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Rapid detection of the alternative *N*-glycosylation pathway using high pH anion exchange chromatography

C. VILLERS, Y. PLANCKE, R. CACAN and A. VERBERT
Laboratoire de Chimie Biologique, U.M.R. no. 111 du C.N.R.S., Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq, Cedex, France

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An alternative *N*-glycosylation pathway using $Glc_{1-3}Man_5GlcNAc_2$ as a donor to be transferred to a protein acceptor is found either in Man-P-Dol synthase deficient cells or in wild type CHO cells grown in energy deprivation conditions. Discrimination between oligomannosides of this alternative pathway and oligomannosides of the major one containing the same number of sugar residues $Man_{6-8}GlcNAc_2$ required structural studies. Taking advantage of the specific chromatographic behaviour of glucosylated oligomannosides, in pellicular high pH anion exchange chromatography, we developed a one-step method for the identification of the alternative *N*-glycosylation pathway compounds differing from those of the major one.

Keywords: HPAEC; alternative *N*-glycosylation pathway

Abbreviations: HPAEC, high pH anion exchange chromatography; endo H, endo beta *N*-acetylglucosaminidase H; PNGaseF, peptide *N*-glycosidase F; M2, $Man_2GlcNAc_2$; M4, $Man_4GlcNAc_2$; M5, $Man_5GlcNAc_2$; G1M5, $Glc_1Man_5GlcNAc_2$; G2M5, $Glc_2Man_5GlcNAc_2$; G3M5 $Glc_3Man_5GlcNAc_2$; M6, $Man_6GlcNAc_2$; M8, $Man_8GlcNAc_2$; M9, $Man_9GlcNAc_2$; G1M9, $Glc_1Man_9GlcNAc_2$; G2M9, $Glc_2Man_9GlcNAc_2$; G3M9 $Glc_3Man_9GlcNAc_2$.

Introduction

N-Glycosylation comprises the transfer *en bloc* of the oligosaccharide moiety linked to a lipid carrier on to a protein acceptor. This reaction is catalysed by an oligosaccharidyltransferase and occurs in the endoplasmic reticulum membrane system. It involves the kinetic adjustment of two metabolic pathways: synthesis of the glycan moiety afforded by the lipid donor ($Glc_3Man_9GlcNAc_2$-PP-Dolichol) and protein acceptor biosynthesis. In addition to the major pathway of glycoprotein biosynthesis ($Glc_{1-3}Man_9GlcNAc_2$, $Man_{5-9}GlcNAc_2$), the existence of an alternative pathway adding three species ($Glc_{1-3}Man_5GlcNAc_2$) has been demonstrated. This alternative pathway has been observed in cells deficient in the synthesis of Man-P-Dol, such as Thy^{-1} [1] or, in our case, B3F7 cells [2, 3]. There remains the problem of the rapid discrimination between glucosylated oligomannosides and non-glucosylated ones possessing the same number of sugar residues; for example, $Glc_3Man_5GlcNAc_2$ and $Man_8GlcNAc_2$. Amino bonded phase chromatography of oligomannose type oligosaccharides [4] does not allow clear identification of glycan moieties from the alternative pathway. Identification of these glycan moieties involves either the structural determination or the use of endo *N*-acetylglucosaminidase H for releasing *N*-glycans from protein, since $Glc_{1-3}Man_5GlcNAc_2$ is endo H resistant in contrast to $Man_8GlcNAc_2$ [5].

Pellicular anion exchange chromatography is becoming increasingly popular in the field of glycan analysis [6–8]. This powerful tool of investigation may be useful for monitoring metabolic events, as for example, the stepwise formation of mannose type oligosaccharides of the dolichol pathway. In this report, using pellicular anion exchange chromatography, we show that the chromatographic behaviour of glucosylated oligomannosides allows their rapid discrimination from non-glucosylated ones possessing the same number of sugar residues. Thus, a one-step chromatography demonstrates the presence or the absence of an alternative pathway in the cell line under study.

