The unfolded protein response in a dolichyl phosphate mannose-deficient Chinese hamster ovary cell line points out the key role of a demannosylation step in the quality-control mechanism of N-glycoproteins

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The CHO (Chinese hamster ovary) glycosylation mutant cell line, B3F7, transfers the truncated glycan $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ on to nascent proteins. After deglycosylation, the resulting $\text{Man}_5\text{GlcNAc}_2$ glycan is subjected to two reciprocal enzymic processes: the action of an endoplasmic-reticulum (ER) kifunensine-sensitive α 1,2-mannosidase activity to yield a $\text{Man}_4\text{GlcNAc}_2$ glycan, and the reglucosylation involved in the quality-control system which ensures that only correctly folded glycoproteins leave the ER. We show that the recombinant secreted alkaline phosphatase (SeAP) produced in stably transfected B3F7 cells, is co-immunoprecipitated with the GRP78 (glucose-regulated protein 78), a protein marker of the unfolded protein response

(UPR). The level of GRP78 transcription has been evaluated by reverse transcription-PCR (RT-PCR) and we demonstrate that B3F7 cells present a constitutively higher level of UPR in the absence of inducers, compared with Pro⁻⁵ cells. Interestingly, a decrease was observed in the UPR and an increase in SeAP secretion in the kifunensine-treated B3F7 cells. Altogether, these data highlight the relationships between the glycan structure, the quality control system and the UPR. Moreover, they support the idea that a specific demannosylation step is a key event of the glycoprotein quality control in B3F7 cells.

Key words: mannosidase activity, N-glycosylation.

INTRODUCTION

The normal process of N-glycosylation corresponds to the transfer *en bloc* of the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide chain on an asparagine residue of a nascent protein. This process takes place in the endoplasmic reticulum (ER) in which newly synthesized glycoproteins encounter a favourable folding environment, essential for the acquisition of their biological properties. This environment comprises enzymes [e.g. the peptidyl disulphide-isomerase (PDI)], molecular chaperones including GRP78 (glucose-regulated protein 78) and lectin-like chaperones such as calnexin (CNX) or calreticulin (CRT) [1]. Moreover, a quality-control system which ensures that only the correctly folded glycoproteins leave the ER is mediated by the soluble UDP-Glc: glycoprotein glucosyltransferase (UGGT) and glucosidase II, resident of the ER. This first enzyme recognizes hydrophobic patches on incorrectly folded or unassembled glycoproteins and, as a consequence, adds a glucose residue on the outermost mannose residue of the α 1,3 arm of oligomannoside glycans. Resulting monoglucosylated glycoproteins can then interact specifically with molecular chaperones CNX or CRT in a folding complex with ERp57 (a PDI-like protein) in order to correct their folding. The removal of the glucose residue by glucosidase II allows the release of glycoproteins from the folding complex. UGGT, which serves as a folding sensor, detects the still misfolded glycoproteins which undergo a new gluco-degluco cycle (for a review, see [2]). Finally, the glycoproteins that fail to acquire a correct folding are directed into the ER-associated degradation (ERAD) pathway [3]. An ER α 1,2-mannosidase, probably the ER mannosidase I, has been shown to play a

pivotal role in this pathway [4–7]. However, there is very little information concerning the relationships between the glycan structure, the quality-control system and the secretion of correctly folded glycoproteins [8].

Accumulation of unfolded proteins in the ER lumen induces a specific response named UPR (unfolded protein response) [9]. This response leads to the transcriptional activation of numerous genes (more than 350 in yeast), most of which encode factors involved in the folding process, such as molecular chaperones or folding enzymes [10], and also in the ERAD process [11,12].

The relationships between the glycan structure and the UPR have been well documented in yeast. Indeed, it has been shown in some glycosylation mutants which transfer truncated glycans on to their nascent glycoproteins that an UPR was triggered in the absence of exogenous stimuli [13]. Conversely, direct relationships between glycan structure and UPR have never been demonstrated in mammalian cells, since the appearance of truncated glycan chains on glycoproteins was always the result of exogenous stimulations such as long-term glucose starvation, or the use of inhibitors of either glycosylation (tunicamycin) or glycan processing (e.g. castanospermine) [14].

