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Truncated N-glycans affect protein folding in the ER of CHO-derived mutant cell lines without preventing calnexin binding

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The involvement of N-glycans in the folding of influenza virus hemagglutinin (HA) was analyzed in two CHO-derived glycosylation mutants exhibiting a thermosensitive defect for secretion of human placental alkaline phosphatase. Truncated $Man_5GlcNAc_2$ oligosaccharides with one or three glucose residues are attached to proteins of the MadIA214 and B3F7AP2-1 mutant cells, respectively. Newly synthesized proteins retained in these cells carry a Man_4 trimmed glycan generated by a mannosidase different from the ER mannosidases I and II and suggesting a recycling through the Golgi complex. The glucosidase inhibitor castanospermine affects the binding of HA folding intermediates to the lectin-like chaperone calnexin in B3F7AP2-1 but not in MadIA214 cells. We demonstrated that calnexin interacts *in vivo* with truncated Man_5 derivatives. In MadIA214 cells, this is only possible when $Man_5GlcNAc_2$ on protein becomes reglucosylated. The pattern of intermediates seen during the folding of HA in the MadIA214 and B3F7AP2-1 mutant cell lines is different than in control cells. We also observed a variable occupancy of the seven glycosylation-sites. However, even under conditions that restore glycosylation of all sites, the folding intermediates of HA in the mutant cells still remain heterogeneous. Our results demonstrate that addition of truncated N-glycans interferes extensively with the folding of newly synthesized proteins *in vivo*.

Key words: calnexin/CHO-glycosylation mutant/influenza hemagglutinin/protein folding

Introduction

N-Linked oligosaccharides are added to specific consensus amino acid sequences of newly synthesized polypeptides during their translocation into the lumen of the endoplasmic reticulum (ER) of eukaryotic cells (Hirschberg and Snider, 1987). Nascent chains destined for the secretory pathway must first acquire a native conformation in this compartment (Gething *et al.*, 1986). A molecular mechanism referred to as ER quality control (Hurtley and Helenius, 1989) prevents further transport of incompletely folded or unassembled proteins which are retained by interaction with ER resident molecular chaperones (Knittler and Haas, 1992; Ou *et al.*, 1993; Vassilakos *et al.*, 1996). This mechanism ensures that only the proteins competent for secretion may leave the ER, whereas malfolded proteins are retained before being degraded.

Much evidence has been obtained for the importance of N-glycosylation in glycoprotein folding (Helenius, 1994), assembly and export (Varki, 1993; Fiedler and Simons, 1995). However, the molecular basis for these processes is not yet fully understood. The maturation of N-linked glycans associated to secretory proteins is quite complex. It results from an exquisitely ordered process taking place within subcompartments of the secretory pathway (ER, Golgi apparatus, trans Golgi network). The core glycan, $Glc_3Man_9GlcNAc_2$, is cotranslationally transferred as a unit onto asparagine-sites of nascent polypeptides and is then subjected to trimming (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987). The three glucose and single mannose residues are removed before the proteins are further transported. Interestingly, some ER chaperones such as calnexin and calreticulin have a lectin-like specificity for the monoglucosylated N-glycans, $Glc_1Man_{7\text{-}9}GlcNAc_2$ (Ware *et al.*, 1995; Spiro *et al.*, 1996). This points to the involvement of this carbohydrate moiety in ER quality control. A model has been proposed from studies on influenza virus hemagglutinin (HA) and vesicular stomatis virus G (VSVG) protein folding (Hammond *et al.*, 1994). Until they acquire a proper conformation, glycoproteins cycle between a deglucosylated and a reglucosylated state maintained by the concerted action of glucosidase II and of UDP-Glc: glycoprotein glucosyltransferase that interacts with malfolded proteins (Labriola *et al.*, 1995).

The removal of N-glycosylation sites by directed mutagenesis and the use of drugs interfering with the transfer or processing of N-linked oligosaccharides were shown in some, but not all, cases to lead either to the aggregation of proteins which are retained in the ER (Marquardt and Helenius, 1992), or to their aberrant trafficking (Lu *et al.*, 1997). We recently described a CHO-derived mutant cell line, MadIA214, which synthesizes truncated N-glycans, $Man_5GlcNAc_2$, as the result of a likely mislocalization of some intermediates required for N-glycan elongation (Ermonval *et al.*, 1997). This mutant was useful in demonstrating the involvement of carbohydrates in

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secretory processes. Indeed, maturation of human placental alkaline phosphatase (SeAP) and of H-2K^d MHC class I proteins is differentially affected in the MadIA214 cell line: secretion of SeAP is thermosensitive in this mutant, while H-2K^d maturation is restricted in a temperature independent way. Here, we study in more detail the fate of glycoproteins in this mutant and in another glycosylation mutant, B3F7, deficient in Dol-P-Man synthase. Both mutants transfer truncated N-glycans onto proteins. In B3F7 cells, the proteins carry $Glc_3Man_5GlcNAc_2$ (Cacan *et al.*, 1992) whereas in MadIA214 the proteins carry $Glc_1Man_5GlcNAc_2$ glycans. Secretion of SeAP is thermosensitive in transfected B3F7 cells (B3F7AP2-1) as it is in MadIA214 cells, further supporting the role of truncated N-glycans in this process.

In addition to an effect on newly synthesized proteins, cellular alterations, such as a strongly dilated ER occur in MadIA214 cells (Ermonval *et al.*, 1997). This ultrastructural modification correlates well with the longer retention of glycoproteins in the ER and the increased degradation of glycoproteins seen in the two mutant cell lines (Villers *et al.*, 1994; Ermonval *et al.*, 1997). It most likely reflects an accumulation of misfolded or unfolded glycoproteins retained in the ER by interaction with molecular chaperones, before being folded properly or degraded when competence for secretion cannot be achieved. The ER has a unique ability to increase in size in response to stress or to a high level of secretion (Pacifici and Lozzo, 1988). This phenomenon also occurs in a large group of human hereditary diseases referred to as ER storage diseases, ERSD (Amara *et al.*, 1992) and in a carbohydrate-deficient glycoprotein syndrome, CDGS (Marquardt *et al.*, 1995). Interestingly, in many of these pathologies the primary defect is thought to relate to protein folding.

We proposed in our earlier study that the addition of a truncated N-glycan could interfere with protein folding (Ermonval *et al.*, 1997). This point was further investigated here by taking advantage of the well characterized HA model. The HA folding intermediates resulting from loop formation by sequential acquisition of disulfide bonds can be visualised (Braakman *et al.*, 1991) and the involvement of calnexin in quality control by interaction with the monoglucosylated N-glycans of HA is well documented (Hammond *et al.*, 1994). Our results using glycosylation mutant cell lines infected with influenza virus show that calnexin binds to partially oxidized HA folding intermediates in both MadIA214 and B3F7AP2-1 cells, demonstrating that *in vivo*, calnexin can bind monoglucosylated Man_5. However, in the presence of glucosidase inhibitor, calnexin interaction with HA folding intermediates differed in the two mutants, depending on their number of glucose residues. In addition, the folding pathway of HA in both mutants is different from that of wild-type cells and will be discussed.