Materials and methods

Cells, cell culture and metabolic labelling

Chinese hamster ovary cells (wild type cells) and a mutant cell line (B3F7) were routinely cultured in monolayer in alpha minimum essential medium (MEM) with 10% fetal calf serum (Gibco laboratories, Grand Island, NY, USA) at

* To whom correspondence should be addressed.

34 °C using 10 cm culture dishes under 5% CO_2. The mutant cell line was a gift from Dr Sharon S. Krag (Johns Hopkins University, Baltimore, USA); these B3F7 cells are deficient in mannosyl pyrophosphodolichol synthase and they use an alternative glycosylation pathway involving a smaller mannosyl core residue with only five mannoses [2, 3]. This allows the preparation of alternative pathway standards.

Madin Darby bovine kidney (MDBK) cells were cultured in monolayer in alpha minimum essential medium with 10% fetal horse serum (Gibco laboratories, Grand Island, NY, USA) at 34 °C using 10 cm culture dishes under 5% CO_2. MDBK and wild type CHO cells possess the mannosyl pyrophosphodolichol synthase activity allowing the synthesis of $Man_9GlcNAc_2$ derivatives.

Cells were labelled 3 days after subculture (90% of confluency: 5–7 × 10^6 cells per dish) with 200 μCi [2-^{3}H] mannose (429 GBq mmol^{-1}) from Amersham International (Bucks, UK), 50 μCi ml^{-1} in alpha MEM containing 0.5 mM glucose and 10% dialysed fetal calf serum. Incubations were performed at 34 °C for the wild type CHO and MDBK cells and at 39 °C for the mutant B3F7 cells [9] in the presence of processing inhibitors. Castanospermine and 1-deoxymannojirimycin (Boehringer Mannheim, Mannheim, Germany) were added 30 min before labelling and were present throughout the incubation period at a final concentration of 50 and 100 μg ml^{-1} for castanospermine and 1-deoxymannojirimycin, respectively. After 1 h, the incubation medium was removed and the cell layer washed with ice-cold phosphate buffer saline. The sequential extraction was then achieved as previously described [9].

Preparation of the oligosaccharide standards

Radioactive standards were prepared after metabolic labelling of cells from lipid intermediates for non-glucosylated oligosaccharides and from glycoproteins for glucosylated oligosaccharides [9]. Wild type or mutant CHO cells and MDBK cells (5–7 × 10^6 cells per dish) were pulsed for 10 min with 200 μCi [2-^{3}H] mannose in the presence of 50 μg ml^{-1} castanospermine and 100 μg ml^{-1} 1-deoxymannojirimycin to avoid any processing.

After the extraction procedure, lipid donors were submitted to a mild acid hydrolysis by 0.1 N HCl in tetrahydrofuran for 2 h at 50 °C.

Glycoproteins obtained after sequential lipid extraction were digested overnight at room temperature with 0.3 mg of TPCK-treated trypsin (Sigma, St Louis, MO, USA), in 0.1 M ammonium bicarbonate pH 7.9 in a final volume of 0.3 ml. The glycan moiety linked to protein was cleaved by peptide N glycosidase F (Boehringer Mannheim, Germany) 0.5 U for an overnight incubation in 20 mM sodium phosphate pH 7.5, 50 mM EDTA, 50% glycerol, 0.02% sodium azide. The released oligosaccharide standards possess two *N*-acetylglycosamine residues at the reducing end.

Oligosaccharide moieties obtained from lipid intermediates or glycoproteins were desalted on a Bio-Gel P-2 column using 0.1 M acetic acid as solvent before HPLC analysis.

HPLC analysis

The amino derivatized column: Supelcosil LC-NH_2, 5 μm, was purchased from Supelco (Bellefonte, PA, USA). The gradient was acetonitrile:water from 70:30 to 50:50 at 1 ml min^{-1} for 80 min.