In the dolichyl phosphate mannose (Dol-P-Man)-deficient Chinese hamster ovary (CHO) mutant cell line B3F7, a truncated $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ oligosaccharide is transferred on to nascent protein chains [15]. Indeed, the newly transferred $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ ($\text{Glc}\alpha 1-2\text{Glc}\alpha 1-3\text{Glc}\alpha 1-3\text{Man}\alpha 1-2\text{Man}\alpha 1-2\text{Man}\alpha 1-3$ [$\text{Man}\alpha 1-6$] $\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}$) is rapidly trimmed by glucosidases I and II [16]. We have recently demonstrated that the generated $\text{Man}_5\text{GlcNAc}_2$ glycans can be either reglucosylated to yield $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$, which is able to interact

Abbreviations used: Ap-Ab, antiserum against human placental alkaline phosphatase; CHO, Chinese hamster ovary; CNX, calnexin; CRT, calreticulin; Dol-P-Man, dolichyl phosphate mannose; ER, endoplasmic reticulum; ERAD, endoplasmic-reticulum-associated degradation; GRP78, glucose-regulated protein 78; α -MEM, α -minimal essential medium; PDI, peptidyl disulphide-isomerase; PNGase F, peptide N-glycanase F; pNPP, p-nitrophenyl phosphate; PTA, phosphotungstic acid; SeAP, secreted alkaline phosphatase; UGGT, UDP-Glc: glycoprotein glucosyltransferase; UPR, unfolded protein response.

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Scheme 1 Processing of oligomannoside bound to glycoproteins in B3F7 cells

In the Dol-P-Man-deficient CHO cell line B3F7, the truncated $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ oligosaccharide which is transferred *en bloc* on to nascent proteins is rapidly deglycosylated to yield the $\text{Man}_5\text{GlcNAc}_2$ glycan. This glycan structure undergoes two reciprocal enzymic actions: (i) the action of a kifunensine-sensitive ER $\alpha 1,2$ -mannosidase activity to generate the $\text{Man}_4\text{GlcNAc}_2$ glycan which cannot be further reglucosylated, and (ii) the reglucosylation by the UGGT to generate $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$ glycans involved in the quality control system. (■, GlcNAc; ●, Man; ◀, Glc and UGGT).

with calnexin [17,18], or are the substrate of a kifunensine-sensitive ER $\alpha 1,2$ -mannosidase activity yielding $\text{Man}_4\text{GlcNAc}_2$ glycans, unable to be reglucosylated [19] (see Scheme 1).

In the present paper we show that, in these glycosylation mutant CHO B3F7 cells, the UPR is constitutively activated compared with the parental CHO cell line, Pro⁻⁵. More interestingly, we demonstrate that, in kifunensine-treated B3F7 cells, the level of the UPR was decreased and the secretion of glycoproteins increased.

EXPERIMENTAL

Materials

[2-³H]Mannose (429 GBq/mmol) and radioactive [³⁵S]Met/Cys mixture (Promix; 37 TBq/ml, 1000 Ci/mol) were from Amersham (Little Chalfont, Bucks., U.K.). Trypsin and p-nitrophenyl phosphate tablets (pNPP) were from Sigma. Peptide N-glycanase F (PNGase F) was from New England Biolabs, Beverly, MA, U.S.A., Kifunensine was from ICN, Orsay, France, and protease-inhibitor-cocktail tablets were from Roche Diagnostics GmbH, Mannheim, Germany and the $\alpha 1,2$ -mannosidase (from *Aspergillus saitoi*) was from Glyko, Novato, CA, U.S.A. Promega, Charbonnière-les-bains, France, supplied dNTPs. Polyclonal anti-serum against human placental alkaline phosphatase (AP-Ab) was purchased from Dako, Glostrup, Denmark, and was coupled directly to Protein A-Sepharose CL4B beads (Pharmacia). Polyclonal antibody against GRP78 was from Stressgen, Biotechnologies Corp., Victoria, BC, Canada. Goat anti-rabbit IgG conjugated to horseradish peroxidase was from Dako, Glostrup, Denmark. Hi-Taq DNA polymerase and RNeasy Mini Kit were from Qiagen GmbH, Hilden, Germany, and First-strand cDNA synthesis kit was from Amersham Pharmacia Biotech. The oligonucleotide primers were synthesized and purified from Eurogentec, Liège, Belgium.