Results

Glycoproteins from both MadIA214 and B3F7AP2-1 cells carry truncated Man_5 N-glycans but the number of glucose residues they contain is different

In the MadIA214 cell line, synthesis of the core oligosaccharide transferred onto the consensus Asn-site is blocked at the level of the $Man_5GlcNAc_2$-P-P-Dol. This glycosylation defect lies in the mislocalization of some molecules needed in the ER to elongate the lipid intermediate from $Man_5GlcNAc_2$-P-P-Dol to $Glc_3Man_9GlcNAc_2$-P-P-Dol (Ermonval *et al.*, 1997). The B3F7AP2-1 cell line is derived from the B3F7 glycosylation defective cell line (Stoll, 1986) and belongs to the Lec15 complementation group (Beck *et al.*, 1990). These cells accumulate Man_5 derivatives. This is caused by a defect in the activity of the Dol-P-Man synthase that generates the Dol-P-Man substrate essential for Man_5 to Man_9 elongation. Therefore, B3F7 cells synthesizes a $Glc_3Man_5GlcNAc_2$-P-P-Dol intermediate.

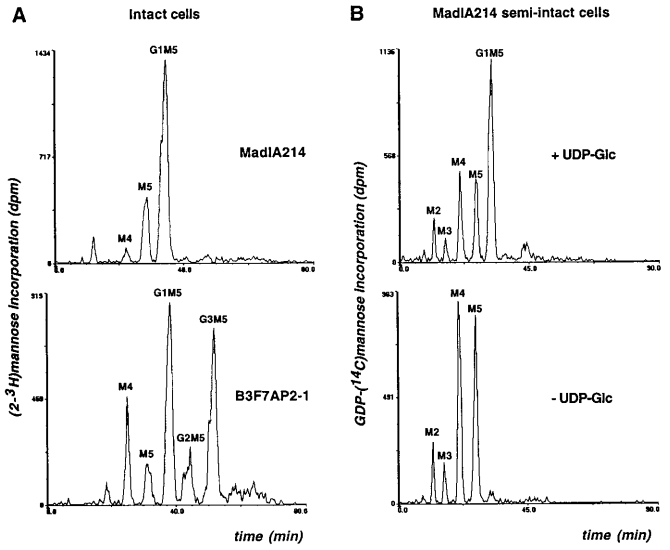

Fig. 1. Proteins carry Glc_1Man_5 in MadIA214 and Glc_3Man_5 in B3F7AP2-1 cells. Intact MadIA214 and B3F7AP2-1 glycosylation mutant cells were metabolically labeled for 30 min at 40°C with (2–^{3}H)Man in the presence of 50 μg/ml of castanospermine (**A**). Digitonin permeabilized MadIA214 cells, also treated with castanospermine, were labeled with GDP-(^{14}C) Man in the presence or absence of UDP-Glc as indicated (**B**). After sequential extraction, oligomannoside species were released from the glycoprotein fractions and analyzed by HPLC. The different radioactive moieties that eluted in an acetonitrile/water gradient were recorded as a function of time. The different peaks correspond to oligosaccharide species possessing two GlcNAc residues at their reducing end, and the indicated number of Man (M) and Glc (G) residues.

To determine the composition of protein-bound oligosaccharides, MadIA214 and B3F7AP2-1 cells were metabolically labeled with 2-(^{3}H)Man. Castanospermine was added to inhibit the trimming of glucose which occurs immediately after transfer of the N-glycans to proteins. Oligosaccharides extracted from glycoproteins were then analyzed by HPLC. The upper panel of Figure 1A shows that MadIA214 proteins accumulated monoglucosylated Man_5 sugars. Since only nonglucosylated Man_5 lipid intermediates were detected in these cells, the monoglucosylated sugars attached to proteins are not expected to be derived by trimming of a triglucosylated core. In contrast, in B3F7AP2-1 cells which synthesize a $Glc_3Man_5GlcNAc_2$-P-P-Dol intermediate, oligosaccharides attached to proteins were enriched in triglucosylated Man_5 moieties (lower panel). The other oligosaccharide moieties ($Glc_{0-2}Man_5$) detected in B3F7AP2-1 cells did not result from a

leaky block of the castanospermine but should be attributed to an ability of the oligosaccharyl transferase *in vivo* to transfer shorter oligomannosides which are not fully glucosylated (our unpublished data). A comparable situation exists in control cells treated with castanospermine (Ermonval *et al.*, 1997).

To prove that the peak eluting at the position of $Glc_1Man_5GlcNAc_2$ in MadIA214 cells was the result of glucose addition rather than another type of processing leading to modification products with the same elution time (i.e., Man_6, $GlcNAc_2Man_3$...), we performed oligomannoside labeling in semi-intact cells. This allows the sugar source used for N-glycan synthesis to be manipulated. A mixture of unlabeled (UDP-GlcNAc, UDP-Glc) and labeled (GDP-(^{14}C)Man) sugar precursors was supplied to digitonin permeabilized MadIA214 cells to allow synthesis and labeling of high mannose glycans. Castanospermine was added to inhibit glucose trimming. Under these conditions, the same carbohydrate moieties were found in the glycoprotein fractions of semi-intact and intact MadIA214 cells (compare upper panels of Figure 1A,B). When unlabeled UDP-Glc was omitted, only nonglucosylated truncated oligomannosides were detected and material no longer eluted at the position of the $Glc_1Man_5GlcNAc_2$ peak (Figure 1B).

These results show that both MadIA214 and B3F7AP2-1 cell lines transfer to proteins a truncated oligomannoside with five mannose residues, which is nonglucosylated and triglucosylated respectively. Interestingly, the protein-bound glycan is triglucosylated in B3F7AP2-1 cells but monoglucosylated in MadIA214 cells.

The immature forms of newly synthesized glycoproteins persist for a longer time in MadIA214 and B3F7AP2-1 than in Cl42 cells

We previously reported that SeAP secretion from MadIA214 cells is drastically reduced at the nonpermissive temperature (40°C) as compared to 34°C. The immature SeAP molecules with altered oligosaccharide structure are associated with the ER chaperone BiP. The amount of coimmunoprecipitated BiP increases when the growth temperature is raised from 34°C to 40°C (Ermonval *et al.*, 1997). Our results suggested that nonsecreted immature forms of SeAP are retained in the ER of MadIA214 cells. In contrast, the maturation of the H-2K^d MHC class I heavy chains is only slowed down in these cells as compared to control cells, independent of temperature (Ermonval *et al.*, 1997).

To characterize in more detail the glycoprotein maturation in the two glycosylation defective cell lines, we performed pulse-chase experiments and analyzed the immunoprecipitated SeAP and H-2K^d proteins by SDS-PAGE. The Cl42 parental line and the MadIA214 and B3F7AP2-1 mutant cell lines were radioactively pulse-labeled with a (^{35}S)Met-(^{35}S)Cys mixture for 15 min at 34°C or 40°C. The radioactivity was then chased at the same temperature for times ranging between 0 h and 8 h. Figure 2A shows that at 34°C (upper gels) the mature forms (m) of SeAP (right panel) were secreted into the chase medium of MadIA214 cells comparable to that of the Cl42 cells. Surprisingly, at this temperature, immature (i) SeAP (left panel) was detected in the MadIA214 cell lysates over an 8 h chase period whereas immature SeAP had practically disappeared from the parental cell line after 4 h of chase. The same difference in persistence of immature SeAP was seen at 40°C