The HPLC system used for high pH separations consisted of a Dionex Bio-LC gradient pump and a CarboPac PA-100 column (4.6 × 250 mm). Elutions were carried out in 100 mM sodium hydroxide under gradient conditions using from 0 to 100 mM sodium acetate over 100 min at a flow rate of 1 ml min^{-1}. As the radiolabelling detection is about a hundred-fold more sensitive than the pulsed amperometric detection and, as we were interested in studying newly synthesized glycoproteins, detection has been achieved by in-line radioactivity detection rather than by pulsed amperometric detection. The detection of the radioactivity was followed with a continuous Flo-one beta detector (Flotec, France) using Luma flow II (Lumac, Netherlands) as the scintillation liquid with a ratio of scintillation liquid versus HPLC flow of 2:1.

Results

Figure 1 shows HPLC analysis of glycan moieties released by PNGase F digestion of short-time pulsed glycoproteins from wild type and mutant CHO cell lines. The separation obtained on an amino bonded column relied on the number of sugar residues rather than on a structural basis. Indeed, the identification of glycan moieties from wild type cell glycoproteins (panel a) is undetermined between $Glc_1Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ as well as between $Glc_3Man_5GlcNAc_2$ and $Man_8GlcNAc_2$. Incubation of wild type CHO cells has been achieved at low glucose concentration (0.5 mM). In glucose depleted culture conditions, the alternative *N*-glycosylation pathway was also observed: smaller oligosaccharides are transferred to protein acceptors and the cells are viable [10, 11]. Thus, we checked whether wild type cells (CHO and MDBK) use the alternative pathway in the presence of 0.5 mM glucose.

The oligosaccharide material obtained by metabolic labelling of wild and mutant CHO cells and of MDBK cells was analysed in HPAEC. As shown in Fig. 2a, the HPAEC analysis of glycan moieties from B3F7 glycoproteins reveals structures which correspond respectively to $Man_4GlcNAc_2$, $Man_5GlcNAc_2$, $Glc_1Man_5GlcNAc_2$, $Glc_2Man_5GlcNAc_2$ and $Glc_3Man_5GlcNAc_2$. In contrast to the amino bonded column for which the separation is based on the number of sugar residues (Fig. 1), in HPAEC, glucosylation affects the retention time of oligomannosides suggesting a possible resolution of glucosylated oligomannosides from non-glucosylated ones.