Cell culture

The Dol-P-Man synthase-deficient mutant CHO cell line (B3F7), derived from parental Pro⁻⁵ (proline auxotroph) CHO cell line was a gift from Dr S. S. Krag (Biochemistry Department, School of Hygiene and Public Health, Johns Hopkins University,

Baltimore, U.S.A.). The AP2-1 cell line was derived from the B3F7 cell line, which was stably transfected by electroporation with plasmid pBC12RSVSeAP encoding a secreted truncated form of human placental alkaline phosphatase (SeAP) [18]. The MadIA214 cell line was a gift from Dr Myriam Ermonval (Institut Pasteur, Paris, France), the Mi8-5 cell line was a gift of Dr S. S. Krag and the Lec23 cell line was a gift from Dr Pamela Stanley (Department of Cell Biology, Albert Einstein College of Medicine, New York, U.S.A.). All these cell lines were grown in the α -minimal essential medium (α -MEM) (Gibco BRL) supplemented with 10% (v/v) fetal-calf serum, at 34 °C under 5% CO_2 .

Protein labelling, immunoprecipitation and gel electrophoresis

Cells (2.5×10^5 per 35 mm-diameter dish) were cultured overnight at 34 °C. Following a pre-incubation period of 45 min in a methionine/cysteine-free medium, cells were pulsed for 30 min with 100 $\mu\text{Ci/ml}$ (0.1 μM) of [³⁵S]Met/Cys. After 2 h of chase in 500 μl of cell culture medium containing unlabelled methionine and cysteine to a final concentration of 14 mg/l and 100 mg/l respectively, supernatant was removed and cells were lysed in NET buffer (150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 5 mM EDTA and 1% Triton X-100). Cell extracts were then immunoprecipitated for 30 min at 4 °C using 50 μl of anti-AP-Ab solution previously mixed with 50 μl of Protein A-Sepharose CL-4B beads. After incubation for 1 h at 4 °C, beads were washed four times with 1 ml of NET buffer containing 0.5 M NaCl followed by two washes with a 40 mM Hepes buffer (pH 7.4) prior to SDS/PAGE analysis. For this purpose, immunoprecipitates were boiled in 25 μl of 10% SDS/1 M 2-mercaptoethanol/25 mM Tris/HCl, pH 8.0, buffer solution and subjected to SDS/PAGE for 4 h. Hyperfilms (Amersham) were used for autoradiography of intensified gels. Unless otherwise indicated, an inhibitor of glycan processing, kifunensine, at a final concentration of 20 μM , was added 45 min before pulse-labelling and throughout the labelling and chase period.

Metabolic labelling of oligosaccharides and chase experiments

For this purpose, B3F7 cells were routinely grown in 10 cm-diameter Petri dishes. Cells were metabolically labelled for 1 h with 100 $\mu\text{Ci/ml}$ (4 μM) of [2-³H]mannose in the culture medium containing 5 mM glucose. For chase experiments, the radioactive culture medium was replaced by the α -MEM supplemented with 5 mM mannose. When used, kifunensine was present throughout the experiment (preincubation, pulse and chase) at a final concentration of 20 μM . Sequential extraction and purification of oligosaccharide materials were achieved as previously described [20].

Analysis of oligosaccharide material

The glycoprotein fraction obtained at the end of the sequential extraction was digested with trypsin (1 mg/ml) in 0.1 M ammonium bicarbonate buffer, pH 7.9, overnight at room temperature. Glycopeptides were then treated with 0.5 unit of peptide-N-glycanase F (PNGase F) in a 50 mM phosphate buffer, pH 7.2, for 4 h to release oligosaccharides, which were subsequently desalted on a Bio-Gel P2-size exclusion chromatography column eluted with a 5% (v/v) acetic acid solution. Then they were fractionated by HPLC (apparatus from Spectra-Physics GmbH, Darmstadt, Germany) on an amino-derivatized Asahipak NH2P-50 (250 mm $\times$ 4.6 mm) column (Asahi, Kawasaki-ku, Japan) with a solvent system of acetonitrile/water from 70:30 to 50:50 (v/v) at a flow rate of 1 ml/min over 80 min. Oligomanno-