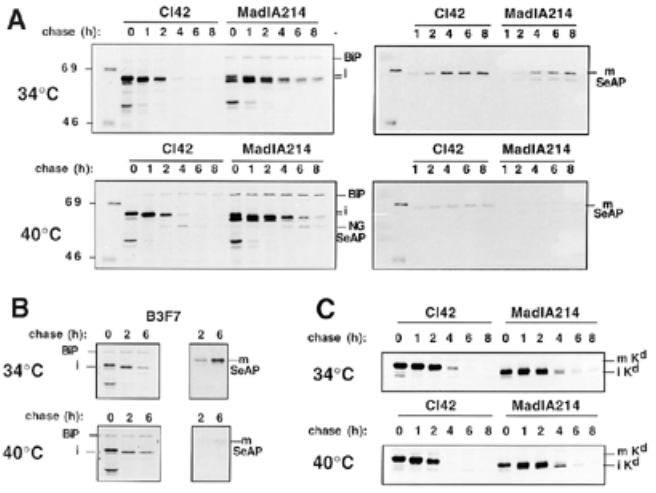

Fig. 2. Immature forms of glycoproteins are retained longer in glycosylation mutant cells than in normal cells. Proteins from Cl42, MadIA214, and B3F7AP2-1 cells were metabolically labeled for 15 min with a (^{35}S)Met-(^{35}S)Cys mixture either at 34°C (upper gels in **A**–**C**) or 40°C (lower gels in **A**–**C**). They were then chased for up to 8 h with unlabeled amino acids for the indicated periods of time. SeAP and H-2K^d were specifically immunoprecipitated from the chase medium or from cell lysates in 1% Triton X-100. SeAP and H-2K^d immunoprecipitates were analyzed using 8% and 10% SDS-PAGE respectively, and the incorporated radioactivity in newly synthesized proteins was visualized by fluorography. (**A**) shows SeAP in MadIA214 as compared to Cl42 cells immunoprecipitated from cell lysates (left gels) or from chase media (right gels). (**B**) shows similar immunoprecipitations for the analysis of SeAP maturation in B3F7AP2-1 cell lysates (left gels) and culture supernatants (right gels). (**C**) shows the H-2K^d molecules immunoprecipitated from Cl42 and MadIA214 cell lysates. The positions for the mature (m) and immature (i) forms of glycoproteins are indicated as well as for the BiP chaperone co-precipitating with SeAP.

(lower gels, left panel). However, at this nonpermissive temperature, SeAP was no longer secreted from MadIA214 cells (right panel), indicating that part of immature SeAP must be degraded. As expected from our previous data, whereas in MadIA214 cells BiP coimmunoprecipitated with immature SeAP at both temperatures, in Cl42 cells it was detected at 40°C only. The maturation of SeAP in the B3F7AP2-1 cell line (Figure 2B) was very similar to that observed in the MadIA214 cells. Immature SeAP remained in the cells during at least 6 h of chase at both 34°C and 40°C (left panels) and was associated with BiP. In both mutant cell lines, SeAP secretion was thermosensitive (right panels). Using the same approach, we investigated the maturation kinetics of H-2K^d which does not display a thermosensitive phenotype. Interestingly, at both temperatures, the immature forms of mouse class I molecules persisted for at least 2 h longer in MadIA214 than in Cl42 cells (Figure 2C).

Altogether, these results indicate that, whatever their fate, proteins loaded with truncated N-glycans are longer retained in the ER of the glycosylation defective cell lines.

Accumulating forms of N-glycans linked to the bulk of immature proteins consist of Man_4 derivatives in both MadIA214 and B3F7AP2-1 cells

The immature forms of SeAP and H-2K^d detected after a 1 h chase had a lower apparent molecular weight (Mw) than the

early precursors seen after a short pulse (see Figure 2). We previously showed that this reduction in Mw is caused by trimming of the glycans (Ermonval *et al.*, 1997). The processing of N-glycans starts in the ER with the removal of the three glucose residues by glucosidases I and II, and subsequent cleavage of one mannose residue by the ER-mannosidase I specific for a single branched α1-2 mannose (Kornfeld and Kornfeld, 1985). This enzyme converts $Glc_{3\text{-}0}Man_9GlcNAc_2$ into $Glc_{3\text{-}0}Man_8GlcNAc_2$, but it cannot remove any mannose residue from a truncated Man_5 glycan. Therefore, in the ER of B3F7AP2-1 and MadIA214 cells glucose is the only monosaccharide residue expected to be removed. In MadIA214 cells this results in a shift from $Glc_1Man_5GlcNAc_2$ to $Man_5GlcNAc_2$.

In order to determine the structure of the oligosaccharides linked to newly synthesized proteins of the glycosylation defective cell lines, N-glycans were radiolabeled with 2-(^{3}H)Man for 30 min. The radioactivity was then chased for various times up to 8 h, as for the protein maturation analysis. Figure 3 shows the HPLC profile of oligosaccharides extracted from glycoprotein fractions of B3F7AP2-1 cells at different chase points. Interestingly, after 1 h of chase a major peak consisting of $Man_4GlcNAc_2$ appeared in the mutant cell line which replaced the Man_5 precursor species seen immediately after the pulse (0 h chase). This $Man_4GlcNAc_2$ peak persisted throughout the chase, as illustrated by the 8 h chase sample. When a ConA column was used before the HPLC analysis of the B3F7AP2-1 protein fraction, the very same profiles were obtained over an 8 h chase. Since ConA only retains oligomannosides, either glucosylated or not, this clearly indicates that the 8 h pattern does not correspond to complex sugars. This is confirmed by the observation that sequential treatment of these oligosaccharides with β-galactosidase and hexosaminidase did not change the profile of the peaks seen in Figure 3 (data not shown). The results were the same for the MadIA214 cells, while in control cells the processing of high mannose chains was much more rapid (half-time around 1 h) and resulted in the presence of different carbohydrate moieties caused by a progression towards complex type sugars (data not shown).

These results indicate that immature glycoproteins retained in the ER of both MadIA214 and B3F7AP2-1 cells carry a trimmed Man_4 oligomannoside.

