

Glycosylation of Human Truncated FcεRI α Chain Is Necessary for Efficient Folding in the Endoplasmic Reticulum*

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The high affinity immunoglobulin E (IgE) receptor is an $\alpha\beta\gamma_2$ tetrameric complex. The truncated extracellular segment (α) of the heavily glycosylated α chain is sufficient for high affinity binding of IgE. Here we have expressed various α mutants in eukaryotic and prokaryotic cells to analyze the role of glycosylation in the folding, stability, and secretion of α . All seven N-linked glycosylation sites in α are glycosylated and their mutations have an additive effect on the folding and secretion of α . Mutation of the seven N-glycosylation sites (Δ 1–7 α) induces misfolding and retention of α in the endoplasmic reticulum. Similarly, tunicamycin treatment reduces substantially the folding efficiency of wild-type α . In contrast, no difference in folding efficiency is detected between wild-type α and Δ 1–7 α expressed in *Escherichia coli*. In addition, maturation of N-linked oligosaccharides and addition of O-linked carbohydrates are not required for either the transport or the IgE-binding function of α . Furthermore, complete enzymatic deglycosylation does not affect the stability and the IgE-binding capacity of α . Therefore, glycosylation is not intrinsically necessary for proper folding of α but is required for folding in the endoplasmic reticulum. Our data are compatible with the concept that specific interactions between N-linked oligosaccharides and the folding machinery of the endoplasmic reticulum are necessary for efficient folding of α in eukaryotic cells.

The high affinity IgE receptor (FcεRI), which is required for the initiation of IgE-mediated allergic reactions (Dombrowicz *et al.*, 1993), is expressed constitutively on mast cells and basophils (Metzger *et al.*, 1986; Kinet, 1990). It is also expressed on Langerhans cells (Bieber *et al.*, 1992; Wang *et al.*, 1992), on monocytes of allergic individuals (Maurer *et al.*, 1994), and on eosinophils of some patients with hypereosinophilia (Gounni *et al.*, 1994). FcεRI is a multimeric complex consisting of an IgE-binding α chain, a β chain, and a dimer of disulfide-linked γ chains which associate through noncovalent interactions (Kinet, 1990; Ravetch and Kinet, 1991). The α chain is a type I integral membrane protein which is homologous to Fc receptors

for IgG (FcγRII, FcγRIII) (Hogarth *et al.*, 1992). The extracellular domain of human α contains 181 amino acid residues, and is organized into two immunoglobulin-like domains defined by two pairs of cysteine residues forming two disulfide bridges (Kochan *et al.*, 1988; Shimizu *et al.*, 1988). Previous studies have shown that expression on the plasma membrane of the extracellular domain of α reconstitutes IgE binding (Blank *et al.*, 1989; Hakimi *et al.*, 1990). Furthermore, truncation of the transmembrane and cytoplasmic domains of human FcεRI α results in a protein (α) efficiently secreted by CHO¹ cells and yet able to mediate high-affinity binding of IgE (Blank *et al.*, 1991). The same α construct has also been expressed in a secreted and active form in *Escherichia coli* (Robertson, 1993). Recently, the second Ig domain has been shown to contain at least a portion of the receptor binding site (Mallamaci *et al.*, 1993; Robertson, 1993). However, this second domain binds IgE only with low affinity. Therefore, it is likely that the first domain is somehow also involved in providing the receptor with a high-affinity binding site.

The extracellular domain of the α chain is heavily glycosylated. The core protein of α has a theoretical molecular weight (M_r) of 19,275, but the apparent M_r , estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is 50,000 (Blank *et al.*, 1991). This increase in M_r is primarily due to N-linked glycosylations, which represent 38–42% of the total M_r of the full α chain, and secondarily to O-linked carbohydrates which represent 4% of the total M_r (La Croix and Froese, 1993). Sequence analysis reveals seven potential N-glycosylation sites in the extracellular part of the human α chain (Kochan *et al.*, 1988; Shimizu *et al.*, 1988). The mouse and rat FcεRI α chains display 6 and 7 N-glycosylation sequences, respectively (Kinet *et al.*, 1987; Ra *et al.*, 1989), but only one sequon position is conserved among murine, rat, and human α chains.

While a role for N-linked oligosaccharides in the folding of glycoproteins is widely recognized, the mechanisms underlying this function are still unclear. Current understanding does not allow accurate predictions of the role of glycosylation for a particular protein. For some glycoproteins, N-linked oligosaccharides are necessary for their overall stability, while for others the presence of the sugars is only needed during the folding process. Some proteins, such as IgD, fold more efficiently without sugars, while the trimming of the N-linked oligosaccharides is essential for others (reviewed in Helenius (1994)). In the present study, we clarify the role of glycosylation in the maturation of α . We show that the main function of

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¹ The abbreviations used are: CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PCR, polymerase chain reaction; WT, wild-type; BiP, immunoglobulin heavy chain-binding protein.

glycosylation is to facilitate the folding process of α t in the endoplasmic reticulum of eukaryotic cells.