sides were identified on the basis of their retention times compared with well-defined standards. Standard oligomannosides with two GlcNAc residues at their reducing end were obtained after PNGase F digestion of glycoprotein fractions from cells labelled with [^3H]mannose. $\text{Man}_3\text{GlcNAc}_2$ was obtained from SF9 (*Spodoptera frugiperda*) insect cells [21]. $\text{Man}_1\text{GlcNAc}_2$ was isolated after extensive digestion of $\text{Man}_3\text{GlcNAc}_2$ with jack-bean (*Canavalia ensiformis*) mannosidase according to [21]. $\text{Man}_4\text{GlcNAc}_2$, $\text{Man}_5\text{GlcNAc}_2$, and $\text{Glc}_{(1-2)}\text{Man}_5\text{GlcNAc}_2$ were obtained after incubation of Lec 15 CHO cells as described in [19]. $\text{Man}_8\text{GlcNAc}_2$, $\text{Man}_9\text{GlcNAc}_2$ and $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ were obtained from Mi8-5 cells [22]. $\text{Glc}_3\text{Man}_8\text{GlcNAc}_2$ and $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ were obtained from Lec23 CHO cells [23]. Elution of the radiolabelled oligosaccharides was monitored by continuous-flow detection of the radioactivity with a Flo-One β Detector (Packard, Rungis Cedex, France).

Western blot analysis of the GRP78

At confluence, the B3F7 and Pro $^{-5}$ cells were lysed in the lysis buffer (Tris/HCl, pH 7.5, and 1% Triton X-100) and protein concentration was measured using the BCA (bicinchoninic acid) Kit (Interchim, Montlucon, France). A 20 μg portion of protein per lane was loaded on to SDS/PAGE gels. After the gel run was complete, proteins were electrotransferred to nitrocellulose and visualized by Ponceau Red staining (Sigma). The nitrocellulose membranes were blocked in 2% gelatin (Sigma) in TBS buffer (15 mM Tris/HCl, pH 8, 140 mM NaCl and 0.05% Tween 20). The proteins were detected by using the polyclonal anti-GRP78 antibody as the primary antibody (1/500 in TBS buffer) and a goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 in TBS buffer). The immunoreactive bands were detected by chemiluminescence using an ECL $^{\text{®}}$ (enhanced chemiluminescence) kit from Amersham Pharmacia (Uppsala, Sweden).

Quantification of secreted glycoproteins

B3F7 cells were pulsed for 30 min with 100 $\mu\text{Ci}/\text{ml}$ (0.1 μM) of [^{35}S]Met/Cys following a pre-incubation for 15 h in the absence or presence of 20 μM kifunensine. Cells were then chased for 6 h and, every hour, culture medium was precipitated by 0.6% phosphotungstic acid (PTA) in 12% (v/v) trichloroacetic acid. After 30 min at 4 $^{\circ}\text{C}$, precipitates were recovered on a glass filter, and successively washed with 10% trichloroacetic acid, water and ethanol. Filters were then dried and counted for radioactivity by liquid scintillation using a Beckman scintillation counter. The enzymic activity of SeAP was determined as previously described [24].

Figure 1 Effect of kifunensine on the processing of glycoproteins in B3F7 cells

(A) AP2-1 cells were pulse-labelled for 15 min with [^{35}S]Met/Cys mixture and chased for 2 h with unlabelled amino acids without (lane 1) or with 20 μM kifunensine (lane 2). At the end of the chase period, cells were lysed in a total volume of 200 μl and the SeAP was immunoprecipitated. In lane 3, a mixture of immunoprecipitated SeAP synthesized in the presence or the absence of kifunensine was pooled, and digested by $\alpha 1,2$ -mannosidase (*A. saitoi*) prior to analysis by SDS/PAGE and autoradiography. (B) AP2-1 was pulse-labelled for 1 h with [^3H]mannose and chased for 2 h in the presence or in the absence of 20 μM kifunensine. Glycoproteins were extracted and the N-glycan species were released with the PNGase F prior to being analysed by HPLC as described in the Experimental section. Oligomannosides were identified on the basis of their retention times compared with standards. M1, M3, M4, M5 and G1M5, G2M5 correspond to $\text{Man}_1\text{GlcNAc}_2$, $\text{Man}_3\text{GlcNAc}_2$, $\text{Man}_4\text{GlcNAc}_2$, $\text{Man}_5\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_5\text{GlcNAc}_2$ and $\text{Glc}_2\text{Man}_5\text{GlcNAc}_2$ respectively. M8, M9, G1M9, G3M8 and G3M9 correspond to $\text{Man}_8\text{GlcNAc}_2$, $\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_3\text{Man}_8\text{GlcNAc}_2$ and $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ respectively.