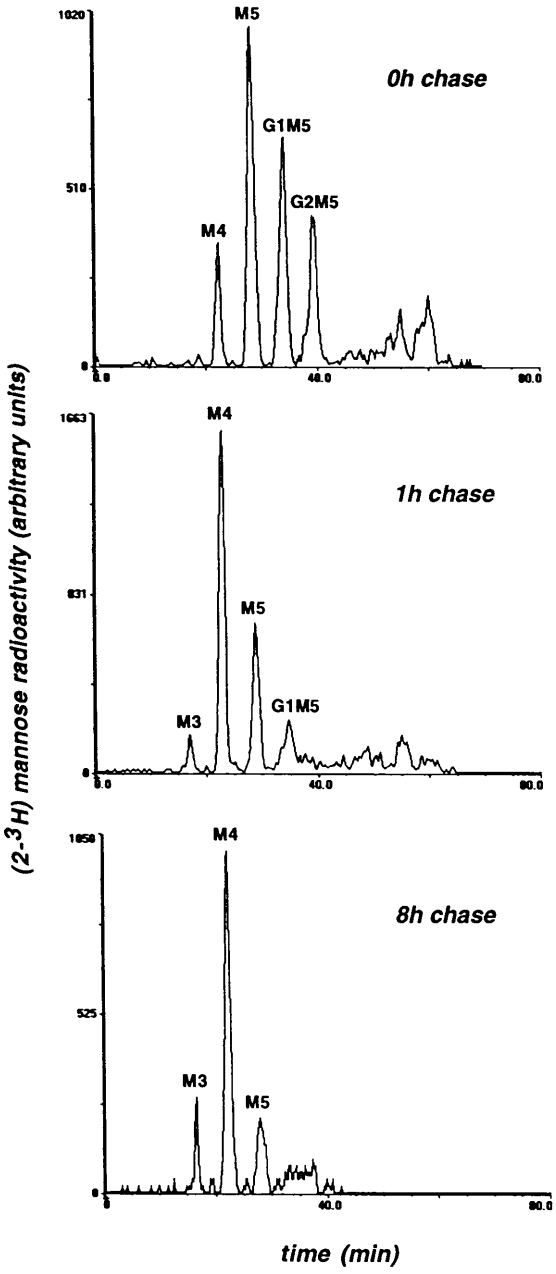

Fig. 3. Proteins loaded with a trimmed Man_4 sugar derivative accumulate in the B3F7AP2-1 mutant. B3F7AP2-1 cells were pulse-labeled for 30 min at 40°C with (2–^{3}H)Man. After a 0 h, 1 h, or 8 h chase, the oligomannosides associated with the glycoprotein fractions were analyzed as described in Figure 1. Each peak corresponds to an oligosaccharide species with two GlcNAc residues at the reducing end in addition to the indicated number of Man (M) and Glc (G) residues.

The profile of HA folding intermediates in the glycosylation mutants is different from that of wild-type cells

The immature forms of SeAP were associated with BiP and retained in the ER of MadIA214 and B3F7AP2-1 cells, which suggests a delay or defect in acquisition of their proper conformation. Since partially oxidized SeAP intermediates cannot be detected (data not shown) and conformation-sensitive antibodies are not available against this protein, we could not study the folding process of SeAP in more detail. We therefore used the well-characterized influenza virus HA as a model protein to test whether addition of truncated N-glycans onto HA would interfere with its folding and trimerization.

The folding intermediates of HA are easily detectable under nonreducing conditions (Braakman *et al.*, 1991). We compared HA folding in the parental Cl42 cells with folding in the MadIA214 and B3F7AP2-1 mutant cells (Figure 4). Five hours after infection with the influenza virus, cells were metabolically labeled at 34°C (lanes 1–2) or 39°C (lanes 3–4) with a (^{35}S)Met-(^{35}S)Cys mixture. Using an antiserum raised against the whole virus, HA was immunoprecipitated from cell lysates prepared either immediately after a short pulse (lanes 1 and 3) of 4 min at 34°C or 2 min at 39°C, or after a chase (lanes 2 and 4) for 60 min. Samples were analyzed by SDS-PAGE under nonreducing and reducing conditions. The viral nucleoprotein NP, also precipitated by the antiserum, was visible in every sample. In Cl42 cells at both temperatures (Figure 4A, lanes 1 and 3), the three expected bands corresponding to the partially

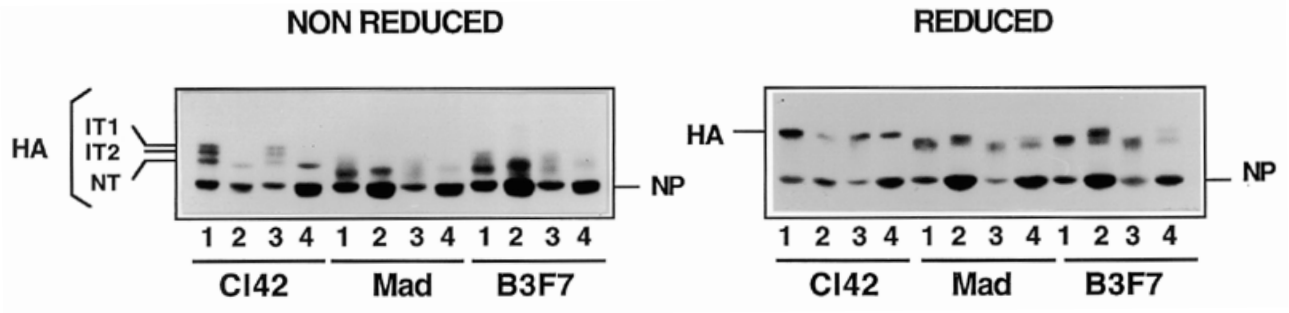

Fig. 4. Glycosylation of HA is incomplete in the mutant cell lines. Five hours after infection with X31 influenza virus, Cl42, MadIA214, and B3F7AP2-1 cells were metabolically labeled with (^{35}S)Met-(^{35}S)Cys amino acids for 4 min at 34°C (lanes 1) or 2 min at 39°C (lanes 3) and then chased for 60 min at the corresponding temperature (34°C, lanes 2 or 39°C, lanes 4). After lysis in 2% CHAPS, total HA was immunoprecipitated from the cell lysates and analyzed using 7.5% SDS-PAGE under non-reducing (left panel) and reducing (right panel) conditions. Partially oxidized intermediates (IT1 and IT2), fully oxidized HA (NT) and reduced HA and NP are designated.

oxidized HA folding intermediates, IT1 and IT2, and the fully oxidized native form NT (Braakman *et al.*, 1991), were observed immediately after the pulse, under nonreducing conditions. In IT2 the major loop-forming disulfide bond between Cys14 and Cys466 has not yet been formed, whereas IT1 lacks in addition the disulfide bond between residues 52 and 277 (I.Braakman, B.Foellmer and A.Helenius, unpublished observations). NT contains at least both large disulfide loops: it consists of monomers and trimers of HA ranging from DTT sensitive forms to completely stably folded molecules. During the chase, IT1 and IT2 disappear and only NT remains (lanes 2 and 4). This and the absence of aggregates indicated that the efficiency of HA folding in this cell line was comparable to that seen in CHO cells (Braakman *et al.*, 1991). Under reducing conditions (Figure 4, right panel), the three HA forms migrated as one sharp band (lanes 1–4).

In MadIA214 and B3F7AP2-1 cells, the HA folding intermediates were not present as sharp bands. Instead, a broad fuzzy smear was seen immediately after the pulse (Figure 4, left panel, lanes 1 and 3), in which discrete bands were absent. The heterogeneous forms in both cell lines did acquire more disulfide bonds during the chase since the amount of NT-like bands increased (lanes 2 and 4). Under reducing conditions, the HA bands were also quite broad in the mutant cell lines as compared to HA in the Cl42 cells (Figure 4, right panel lanes 1–4). These HA molecules had a higher mobility than HA in the Cl42 cells because of their smaller oligosaccharides combined with hypoglycosylation of HA in the mutant cell lines. The absence of extensive aggregation under the nonreducing conditions was especially striking, indicating that HA did not misfold substantially (Braakman *et al.*, 1992a). The pattern of folding intermediates was not significantly different at 34°C and 39°C in the glycosylation defective cell lines and a comparable pattern was also observed at 37°C (data not shown).

Our results indicate that despite the truncation of its N-glycans, HA could oxidize into an NT-like band without obvious misfolding. On the other hand, folding intermediates of HA could not be distinguished in the mutant cell lines and reduced HA still ran with heterogeneous mobilities.