MATERIALS AND METHODS

Antibodies—The following antibodies were used: mAb 15-1, a mouse monoclonal antibody anti-human Fc ϵ RI α (Wang *et al.*, 1992); mouse monoclonal IgE anti-dinitrophenyl antibody; rabbit anti-mouse IgE previously described (Letourneur *et al.*, 1991); 974, a rabbit antibody raised against the peptide VSLNPPWNRI, a sequence corresponding to the amino acids 35 to 44 on the human Fc ϵ RI α chain; and 997, a rabbit polyclonal anti- α t generated against purified human α t. Preparation of peptide and immunization of rabbits were as described previously (Letourneur *et al.*, 1991).

Site-directed Mutagenesis—The cDNA encoding human α t was excised from the plasmid pGEM-3Z (Blank *et al.*, 1991) and subcloned into the HindIII-EcoRI sites of the expression vector pEE14 (Celltech, Berkshire, United Kingdom). Polymerase chain reactions (PCR) (Higuchi *et al.*, 1988) were used to mutate α t. For each mutant, the asparagine, serine, or threonine residues in the Asn-X-Ser/Thr sequence were changed to alanine using one or two nucleotide substitutions. For all constructs, PCR was used to engineer two additional restriction sites: XbaI upstream of the ATG start codon and EcoRI downstream of the TAA stop codon. The PCR products were digested using EcoRI and XbaI, agarose gel purified, and subcloned into the EcoRI and XbaI sites of the expression vector pCDL-SR α 296 (Takebe *et al.*, 1988). Double mutants were generated by PCR using single mutated α t constructs as templates (Higuchi *et al.*, 1988). The subsequent double mutants were then used as PCR templates to construct triple and multiple mutants using the same procedure. All constructs were sequenced using the Sequenase II kit (U. S. Biochemicals, Cleveland, OH). The constructs were designated by numbers defining the N-glycosylation sites mutated, e.g. a mutant of α t lacking glycosylation sites 1, 2, and 3 was referred as Δ 1-3 α t.

Expression of α t in *E. coli* and Periplasmic Preparation—The WT α t and Δ 1-7 α t cDNAs were fused to the pel-B leader and then ligated into the pUC119 vector (Robertson, 1993). *E. coli* strain HB2151 was transformed by electroporation using a Gene Pulser (Bio-Rad), and single colonies isolated on 2 $\times$ YT-agarose plates containing carbenicillin. Cultures from a single transformation colony were grown in 2 $\times$ YT broth containing 0.1% glucose and 100 μ g/ml carbenicillin at 37 $^{\circ}$ C with shaking until an optical density at 600 nm (A_{600}) of 0.5 was reached. Cultures were then equilibrated to room temperature with continuous shaking until A_{600} of 0.9–1.0 was obtained and then induced by adding isopropyl- β -D-thiogalactopyranoside to a final concentration of 0.25 mM. The cultures were harvested after 9 h of induction and centrifuged for 10 min at 3000 $\times$ g. Periplasmic extracts were prepared as follows: the cell pellet was resuspended in 8 ml of 30 mM Tris-HCl, pH 8.0, 20% sucrose, 1 mM EDTA per 0.1 g of cell pellet and incubated at room temperature for 10 min before centrifugation at 8000 $\times$ g at 4 $^{\circ}$ C for 10 min; the resulting pellet was resuspended in 4 ml of 5 mM MgSO $_4$ per 0.1 g of cell pellet for 10 min on ice and centrifuged as before. Both culture supernatants and periplasmic extracts were assayed for IgE binding.

Immunoprecipitation and Western Blotting of α t from *E. coli*—Periplasmic preparations were incubated for 1 h at 4 $^{\circ}$ C with mAb 15-1 or 974 antibodies bound to protein A-Sepharose (Pharmacia Biotech Inc.). For the immunoprecipitation with mAb 15-1 the beads were first loaded with rabbit polyclonal anti-IgG (Jackson ImmunoResearch Labs, West Grove, PA). After washing, the immunoprecipitated proteins were eluted by boiling for 10 min in Laemmli sample buffer (Laemmli, 1970). Samples were then separated by 12% SDS-PAGE under nonreducing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked in 20 mM Tris, pH 7.6, 137 mM NaCl (TBS) containing 2.5% bovine serum albumin for 2 h and then incubated for 2 h at room temperature with a 1:10,000 dilution of rabbit serum 997 in TBS containing 0.1% bovine serum albumin. After washing with TBS containing 0.1% Tween 20, the membranes were incubated for 20 min at room temperature in the same buffer with a 1:20,000 dilution of goat anti-rabbit antibody conjugated with horseradish peroxidase (Bio-Rad). Membranes were developed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Corp.).