Figure 2 Kinetics of the PCR amplification of GRP78 and β -actin mRNAs isolated from B3F7 and Pro⁻⁵ cells

For each experiment, the PCR products obtained as described in the Experimental section were run on a 1% agarose gel, stained with ethidium bromide (**A**) and quantified (**B**). Arrows indicate the cycle number chosen for relative quantitative RT-PCR (20 cycles for GRP78 and β -actin).

RNA extraction, cDNA synthesis and reverse transcription-PCR (RT-PCR)

Total RNA was extracted from B3F7 or Pro⁻⁵ cells using the RNeasy Mini Kit and quantified by spectrophotometry at 260 nm.

Total cellular RNA (5 μ g) was denatured at 65 °C for 10 min and placed on ice for 2 min. RT into cDNA was achieved using the First-Strand cDNA Synthesis kit, according to the manufacturer's protocol. Oligonucleotide primer pairs corresponding to *Cricetulus griseus* GRP78 and β -actin nucleotide sequences found in a public domain database (Genbank® accession number M97169 and U20114 respectively) were designed for the specific PCR amplification of two cDNA fragments of 399 bp and 265 bp respectively. In addition, these primers were designed such that they could be used under identical PCR conditions [i.e. similar GC content and T_m (melting temperature) values]. Of each primer (GRP78 sense primer 5'-CGCCTCATCGG-ACGCACTTG-3', GRP78 antisense 5'-AGGTTCCACCGCC-CAGGTCA-3', β -actin sense 5'-GGCCGTCTTCCCATCCA-TCG-3', β -actin antisense 5'-CACCCTTGGCCTTGGGGTTC-3') 150 pmol was combined with 0.7 unit of Hi-Taq DNA polymerase, 100 μ M dNTPs, in the Hi-Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris/HCl, pH 9, 0.1 % TritonX-100, 2.5 mM MgCl₂ and 150 ng of the first-strand cDNA) in a 50 μ l reaction volume. PCR was performed as follows: after 1.5 min of a denaturation step at 95 °C, a variable number of cycles were achieved at 93 °C for 30 s, 66 °C for 30 s and 72 °C for 45 s, followed by a 10 min extension step at 72 °C. In all experiments,

negative control reactions were done in which cDNA templates were replaced with sterile water. A 10 μ l portion of amplification products were size-separated on a 1% agarose gel containing ethidium bromide, visualized under UV light, photographed using Polaroid film and analysed using Quantiscan Software (Bio-Rad, Hemel Hempstead, Herts., U.K.).

RESULTS

Processing of glycoproteins in B3F7 cells

In order to study the role of the kifunensine-sensitive α 1,2-mannosidase activity in the *N*-glycosylation process of B3F7 cells, a reporter recombinant glycoprotein SeAP, stably expressed in these cells (named AP2-1), was used as a model. As observed in Figure 1(A), the electrophoretic mobility study of intracellular SeAP in the absence or presence of kifunensine shows that, in the control (lane 1), SeAP migrates as a single band (approx. 66 kDa) whereas, when cells were treated with 20 μ M kifunensine, the migration was slightly reduced (lane 2). To analyse the oligomannosides bound to proteins, AP2-1 cells were pulse-labelled with [2-³H]mannose as described in the Experimental section and chased for 2 h. HPLC analysis (Figure 1B) reveals a major peak of Man₅GlcNAc₂ in the presence of kifunensine compared with the control incubation, for which the major peak was represented by Man₄GlcNAc₂ species.