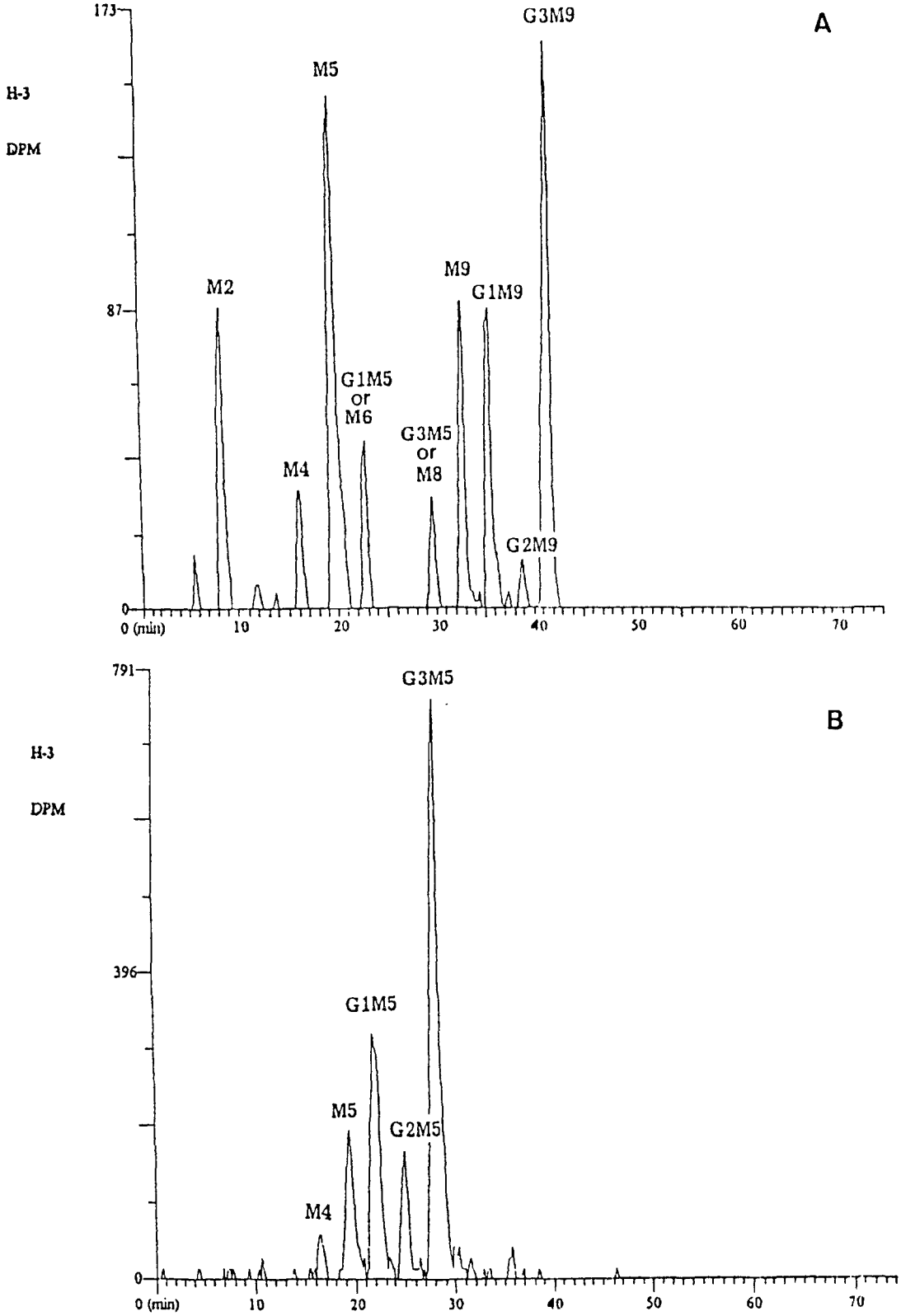

Figure 1. Amino bonded chromatography of glycan moieties from newly synthesized glycoproteins from CHO cell lines. Cells were incubated with [2-^{3}H] mannose in the presence of 0.5 mM glucose and processing inhibitors (castanospermine 50 μg ml^{-1} and 1-deoxymannojirimycin 100 μg ml^{-1}). Glycoproteins from wild type (panel A) and mutant B3F7 (panel B) cell lines were digested with PNGase F and glycans were analysed by amino bonded phase chromatography using a continuous detection of the radioactivity as described in the Materials and methods section. See abbreviations for details of M_2, M_4, M_5, M_6, M_8, M_9, G_1M_5, G_2M_5, G_3M_5, G_1M_9, G_2M_9 and G_3M_9.