Reduction by DTT improves glycosylation of HA in the MadIA214 and B3F7AP2-1 cells

Part of the heterogeneity of HA in the MadIA214 and B3F7AP2-1 cells most likely was caused by a variable occupation of the seven N-linked glycosylation sites as indicated by the persistence of HA's heterogeneity in reducing gels. N-linked oligosaccharide side chains are known to be important for the folding and maturation of HA (Gallagher *et al.*, 1992; Roberts *et al.*, 1993; Hebert *et al.*, 1997). Since both a partial occupation of the glycosylation sites and the transfer of a truncated N-glycan could be responsible for an alteration of HA folding, additional assays were performed to analyze this process in detail. To eliminate the heterogeneity caused by hypoglycosylation of the proteins in the mutant cells, we added DTT during the pulse-labeling. This treatment improves homogeneity of protein glycosylation by facilitating accessibility of the Asn residues on newly synthesized polypeptides to the oligosaccharyl transferase (Allen *et al.*, 1995). An 11 min pulse in the presence of DTT which prevent formation of disulfide bonds in newly synthesized HA (Braakman *et al.*, 1992a), was followed by a chase of 0, 2, or 120 min without DTT to allow folding of HA to proceed (Figure 5). In the reducing gel (left panel, lane 0′), HA from both mutant lines ran as a single band indicative of more homogeneous glycosylation (lanes 4 and 7). The slightly higher mobility than HA from the control cells (lane 1) is consistent with their truncated glycans.

After 2 h of chase, an additional form of HA with lower mobility appeared in both mutant cell lines (Figure 5, lanes 6 and 9). These bands represent HA molecules that have proceeded to the Golgi complex and carry modified complex glycans. To confirm that the occupation of glycosylation sites in HA was identical in all three cell lines, we compared the electrophoretic mobility of the HA Golgi bands from the three cell lines by running the 120 min chase samples side by side (lanes 10–12). HA from the MadIA214 cell line now had identical mobility to that of the Cl42 cells, and HA from the B3F7AP2-1 cells displayed an even lower mobility. Since Cl42 is not the parent cell line for the B3F7 cells, modifications of oligosaccharides in the Golgi may be different.

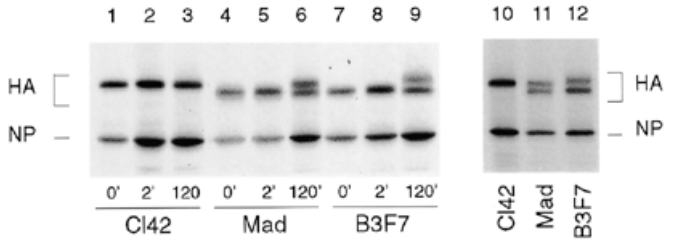

Fig. 5. DTT restores glycosylation of HA in the mutant cell lines. Cl42, MadIA214, and B3F7AP2-1 virus infected cells were pulse-labeled at 37°C with (^{35}S)Met-(^{35}S)Cys amino acids for 11 min in the presence of 5 mM DTT. The reducing agent was removed and folding of HA was allowed to proceed for 2 or 120 more minutes. Cells were lysed in CHAPS. Immunoprecipitation and analysis of HA was performed as in Figure 4 under reducing conditions. To allow direct comparison of the 120 min samples (lanes 3, 6, and 9), they were also analyzed side by side (lanes 10–12).

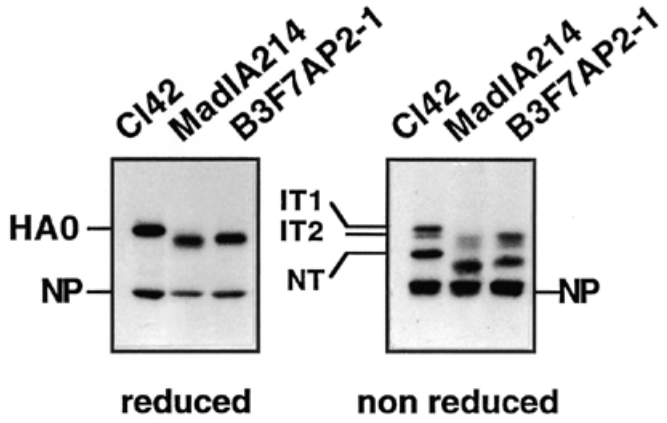

Fig. 6. Folding of HA remains different in the mutant cell lines after DTT treatment. Cl42, MadIA214 and B3F7AP2-1 virus infected cells were pulse-labeled at 34°C with (^{35}S)Met-(^{35}S)Cys amino acids for 5 min in the presence of 5 mM DTT. The reducing agent was removed and folding of HA was allowed to proceed for 2 min. Imunoprecipitation and analysis of HA were performed as in Figure 4. On the nonreducing gel, reduced HA (R) and IT1 ran with overlapping mobilities.

We concluded that the number of glycans added to HA was identical in the Cl42 and MadIA214 cell lines incubated in DTT, and very likely in the B3F7AP2-1 cells as well.

Addition of truncated N-glycans interferes with the folding of HA in MadIA214 and in B3F7P2-1 cells

The DTT treatment allowed us to examine the role of truncated glycans alone on the folding of HA without the effect of hypoglycosylation. A 5 min pulse in the presence of 5 mM DTT, was followed by a 2 min chase in the absence of DTT for up to 30 min. Figure 6 shows that the chase in the absence of DTT allowed oxidation and folding of HA (Braakman *et al.*, 1992b). In the Cl42 control cells (right panel), IT1, IT2, and NT appeared (adding to some reduced HA that had not disappeared and ran overlapping with IT1). Because HA in reducing gel run as a sharper band (left panel) as expected from the results in Figure 5, more discrete bands were also seen for the HA folding intermediates in the mutant cell lines (right panel). However, the folding pattern still was not the same as that in the parental cell line. In particular, IT2 had a different mobility and the bands were much more fuzzy in MadIA214 and B3F7AP2-1 as compared to Cl42 cells. Two out of the six disulfide bonds in HA contribute most to the visualisation of the IT1, IT2, and NT bands in nonreducing gels. With the formation of the two major loops in HA from wild-type cells, the folding intermediates always appeared as discrete bands detected very early (2 min) and not obviously affected in their migration by the formation of the other disulfide bonds. This indicated that, in the glycosylation defective cell lines, either at least some disulfide bonds were formed in a different order from those observed in the Cl42 and other cell lines (Braakman *et al.*, 1991), or non-native disulfide bonds were formed during the folding process.

Taken together, our observations indicate that the folding pathway of HA in MadIA214 and B3F7AP2-1 cells did not follow exactly the same route as in the parental cells.

Calnexin interacts in vivo with the truncated $Glc_1Man_5GlcNAc_2$ sugar

Truncated sugars added onto Asn-sites could interfere with protein maturation at two levels: (1) by a direct effect on protein folding, (2) by an alteration of the specific interaction with the molecular chaperones: calnexin and calreticulin, which display lectin-like activity. We thus investigated whether calnexin could interact with monoglucosylated Man_5 *in vivo* in the glycosylation defective cell lines. This chaperone was demonstrated to transiently associate with HA through N-glycans and to play a role in folding and retention of HA in the ER (Hebert *et al.*, 1996). Calnexin was immunoprecipitated from radiolabeled virus-infected cells that were lysed under conditions known to maintain the interaction of the chaperone with HA (Figure 7). When analyzed by nonreducing SDS-PAGE (Figure 7A), immediately after the 4 min pulse (p), the two folding intermediates of HA and a fraction of folded native HA (NT) were seen to coprecipitate with calnexin in Cl42 cells. Native HA present after a 60 min chase (c) hardly associated with calnexin. Immunoprecipitation with an anti-calreticulin antibody gave similar results (data not shown). Although HA in the mutant cell lines was loaded with truncated N-glycans containing Man_5, the association of HA folding intermediates with calnexin was very similar to that in the control cells (Figure 7A, MadIA214 and B3F7AP2-1). To clarify whether calnexin in the mutant cell lines was bound to HA through a sugar interaction, we performed the pulse-labeling in the presence of castanospermine (Figure 7B). By blocking glucose trimming in wild-type cells, this glucosidase inhibitor prevents the interaction of HA folding intermediates with calnexin which only takes place with N-glycans carrying one glucose residue (Hammond *et al.*, 1994). Figure 7B shows that in both Cl42 and B3F7AP2 cells, the interaction of HA with calnexin indeed was inhibited in the presence of castanospermine (+CST). However, in the MadIA214 cell line, the interaction between HA and calnexin was maintained. This is in agreement with our observation that in these cells a monoglucosylated N-glycan is attached to proteins.