A mixture of control SeAP bearing Man₄GlcNAc₂ species plus SeAP synthesized in the presence of kifunensine-bearing Man₅GlcNAc₂ species was treated *in vitro* with the α 1,2-manno-

Figure 3 Transcriptional and transductional levels of GRP78 expression in B3F7 cells are higher than those in the parental Pro⁻⁵ cells

(A) Multiplex RT-PCR analysis of GRP78 and β -actin mRNA expression in B3F7 and Pro⁻⁵ cells cultured under physiological conditions in the absence (–) or in the presence (+) of 10 μ g/ml of tunicamycin (Tu) during 8 h. RNA was then extracted and amplified by 20 cycles of PCR. (B) Signals obtained from three separate experiments were integrated by Quantiscan software. The relative GRP78 level is expressed as the ratio of the densitometric data GRP78/ β -actin, which is arbitrarily set to 1 in the Pro⁻⁵ cells treated in the absence of tunicamycin. (C) Western-blotting analysis of GRP78 protein after electrophoresis of 20 μ g of protein of B3F7 and Pro⁻⁵ cells respectively as described in the Experimental section.

sidade from *A. saitoi*. As shown in Figure 1A (lane 3), autoradiography revealed a single band with a higher electrophoretic mobility. As already demonstrated [19], this treatment led to the formation of Man₃GlcNAc₂ glycans from both Man₅GlcNAc₂ and Man₄GlcNAc₂ species. This result allowed us to conclude that, indeed, the difference in the electrophoretic migration previously observed between lane 1 and 2 was due to the loss of one α 1,2-linked mannose residue. Altogether, these data indicated that, after 2 h chase, the oligomannoside species bound to SeAP were mainly Man₄GlcNAc₂.

Interestingly, the electrophoregram (Figure 1A, lane 1) also revealed that SeAP was co-immunoprecipitated with a protein with an apparent molecular mass of 78 kDa which has been clearly identified as the GRP78 [18,24]. Moreover, this co-immunoprecipitation was not observed when cells were incubated in the presence of kifunensine (Figure 1A, lane 2). Since it is well established that the GRP78 is one of the major molecular chaperones produced during the UPR [25], we decided to examine first the level of UPR in B3F7 cells and to compare it with that of parental Pro⁻⁵ cells and then the consequence of the inhibition of the mannose trimming on this response.

Figure 4 Newly synthesized glycoproteins in B3F7 are not underglycosylated

AP2-1 cells (lane 1 and 2) and MadIA214 cells (lanes 3 and 4) were metabolically labelled with [³⁵S]Met/Cys amino acids for 15 min. After cell lysis with 1% Triton, the total SeAP was immunoprecipitated from the cell lysates, digested with (lane 2 and 4) or without (lane 1 and 3) PNGase F and analysed using SDS/10% PAGE. 0G, 1G and 2G correspond to non-, mono- and diglycosylated forms of SeAP respectively.

The UPR occurs in B3F7 cells at a higher level than in the parental Pro⁻⁵ cells

The expression of GRP78 mRNA was estimated in B3F7 cells by semi-quantitative RT-PCR [26] and compared with that of the parental Pro⁻⁵ cells. The optimal number of PCR cycles was determined in order to work in the linear phase of amplification. To avoid variation from one tube to another, the amplification of GRP78 mRNA was simultaneously carried out with that of β -actin mRNA used as an internal control. Electrophoregrams (Figure 2A) and densitometry graphs (Figure 2B) indicate that the optimum number of cycles for GRP78 and β -actin PCR co-amplification ranged from 18 to 23 cycles. Thus 20 PCR cycles were chosen. Under these conditions it clearly appears that the expression level of GRP78 mRNA is higher in B3F7 cells than that observed in Pro⁻⁵ cells (compare Figure 3A, lanes 2 and 3). On the basis of β -actin level, densitometric analysis revealed that the expression of GRP78 in B3F7 cells was 2.5-fold higher compared with that in Pro⁻⁵ cells (Figure 3B, control). As a positive control, the two cell lines were treated for 8 h with tunicamycin, which is known to trigger a strong UPR. Figure 3A (lanes 1 and 4) shows that, in both cell lines, a high transcription level of GRP78 was induced by this inhibitor (a 2-fold increase compared with the basal level in both cell lines; Figure 3B).

Taken together, these results indicate that the UPR pathway was constitutively higher in the Man-P-Dol synthase-deficient cell line B3F7, in the absence of exogenous stimulation. The fact that this transcriptional response was accompanied by an increase of protein synthesis was demonstrated by Western blotting using a polyclonal GRP78-specific antibody. Figure 3(C) shows an increase of GRP78 protein in B3F7 cells compared with Pro⁻⁵ cells. The densitometric analysis revealed that the expression of GRP78 protein in B3F7 cells is 2-fold higher compared with that in Pro⁻⁵ cells.