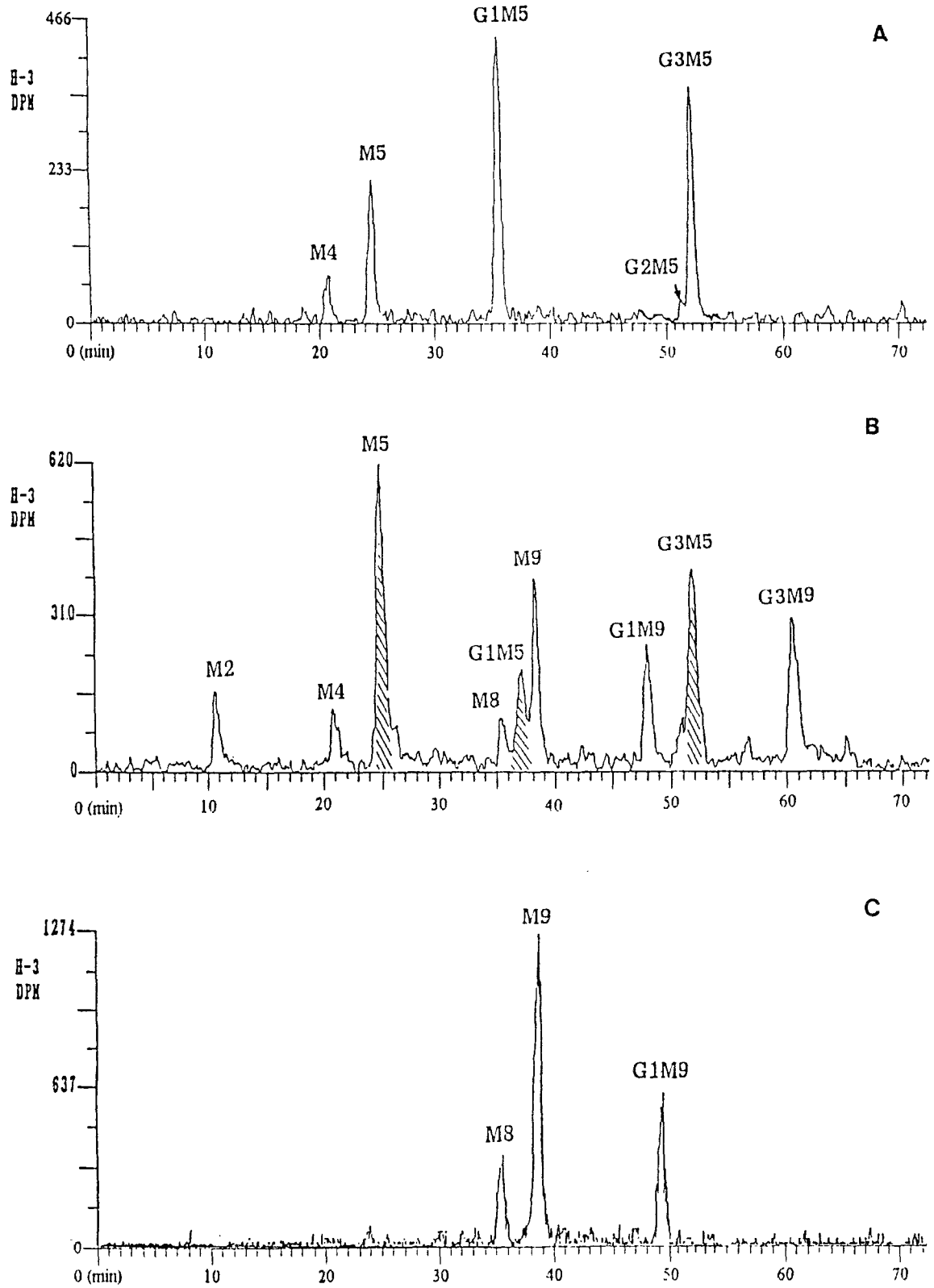

Figure 2. HPAEC of glycans from newly synthesized glycoproteins from CHO and MDBK cell lines. Cells were incubated with [2-^{3}H] mannose in the presence of 0.5 mM glucose and processing inhibitors (castanospermine 50 μg ml^{-1} and 1-deoxymannojirimycin 100 μg ml^{-1}). Glycoproteins were extracted and digested by PNGase F. HPAEC analysis and detection mode of the radioactivity were as described in the Materials and methods section. *Panel A*: Mutant B3F7 cells. *Panel B*: Wild type CHO cells. Peaks from the alternative pathway of *N*-glycosylation are represented as hatched area. *panel C*: MDBK cells. See Abbreviations for details of M_2, M_4, M_5, M_8, M_9, G_1-, G_2- and G_3M_5, G_1-, G_2- and G_3M_9.