These results directly demonstrate that calnexin interacted *in vivo* with monoglucosylated Man_5 derivatives.

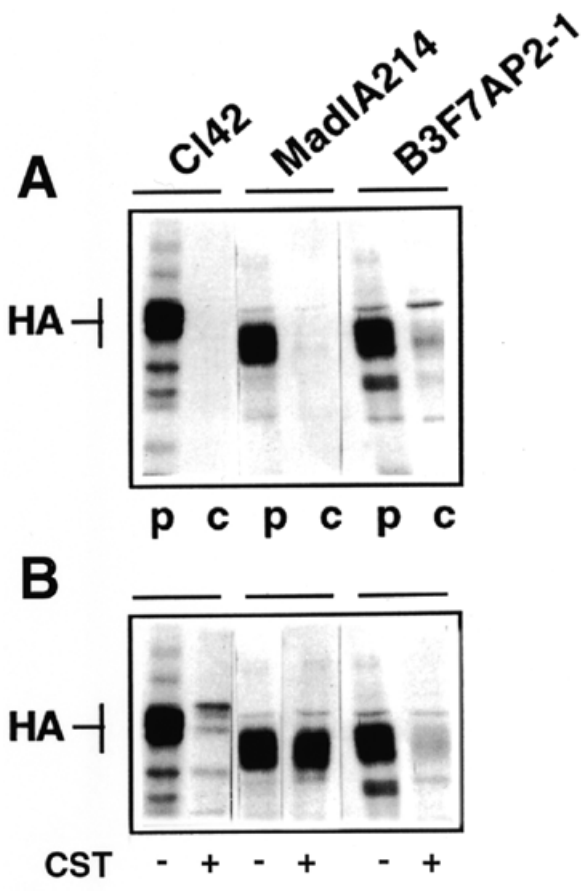

Fig. 7. Calnexin interacts with HA folding intermediates in both mutant cell lines but is dissociated by castanospermine in B3F7AP2-1 cells only. Lysates from pulse-labeled Cl42, MadIA214, and B3F7AP2-1 cells were generated as in Figure 4, using CHAPS as detergent. Calnexin was immunoprecipitated and the coprecipitated labeled HA folding intermediates were analyzed by nonreducing 7.5% SDS-PAGE (**A**). The folding intermediates coprecipitated with calnexin immediately after the pulse, while the more native forms obtained after the 30 chase did not. The pulse was repeated in the presence of 200 μg/ml of castanospermine (CST) to inhibit glucose trimming (**B**) and HA coprecipitating with calnexin was analyzed as described above.

Discussion

Using two different CHO-derived glycosylation defective cell lines, we show in the present study that the addition of truncated N-glycans ($Glc_{0-3}Man_5GlcNAc_2$) to newly synthesized influenza hemagglutinin influences its folding. We also demonstrate that such incomplete oligomannosides do not impair the interaction of HA with the molecular chaperone calnexin.

We recently described that maturation of two glycoproteins is differentially affected in the MadIA214 mutant cell line (Ermonval *et al.*, 1997). The comparison in this study with a second glycosylation defective cell line, B3F7AP2-1, confirms that the thermosensitivity in SeAP secretion indeed resulted from the addition of a truncated N-glycan onto proteins. The glycosylation defect in both mutants leads to a blockade in the elongation of the core oligomannoside. The two cell lines differ only in the number of glucose residues present on the truncated $Man_5GlcNAc_2$ core that is attached to proteins: one Glc residue in MadIA214 and three Glc residues in B3F7AP2-1 cells.

In the MadIA214 cell line, the Man_5 block is caused by a mislocalization of some intermediates of the N-glycan synthesis pathway (Ermonval *et al.*, 1997). All the glycosylation enzymes are functional as shown by reconstitution of a complete $Glc_3Man_9GlcNAc_2$ core in MadIA214 derived microsomes. The same was shown in a similar mutant cell line, Lec35 (Zeng and Lehrman, 1990). Proteins in the B3F7AP2-1 cell line are loaded with triglucosylated $Man_5GlcNAc_2$ oligomannosides. Therefore, truncated Man_5 moieties can be glucosylated *in vivo* by the Dol-P-Glc glucosyltransferases. This raises the question why do proteins in MadIA214 cells carry carbohydrate species containing only one glucose while the Dol-P-Glc glucosyltransferase(s) should be functional (Ermonval *et al.*, 1997). Nonglucosylated Man_5-P-P-Dol intermediates are only detected in MadIA214 cells. A defect in a flippase activity or in an accessory molecule required for the translocation of both Dol-P-Man and Dol-P-Glc into the ER could account for this N-glycan structure. According to this hypothesis the monoglucosylated $Man_5GlcNAc_2$ carbohydrates bound to MadIA214 proteins, interestingly, should derive only from reglucosylation by the UDP-Glc: glycoprotein glucosyltransferase from the ER which uses soluble UDP-Glc as substrate (Trombetta *et al.*, 1991).

As long as glycoproteins have not acquired the proper conformation, they are a substrate for reglucosylation by UDP-Glc: glycoprotein glucosyltransferase (Sousa *et al.*, 1992). The monoglucosylated N-glycan mediates interaction with the lectin-chaperones calnexin and calreticulin. This interaction is maintained by cycling of glycoproteins between the nonglucosylated state (following action of glucosidase II) and the monoglucosylated state (Helenius, 1994). Interaction of calnexin through monoglucosylated N-glycans is required for efficient HA folding (Hammond *et al.*, 1994; Hebert *et al.*, 1996). The fact that calnexin binds to HA and a range of other cellular proteins (data not shown) in the two mutant lines indicates that calnexin can bind truncated Man_5 derivatives *in vivo*. However, calnexin was shown to interact poorly with Man_5 moieties *in vitro* (Ware *et al.*, 1995) in contrast to calreticulin (Spiro *et al.*, 1996). Recent *in vitro* studies have shown that these two lectins exhibit an essentially identical substrate specificity and a similar affinity for $Glc_1Man_{7-9}GlcNAc_2$ oligomannosides (Vassilakos *et al.*, 1998). In B3F7AP2-1 cells, the binding of calnexin to folding intermediates of HA was inhibited by castanospermine. This establishes that the calnexin interaction, in B3F7AP2-1 as in control cells, involves monoglucosylated Man5 binding rather than a glycan-independent interaction (Arunachalam and Cresswell, 1995) or entrapment in an aggregate (Cannon *et al.*, 1996). As expected, the association of HA to calnexin was maintained in MadIA214 cells treated with castanospermine since the proteins in this cell line already carry monoglucosylated sugar moieties. The same was observed in the similar Lec35 mutant cells (data not shown), indicating that glucosylation of $Man_5GlcNAc_2$ moieties was not a peculiarity of the MadIA214 cell line. The possibility for truncated Man_5 species to be reglucosylated *in vivo*, in contrast to what is observed in cell-free

assay (Sousa *et al.*, 1992), is also supported by the N-glycan dependent binding of calnexin in the mutant cells. Apparently, *in vivo* conditions allow some enzymes to function on substrates with low affinity when a better ligand is absent.