Glycoproteins synthesised in B3F7 cells are not underglycosylated

The increase of UPR observed in B3F7 cells reflects an increase in misfolded newly synthesized glycoproteins. In our case, this response could be explained either by the influence of truncated glycans on the protein folding process, or by an underglycosylation due to a lower affinity of the oligosaccharyltransferase for truncated oligosaccharides, as already reported for MadIA214 cell line, another glycosylation mutant which transfers only non-glycosylated Man₃GlcNAc₂ glycans [24]. To verify that glycopro-

Figure 5 Action of kifunensine on GRP78 mRNA expression in B3F7 cells

(A) The B3F7 cells were incubated in the absence (Control) or in the presence of 20 μ M kifunensine for 15 h. Total RNA was extracted and amplified by 20 cycles of relative quantitative RT-PCR. The PCR products were run on 1% agarose gel, ethidium bromide-stained and signals obtained from three individual experiments were integrated by Quantiscan Software. (B) The transcription level of GRP78 mRNA was established as described in Figure 3. The densitometric data GRP78/ β -actin was arbitrarily set to 1 in the B3F7 cells treated in the absence of kifunensine.

teins are not underglycosylated, we used SeAP, which possesses two *N*-glycosylation sites. The intracellular SeAP was then immunoprecipitated after a 15 min pulse in the presence of [35 S] Met/Cys, and analysed by SDS/PAGE. In contrast with the two bands of SeAP produced in MadIA214 (Figure 4, lane 3), which corresponds to the two glycoforms with one and two glycans respectively [18], SeAP produced by AP2-1 cells revealed only one band, corresponding to the fully glycosylated glycoproteins (Figure 4, lane 1). To verify that all these bands corresponded to different extents of glycosylation, immunoprecipitated SeAP produced in both AP2-1 and MadIA214 cells was treated with PNGase F prior to the analysis by SDS/PAGE. In both cases (Figure 4, lanes 2 and 4), the resulting SeAP molecules exhibit an identical electrophoretic mobility in accordance with the lack of their glycan chains.

Kifunensine decreases the UPR and increases the secretion of SeAP in B3F7 cells

As depicted in Figure 1(A), lane 2, GRP78 did not co-immunoprecipitate with SeAP when cells were treated with kifunensine. To ascertain whether this absence was due to a decrease in UPR, B3F7 cells were cultured for 15 h with 5 mM glucose in the absence or presence of kifunensine, and the level of GRP78 mRNA was analysed using the RT-PCR technique previously described. As shown in Figure 5, GRP78 transcript expression was lower in kifunensine-treated cells. Densitometric analysis indicates a 2.5-fold decrease of GRP78 mRNA in the presence of 20 μ M kifunensine. This effect was not detectable in the parental

Figure 6 Effect of kifunensine on the SeAP secretion

(A) AP2-1 cells were preincubated for 15 h in α -MEM culture medium in the absence (□) or presence (■) of 20 μ M kifunensine. Culture medium was then recovered at various times to determine the enzymic activity of the secreted SeAP. (B) AP2-1 cells were preincubated for 15 h in α -MEM culture medium in the absence (□) or presence (■) of 20 μ M kifunensine and metabolically labelled with [35 S]Met/Cys amino acids for 15 min. Culture media were recovered and the protein material was precipitated by 1 ml of a mixture of 0.6% PTA in 12% (v/v) trichloroacetic acid. Radioactivity associated with each precipitate was then counted by liquid scintillation.

Pro⁻⁵ cells (results not shown). This result strongly suggests that the inhibition of ER α 1,2-mannosidase activity by kifunensine leads to a decrease in the ER pool of misfolded glycoproteins, as reflected in the decrease of the UPR.

We thus decided to monitor the secretion of SeAP in the presence or absence of kifunensine. For this purpose, the SeAP secreted in the culture medium was determined both by measuring enzymic activity (Figure 6A), and the level of 35 S radioactivity associated to acid-precipitated glycoproteins (Figure 6B) in which the SeAP was found to represent approx. 90% of the bulk of secreted glycoproteins (results not shown). In both cases, it clearly appears that the secretion of SeAP is enhanced when B3F7 cells are pre-incubated with kifunensine.

