]
	4.692 (β)	GlcNAc-2	3.76 [T]
GlcNAc-2	4.592 (α + β)	Man-3	3.70 [T]
Man-3	4.79 [T]	GlcNAc-9	n.d.
Man-4	4.877		
Man-5	5.368	H-5	
Man-6	4.911	GlcNAc-2	3.60 [T]
Man-7	5.092	Man-3	3.66 [T]
Man-8	5.021	Man-8	3.88 [T]
GlcNAc-9	5.479	GlcNAc-9	3.80* [T]
H-2		H-6a	
GlcNAc-1	3.87 (α) [C]	Man-3	3.95 [T,N]
	3.69 (β) [C]	Man-4	3.99 [N]
GlcNAc-2	3.79 [C,T]	Man-8	4.176 [1DP,N,T]
Man-3	4.256		
Man-4	4.150 [C]	H-6b	
Man-5	4.100 [C,N,T]	Man-3	3.78 [T,N]
Man-6	4.00 [C]	Man-4	3.76 [N]
Man-7	4.065 [C]	Man-8	4.004 [1DP,N,T]
Man-8	4.080 [C]		
GlcNAc-9	3.96 [C,N,T]	N-Ac	
		GlcNAc-1	2.037
H-3		GlcNAc-2	2.063
GlcNAc-1	3.87 (α) [T]	GlcNAc-9	2.074
	3.62 (β) [T]		
GlcNAc-2	3.76 [T]		
Man-3	3.81 [C,N]		
Man-4	3.93 [C,N,T]		
Man-5	4.01 [C,T]		
Man-6	3.86 [C,T]		
Man-7	3.89 [C,T]		
Man-8	3.84 [C]		
GlcNAc-9	3.88* [T]		

T: TOCSY, N: NOESY, C: COSY; 1DP: 1-dimensional heteronuclear ^{1}H-^{31}P correlation.
*may have to be interchanged

maglycoproteins from mammalian hosts with defined carbohydrate structures and reproducible batch-to-batch consistency is desired.

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References

[1] Kornfeld, S. (1992) Annu. Rev. Biochem. 64, 307–330.
[2] Lang, L., Reitman, M., Tang, J., Roberts, R.M. and Kornfeld, S. (1984) J. Biol. Chem. 259, 14663–14671.
[3] Nimtz, M., Martin, W., Wray, V., Klöppel, K.-D., Augustin, J. and Conradt, H.S. (1993) Eur. J. Biochem. 213, 39–56
[4] Chaplin, M.F. (1982) Anal. Biochem. 123, 336–341.
[5] Hakomori, S. (1964) J. Biochem. (Tokyo) 55, 205–207.
[6] Nimtz, M., Noll, G., Pâques E.-P. and Conradt, H.S. (1990) FEBS Lett. 271, 14–18.
[7] Vliegenthart, J.F.G., Dorland, L. and van Halbeek, H. (1983) Adv. Carbohyd. Chem. Biochem. 41, 209–374.
[8] Braunschweiler, L. and Ernst, R.R. (1983) J. Magn. Reson. 53, 521–528.
[9] Macura, S. Huang, Y. Sutter, D. and Ernst, R.R. (1981) J. Magn. Reson. 43, 59–281.
[10] de Ward, P., and J.F.G. Vliegenthart (1989) J. Biol. Chem. 264, 12141–12144.
[11] Ferguson, M.A.J., Homans, S.W., Dwek, R.A. and Rademacher, T.W. (1988) Science 239, 753–759.
[12] Sasaki, H., Bothner, B., Dell, A. and Fukuda, M. (1987) J. Biol. Chem. 262, 12059–12076.
[13] Takeuchi, M., Takasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N. and Kobata, A. (1988) J. Biol. Chem. 263, 3657–3663.
[14] Tsuda, E., Goto, M., Murakami, A., Akai, K., Ueda, M., Kawanishi, G., Takahashi, N., Sasaki, R., Chiba, H., Ishihara, H., Mori, M., Tejima, S., Endo, S. and Arata, Y. (1988) Biochemistry 27, 5646–5654.
[15] von Figura, K. and Hasilek, A. (1986) Annu. Rev. Biochem. 55, 167–193.
[16] Dustin, M.L., Baranski, T.I., Sampath, D., and Kornfeld, S. (1995) J. Biol. Chem. 270, 170–179.
[17] von Figura, K., Hille-Rehfeld, A., Lehmann, L., Peters, C. and Prill, V. (1993) in: Glyco- and Cellbiology, 44, Colloquium-Mosbach (Wieland, F. and Reutter, W. eds.) pp. 33–44, Springer-Verlag, Berlin/Heidelberg/New York.
[18] McKeehan, W.L. (1986) in: Carbohydrate Metabolism in Cells (Morgan, J. ed.) pp. 111–150, Plenum Press, New York.
[19] Ryll, T., Valley, U. and Wagner, R. (1994) Biotechnol. Bioeng., 40, 184–193.
[20] Gawlitzek, M., Valley, U., Nimtz, M., Wagner, R. and Conradt, H.S. (1995) in: Animal Cell Technology: Developments towards the 21st Century (Spier, R.E., Griffiths, J.B. and Murakami, H. eds.) Kluwer Academic Publishers, Dordrecht, The Netherlands, in press.
[21] Hasilik, A. and Neufeld, E. (1980) J. Biol. Chem. 255, 4937–4945.
[22] Horst, M., Mares, M., Zabe, M., Hummel, M., Wiederanders, B., Kirschke, H. and Hasilik, A. (1993) J. Biol. Chem. 268, 19690–19696.