The analysis of the oligosaccharide side chains bound to the proteins of both glycosylation mutants suggested that proteins retained in the ER actually cycle through the Golgi complex. The accumulation of trimmed Man_4 species on proteins in both mutants requires the action of an early Golgi mannosidase. It could be the Golgi Mannosidase I (Velasco *et al.*, 1993) but not the early Golgi endomannosidase (Lubas and Spiro, 1987) recently shown to be absent from CHO cells (Karaivanova *et al.*, 1998). Recycling of trimmed intermediates from the Golgi back to the ER most likely occurs by association with a chaperone that is retrieved. The association with BiP suggests that at least some immature intracellular forms of SeAP fit that profile. Recycling of a misfolded form of the vesicular stomatitis virus G protein (VSVG) was also observed in studies using the ts045 mutant of the virus (Hammond and Helenius, 1994). However, we cannot exclude that some unknown mannosidase or the ER Man_9-mannosidase (Bause *et al.*, 1992) could account for such trimming.

Our observations on the influence of a truncated N-glycan on protein maturation were very similar for the MadIA214 and B3F7AP2-1 cell lines but differed with glycoproteins. In both mutant cell lines, the secretion of SeAP was thermosensitive. In contrast, the transport of H-$2K^d$ to the surface of MadIA214 cells was slowed down in a temperature independent way (Ermonval *et al.*, 1997). Whatever the temperature and whatever their fate, the immature forms of these glycoproteins persisted for longer in the ER of the two glycosylation defective cell lines, and immature SeAP was associated with the ER chaperone BiP. Finally, the folding pathway of HA in the two mutant cell lines was different to that in the control cells. Altogether, these results indicate that truncated N-glycans may affect protein maturation by interfering directly with folding of newly synthesized proteins. We demonstrated here that the truncation indeed affects HA folding at two levels: it can cause incomplete use of glycosylation sites, and it interfered with disulfide bond formation even when all seven consensus sites were glycosylated.

Hypoglycosylation of proteins has also been described in yeast mutant strains synthesizing truncated N-glycans (Burda *et al.*, 1996; Reiss *et al.*, 1996). A variable accessibility of the seven potential N-linked glycosylation sites was seen for HA in both glycosylation defective cell lines. The same was seen for the envelope glycoprotein gp160 of the human immunodeficiency virus type I and its soluble subunit gp120 (data not shown). SeAP in MadIA214 cells also consists of a mixed population with one or two N-glycans (Ermonval *et al.*, 1997). However, the population with two N-glycans is predominant in B3F7AP2-1 cells when the only difference is the three glucose residues on the oligosaccharide side chain transferred onto proteins. The addition of a first truncated sugar might have introduced a different conformation, possibly resulting in the formation of nonnative disulfide bonds, thereby decreasing the accessibility of downstream N-glycosylation sites. Disulfide bond formation in a viral glycoprotein was shown to block access to the oligosaccharyl transferase (McGinnes and Morrison, 1997). Another likely explanation for hypoglycosylation is a lower affinity of the oligosaccharyltransferase for the truncated sugars (Turco *et al.*, 1977; Sharma *et al.*, 1981; Breuer and Bause, 1995). This could also lead to a variable occupancy of N-linked glycosylation sites that could be restored by DTT-treatment.

More importantly, our results imply that truncated N-glycans interfere with protein folding and maturation even when all consensus sites were occupied. Both SeAP in B3F7AP2-1 and H-$2K^d$ in MadIA214 were fully glycosylated with truncated glycans, but their maturation was still affected. The glycosylation of HA in the mutant cell line could be restored completely by preventing disulfide bond formation during HA synthesis, as shown for t-PA (Allen *et al.*, 1995). Under such conditions, the effect of truncated sugars on the maturation of HA were striking. The folding intermediates of HA were different from the IT1 and IT2 we always observe, indicating that the folding pathway of HA had changed. This change must reflect abnormal disulfide bond formation during the folding process.

The importance of N-glycans in the acquisition of glycoprotein tertiary structure was shown in models where glycosylation sites were occupied or not. For example, the refolding *in vitro* of bovine pancreatic RNAse B is much faster than the folding of the nonglucosylated RNAse A form (Yamaguchi and Uchida, 1996). Similar observations were made using synthetic peptides and glycopeptides (Imperiali and Rickert, 1995; Live *et al.*, 1996). Moreover, inhibition of N-glycosylation *in vivo* by tunicamycin treatment induces protein misfolding (Hurtley and Helenius, 1989). Our results show that the oligosaccharide structure also is important for protein folding *in vivo* and are consistent with truncated glycans altering the initial conformation of growing nascent chain during their translation and translocation.

During their folding, proteins interact with many different folding factors of the ER (for review, see Leitzgen and Haas, 1998). Therefore, it has to be considered that the truncated sugar could also affect protein folding indirectly by a difference in binding to chaperones or to other folding factors. The truncated glycans may also slow down the folding process by influencing the accessibility or the affinity of each folding factor, either by a change in conformation or by steric hindrance.

Despite the glycosylation defect in the mutant cell lines, the binding per se of calnexin to HA was not impaired. However, a lower affinity of this chaperone for truncated sugars may have altered kinetics of binding and release and thereby the folding process. We did not detect any interaction of SeAP with calnexin or calreticulin. Instead SeAP was retained in the ER of the glycosylation defective cell lines, associated to BiP as a reflection of a folding problem. The folding alteration induced by truncated oligomannosides can be overcome in some but not all cases (e.g., thermosensitive secretion of SeAP). An alternative folding pathway may be used in some situations and could be influenced by the set of ER chaperones recruited by a given glycoprotein, as was shown for artificially truncated chains of HA (Zhang *et al.*, 1997). Interestingly, the deviation in HA's folding pathway in the two glycosylation defective cell lines still allowed HA to adopt a native trimerization-competent state that could pass the quality control system of the ER and reach the cell surface (data not shown). Never before did we see normal trimerization and transport in the case of HA0 misfolding (Hurtley and Helenius, 1989); the difference always concerned rate and efficiency of folding,

never the nature of the folding intermediates (Braakman *et al.*, 1991, 1992a,b; Tatu *et al.*, 1993). Our results show for the first time that an incomplete N-glycan can interfere with disulfide bond formation during the folding of HA. An alternative pathway may eventually correct this phenomenon and lead to maturation with a delay as observed for H-2K^d (Ermonval *et al.*, 1997). This correlates well with the long retention of immature glycoproteins observed in the glycosylation defective cell lines.

In conclusion, we show that calnexin can bind $Glc_1Man_5GlcNAc_2$ carbohydrates in vivo and also that Man_5 moieties can be reglucosylated by the UDP-Glc: glycoprotein glucosyltransferase. Our results also suggest that retained proteins recycle and accumulate as Man_4 derivatives in the glycosylation defective cell lines. Finally, we show that truncated N-glycans can interfere directly with the folding of glycoproteins with or without further consequences for their maturation.