Altogether, these results reveal that, in addition to a decrease of the UPR, the kifunensine treatment leads to an increase in the secretion of glycoproteins in the B3F7 cells.

DISCUSSION

The fate of newly synthesized glycoproteins is under the control of two different processes in wild-type cells. The first one concerns the cleavage of α 1,2-linked mannose residue and the second one involves the so-called gluco-degluco cycle, which allows monoglucosylated glycoproteins to transiently interact with lectin-like chaperone molecules CNX or CRT for efficient folding [27]. In wild-type cells, the two above-described processes can occur concomitantly, since Man₆ to Man₇ oligomannoside species can be reglucosylated [28]. In contrast, compared with the wild-type cells, the B3F7 cell model is much simpler, since the glycans can be the substrate of a kifunensine-sensitive α 1,2-mannosidase activity, leading to a unique Man₄ isomer which cannot be further reglucosylated. Therefore, demannosylation and glucosylation in these cells appear to be two reciprocal processes. The key point of the quality control of N-glycoproteins in mammalian cells is the detection of unfolded glycoproteins by an enzyme, UGGT, or molecular chaperones such as GRP78 [29]. In the present study, we used the UPR to assess the folding state of proteins in the ER lumen. This response activates the transcription of several genes, particularly that of GRP78. Thus we monitored the level of GRP78 mRNA by a semi-quantitative RT-PCR technique. Compared with β -actin used as an internal standard, we demonstrated that the level of transcription of GRP78 was approx. 2.5-fold higher in B3F7 than in the parental Pro⁻⁵ cells. Furthermore we demonstrated, using Western blotting, that GRP78 protein was 2-fold more expressed in B3F7 than in Pro⁻⁵ cells. To our knowledge, this is the first observation in mammalian cells of a spontaneous UPR without external stress such as glucose starvation or use of drugs interfering with either the glycosylation process or the glycan processing.

The defect in glycoprotein folding could be induced by three parameters: (i) underglycosylation due to the presence of truncated oligosaccharide lipid donor, (ii) a local effect of the truncated glycan on the protein backbone itself or (iii) the efficient action of the ER α 1,2 kifunensine-sensitive mannosidase activity, thus preventing further glucosylation needed for efficient folding via interaction with CNX or CRT. We clearly demonstrated that glycoproteins synthesized in B3F7 cells are not underglycosylated, in contrast with those observed in a similar glycosylation mutant MadIA214 (transferring only non-glucosylated Man₅GlcNAc₂ glycans), for which a strong underglycosylation has been demonstrated [18,24]. The question was whether the high level of misfolded glycoproteins in B3F7 is due to the truncated nature of oligosaccharide bound to proteins or the appearance of Man₄GlcNAc₂ species. The results obtained in the present study show that, when the transformation of Man₅GlcNAc₂ to Man₄GlcNAc₂ is prevented, a decrease in the level of misfolded glycoproteins is observed as reflected by the decrease of UPR. In this glycosylation mutant cell line B3F7, we propose that the formation of Man₄GlcNAc₂ oligomannoside species, which cannot be further reglucosylated, prevents numerous glycoproteins from entering the gluco-degluco cycle. This was supported by two observations: first, we showed that the Man₄GlcNAc₂ was generated at the expense of Glc₁Man₅GlcNAc₂ species [19] and, secondly, it has been recently demonstrated, by using truncated ribophorin as a model glycoprotein which was rapidly degraded, that the Man₄GlcNAc₂ glycans could act as a degradation signal by triggering its degradation [30].

Another effect of kifunensine was the increase of the secretion of the reporter glycoprotein SeAP. Such an effect has already been observed in mammalian wild-type cells [5,8], but the relationships between the inhibitory effect of kifunensine on the ER α 1,2-mannosidase activity, the quality control system

and secretion were not clearly established. In our model, a clear correlation can be made between the positive effect of kifunensine on the folding process and the enhanced secretion of SeAP. The action of kifunensine makes the ER folding machinery more efficient as revealed by an increase of the secretion of glycoproteins.

Although the nature of the α 1,2-mannosidase remains to be determined, in our model this enzyme acts as a timer in the folding of newly synthesized glycoproteins, as already proposed by several workers [4,31] for the ER mannosidase I in mammalian wild-type cells.

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