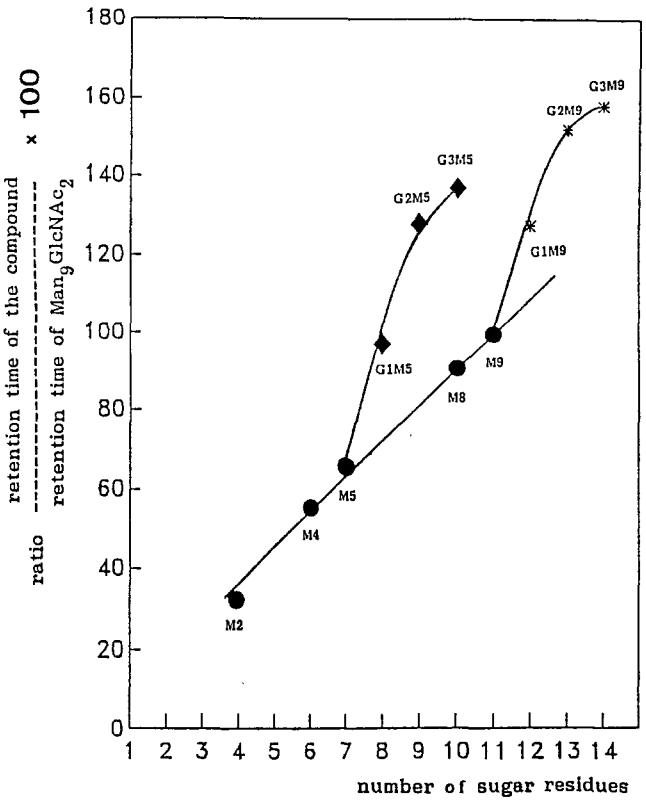

Figure 3. Influence of glucose residues on high mannose oligosaccharides retention time using HPAEC. The diagram represents data from the library constituted by glycan moieties from mutant and wild type cells. The plot is the ratio of retention time of oligomannose oligosaccharide ($\times 100$) ($Man_9GlcNAc_2$ taken as a reference) versus the number of sugar residues. (●) M_2, M_4, M_5 are produced from oligosaccharides isolated from lipid intermediates and M_8, M_9 are derived from wild type CHO glycoproteins. (◆) $G_{1-3}M_5$ are derived from B3F7 glycoproteins. (*) $G_{1-3}M_9$ are derived from wild type CHO glycoproteins. See Abbreviations for details of M_2, M_4, M_5, M_6, M_8, M_9, G_1M_5, G_2M_5, G_3M_5, G_1M_9, G_2M_9 and G_3M_9.

Figure 2b shows an analysis of wild type CHO cell glycoproteins obtained after metabolic labelling at low glucose concentration. We observed the alternative way of glycosylation together with the major one. HPAEC allows the discrimination of the alternative pathway derivatives from Man_5- to $Glc_{1-3}Man_5GlcNAc_2$ among the major derivatives from Man_9- to $Glc_{1-3}Man_9GlcNAc_2$. This indicates that donor oligosaccharides from the two pathways can be simultaneously transferred to cellular proteins. Thus, the composition of glycans of glycoproteins obtained after incubation at low glucose concentration has to be checked since it depends on culture conditions and also on cell type. For example, Fig. 2c shows an analysis obtained with MDBK cells glycoproteins when incubated with [2-^{3}H] mannose in the presence of 0.5 mM glucose and processing inhibitors as described in the Materials and methods section. With MDBK cells, no derivatives from the alternative pathway of *N*-glycosylation were obtained: $Man_8GlcNAc_2$ instead of $Glc_3Man_5GlcNAc_2$ is recovered on glycoproteins.

Figure 3 shows the chromatographic behaviour of glycan moieties of lipid intermediates and glycoproteins from mutant and wild type cells. This gives data showing the influence of glucose residues on oligomannose oligosaccharide retention times. The two curves distinguish clearly the two metabolic pathways: the $Glc_{1-3}Man_5GlcNAc_2$ series whch parallels the $Glc_{1-3}Man_9GlcNAc_2$ series. The glucosylation of the $Man_9GlcNAc_2$ and $Man_5GlcNAc_2$ compounds induces a well-defined shift of the retention times.