Materials and methods

Cells and virus

The CHO derived hamster cells used in this study were grown in alpha-MEM medium (ATGC and Gibco BRL), supplemented with 6% FCS (or 8% for the HA assay) and additives (Hepes, glutamine, G418 at 1 mg/ml) from Gibco BRL.

The Cl42 parental clone is a CHO cell expressing the mouse MHC class I heavy chain H-2K^d on its cell surface and a secreted form of the human placental alkaline phosphatase (SeAP). The MadIA214 glycosylation mutant has been isolated after mutagenesis of Cl42 cells and exhibits a thermosensitive phenotype for SeAP secretion: the enzyme is detected in the cell supernatant at 34°C (referred to as the permissive temperature) and not at 40°C (the nonpermissive temperature). The mutant cell line synthesized a truncated N-glycan consisting of $Man_5GlcNAc_2$. The characteristics of these cell lines have been described previously (Ermonval *et al.*, 1997).

The B3F7AP2-1 clone was derived from the B3F7 (a kind gift from S.S.Krag, John Hopkins University, Baltimore). B3F7 is also a glycosylation defective CHO cell line (Stoll, 1986), in which we have stably expressed the SeAP as described for Cl42 (Ermonval *et al.*, 1997). The B3F7 mutant cell line is deficient in Dol-P-Man synthase and exhibits a Man_5 block.

To study HA folding, cells were infected with X31 influenza virus and subjected to pulse chase analysis 5–7 h postinfection (Braakman *et al.*, 1991).

Antibodies

All the antibodies used for immunoprecipitations were rabbit polyclonal antisera and were coupled directly to Protein A Sepharose CL4B or CLFF beads (Pharmacia).

The anti-human placental alkaline phosphatase serum was purchased from Dako. The H-2K^d heavy chains were precipitated with an anti-exon 8 antiserum (a kind gift from J.Neefjes, Netherlands Cancer Institute, Amsterdam, The Netherlands). These polyclonal antibodies are specific for a C-terminal peptide encoding the exon 8 of H-2K^b molecules (Smith *et al.*, 1986) cross-reacting with the H-2K^d class I. The antiserum used to precipitate HA was prepared against complete influenza virus (Copeland *et al.*, 1986; Braakman *et al.*, 1991). It recognizes all forms of HA as well as the viral nucleoprotein (NP) and the matrix protein. The anti-X31 and the anti-calnexin (Hammond *et al.*, 1994) antisera were kind gifts from Dr. Ari Helenius.

Pulse-chase analysis of secretory proteins

Conditions used to study maturation of SeAP and H-2K^d proteins have been described (Ermonval *et al.*, 1997). Briefly, 2.5×10^5 plated cells were cultured overnight in 24 well plates at the indicated temperatures, then pulsed for 15 min in RPMI medium without FCS, Met and Cys (ICN) and containing 100 μCi of a radioactive [^{35}S]Met-[^{35}S]Cys amino acid mixture (NEN). Radioactivity was chased as indicated, over an 8 h period of time. Culture supernatants were recovered, and cells were lysed with 1% Triton X-100 (Merck) in NET buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM EDTA). Specific immunoprecipitations were performed overnight at 4°C with 25 μl per sample of protein A–Sepharose beads (Pharmacia) precoated with 1 μl of antiserum. After washing in high salt (0.5 M NaCl) NET buffer the immunoprecipitates were eluted from the beads with reducing sample buffer containing SDS, boiled, then analyzed by SDS-PAGE. Amersham Hyperfilm was used for fluorography of the gels.

Analysis of HA folding

Influenza virus infected cells were used to analyze the folding of HA as previously described (Braakman *et al.*, 1991). Subconfluent cells were pulse-labeled 5 h postinfection for several min at 34°C, 37°C, or 39°C as indicated with 50 μCi of [^{35}S] *in vitro* labeling mix (Amersham), then chased up to 2 h at the same temperature. When indicated, 5 mM DTT was included in the pulse medium to improve the efficiency of the N-linked glycosylation process (Allen *et al.*, 1995). Cells were lysed in 2% CHAPS or 0.5% Triton X-100 in MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl pH 7.4) as indicated and immunoprecipitated as described below.

Cell lysates were immunoprecipitated at 4°C with anti-X31 antiserum to precipitate all forms of HA. Immunoprecipitates were washed twice with 0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl in 10 mM Tris-HCl, pH 8.6 at room temperature, and analyzed by 7.5% SDS-PAGE either under reducing conditions, or for folding studies under nonreducing conditions (Braakman *et al.*, 1991).

Calnexin association with HA

To maintain an interaction between HA or SeAP and calnexin or calreticulin, cells were lysed in 2% CHAPS (Pierce) in Hepes-buffered salt solution (HBS). In addition, EDTA was excluded from the lysate. Cell lysates were precleared with heat-killed and fixed *Staphylococcus aureus* cells (Zymed). Immunoprecipitates were washed in 0.5% CHAPS in HBS. When indicated, the glucosidase inhibitor castanospermine was added to a concentration of 200 μg/ml for 1 h before the pulse and kept throughout all the pulse and chase. Calnexin immunoprecipitates were washed with 0.5% CHAPS in HBS (Hammond *et al.*, 1994), and analyzed using 7.5% SDS-PAGE as described above.

HPLC analysis of oligosaccharides

Cells ($5–10 \times 10^6$) were metabolically labeled for 30 min at 40°C in culture medium containing 5% dialyzed FCS, 0.5 mM Glc, and 100 μCi of 2-(^{3}H)Man (42.9 Gbq/mmol) from Amersham. After washing the cells with cold PBS the radioactivity was chased in the same medium supplemented with 5 mM Man. To further determine the identity of the sugars incorporated in the N-glycan core, we also used semi-intact cells permeabilized with digitonin (Plutner *et al*., 1992). Semi-intact cells were labeled for 30 min at 34°C with 5 μCi of GDP-(^{14}C)Man (1.4 Gbq/mmol, Amersham) in 100 ml of TKM incubation buffer (30 mM Tris-HCl pH 7.5, 120 mM KCl, 4 mM Mg acetate) containing 50 μg/ml of castanospermine (Boehringer), 5 mM AMP, 2 mM $MnCl_2$, 5 mM $MgCl_2$, 50 μM UDP-GlcNAc with or without 50 μM UDP-Glc as indicated.

After a sequential extraction of either the intact or semi-intact cell monolayers with organic solvents as already described (Cacan *et al.*, 1993), the protein pellet was digested overnight at room temperature with 0.2 mg of TPCK-treated trypsin in 300 μl of 0.1 M ammonium bicarbonate pH 7.9. Trypsin was destroyed by boiling for 10 min, and the oligosaccharides were cleaved from the peptides by incubating overnight with 0.5 U of Peptide N-Glycanase F (Boehringer). After passage through a biogel-P2 column, the desalted oligosaccharides released from the protein fraction were analyzed by HPLC on an ASAHIPAK-NH2-P-50 column (Asahi, Kawasaki-ku, Japan). The oligomannosides eluted in a 70/30 to 50/50 (v/v) gradient of acetonitrile/water at a flow rate of 1 ml/min over an 80 min time period and were monitored with a Flo-one β detector (Flotec).

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