Discussion

The main feature of the mapping method proposed here is the clear separation of the oligomannosides involved in the dolichol pathway in one single chromatographic run. This allows a method of quantifying clearly any one of more than fifteen compounds from the glycosylation pathway.

The alternative *N*-glycosylation pathway was observed as the only way of *N*-glycosylation for cells such as Thy^{-1} or B3F7 which are deficient in Man-P-Dol synthase [1, 9]. This alternative pathway has been described in CHO cell line when incubated in energy deprivation conditions such as low glucose concentration [10, 11]. The identification of $Glc_1Man_5GlcNAc_2$ to $Glc_3Man_5GlcNAc_2$ in wild type CHO cells using amino bonded phase chromatography requires the action of endo *N*-acetylglucosaminidase H to be differentiated from $Man_6GlcNAc_2$ to $Man_8GlcNAc_2$. HPAEC allowed us to demonstrate the presence of such a pathway in wild type CHO cells with only one run.

It is interesting to note that glucosylation affects the retention times of both $Man_9GlcNAc_2$ and $Man_5GlcNAc_2$ compounds. It has been reported that the three glucose residues bound to oligosaccharide lipids could modulate the activity of the oligosaccharidyltransferase [12]. In addition, it has been observed that the removal of the two external glucose residues by glucosidases I and II is easier than that of the internal glucose residue although it requires the action of the same glucosidase II. This difference could be due to a change in the conformation. It has been reported that the two glucose residues added to $Glc_1Man_5GlcNAc_2$ lead to the $Glc_3Man_5GlcNAc_2$ structure which adopts an helical configuration [13] which could modify the glucosidase action. This conformational change induced by glucosylation could involve a stronger interaction with the pellicular exchange resin. This indicates that, in contrast to amino bonded chromatography, conformational changes can be detected by HPAEC.

Moreover, this chromatography may be useful to check a mutant reversion or any change in cell differentiation, especially for cell batch and continuous large scale production. Using this potential, it is possible to study the

metabolism of the cell during kinetic or pulse-chase experiments in different culture conditions, as is required for monitoring the glycosylation of recombinant glycoproteins.

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References

1. Chapman A, Fujimoto K, Kornfeld S (1980) *J Biol Chem* **255**:4441–46.
2. Stoll J, Krag SS (1983) *J Cell Biol* **97**:443a.
3. Stoll J (1986) PhD Thesis. School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland, USA.
4. Turco SJ (1981) *Anal Biochem* **118**:278–83.
5. Turco SJ, Stetson B, Robbins PW (1977) *Proc Natl Acad Sci USA* **74**:4411–14.
6. Hernandez LM, Ballou L, Ballou CE (1990) *Carbohydr Res* **203**:1–11.
7. Hermentin P, Witzel R, Vliegenthart JFG, Kamerling JP, Nimtz M, Conradt HS (1991) *Anal Biochem* **203**:281–89.
8. Nimtz M, Martin W, Wray V, Kloppel KD, Augustin J, Conradt HS (1993) *Eur J Biochem* **213**:39–56.
9. Cacan R, Villers C, Bélard M, Kaiden A, Krag S, Verbert A (1992) *Glycobiology* **2**:127–36.
10. Rearick JI, Chapman A, Kornfeld S (1981) *J Biol Chem* **256**:6255–61.
11. Chapman AE, Calhoun JC (1988) *Arch Bioch Biophys* **260**:320–33.
12. Maley F, Trimble RB, Tarentino AL, Plummer TS, Jr (1989) *Anal Biochem* **180**:195–204.
13. Alvarado E, Nukada T, Ogawa T, Ballou CE (1991) *Biochemistry* **30**:881–86.