Transient Transfection in COS-7 Cells—The COS-7 cell line (American Type Culture Collection, Rockville, MD) was grown to 70–80% confluence in Dulbecco's modified Eagle's medium (Biofluids Inc., Rockville, MD) supplemented with 5% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin (COS medium) at 37 $^{\circ}$ C in a humidified 5% CO $_2$ incubator before transfection by electroporation.

The cells (8×10^6 COS-7 cells at 10^7 cell/ml) were resuspended in COS medium, placed in a Gene Pulser cuvette in the presence of 20 μ g of plasmid DNA and electroporated at 250 V, 500 microfarads. The medium was replaced with fresh COS medium 18 h after transfection and the cells incubated for an additional 24 h.

Metabolic Labeling and Immunoprecipitation—Forty-two h after transfection, COS-7 cells were washed and preincubated for 30 min at 37 $^{\circ}$ C in methionine-cysteine-free Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal calf serum and 2 mM glutamine. Cells were then metabolically labeled with 0.1 mCi/ml [^{35} S]methionine-cysteine (DuPont, Boston, MA) for 3 or 16 h at 37 $^{\circ}$ C. Cells and supernatants were collected separately. Cells were lysed in ice-cold lysis buffer containing 0.5% (w/v) Triton X-100, phosphate-buffered saline, pH 7.4, 10 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and kept on ice for 10 min. Insoluble material was removed by centrifugation at 15,000 $\times$ g for 30 min at 4 $^{\circ}$ C. Cell lysates and culture supernatants were precleared by incubation with protein G-Sepharose (Pharmacia Biotech Inc.) for 30 min at 4 $^{\circ}$ C and α t proteins were immunoprecipitated for 3 h at 4 $^{\circ}$ C with mAb 15-1 or the 974 antibody prebound to protein G-Sepharose. Alternatively, α t proteins were incubated for 1 h with mouse IgE and immunoprecipitated for 2 h with rabbit anti-mouse IgE bound to protein G-Sepharose. Immunoprecipitated proteins were eluted from washed immunoprecipitates with Laemmli sample buffer and resolved by SDS-PAGE. Immunoprecipitation of unfolded α t was as follows: after immunoprecipitation using IgE, α t was eluted by boiling for 5 min in phosphate-buffered saline containing 2% β -mercaptoethanol, 1% SDS, 20 mM iodoacetamide; the supernatant was recovered and diluted to a final concentration of 0.014% SDS and 0.025% β -mercaptoethanol; the diluted sample was divided into 3 aliquots which were immunoprecipitated with IgE, mAb 15-1, or 974 using the conditions described above.

Pulse-Chase Experiments—Transfected cells were metabolically labeled with 0.5 mCi/ml [^{35} S]methionine-cysteine for 20 min at 37 $^{\circ}$ C. Pulse-labeled cells were chased for different periods of time in growth medium. At each time point cells were washed in cold phosphate-buffered saline, centrifuged, and the cell pellets were stored at -80° C. Lysis of the cells and immunoprecipitations were performed as described above. Where indicated, immunoprecipitates were treated with endo- β -N-acetylglucosaminidase H (Endo H) (New England Biolabs) as follows: after washing of the beads, the immunoprecipitated proteins were eluted by boiling for 10 min in 40 μ l of 50 mM sodium citrate buffer, pH 5.5, containing 0.5% SDS and 1% β -mercaptoethanol, and then 20 μ l of the eluate were treated with 400 units of Endo H for 2 h at 37 $^{\circ}$ C.

Stable Transfection of CHO Cells and Selection of Clones—Wild type Chinese hamster ovary (CHO) cells (CHOK1, clone Pro-5;25) were obtained from the American Type Culture Collection. The glycosylation deficient CHO cell lines (Lec3.2.8.1 and ldlD.Lec1) were kindly provided by Dr. P. Stanley (Albert Einstein College of Medicine, New York). Before transfection and during selection, CHO cells were maintained in GEMEM-S (Life Technologies, Inc./BRL) containing 10% fetal calf serum at 37 $^{\circ}$ C in a humidified 5% CO $_2$ incubator. CHO cells (1×10^7) were electroporated (250 V, 500 microfarads) with 20 μ g of SalI linearized pEE14 plasmid containing WT or mutant α t cDNAs. Cells were plated directly into 96-well plates. The medium was aspirated 24 h after transfection and replaced by fresh media containing 25 μ M methionine sulfoximine (Sigma) which inhibits endogenous glutamine synthetase. Clones were detected after 2 weeks (for CHOK1 cells) or 5–6 weeks (for Lec3.2.8.1 and ldlD.Lec1 cells) and assayed for secretion of α t. Positive clones were subjected to gene amplification by progressively increasing the concentration of methionine sulfoximine, up to 100 μ M.

Purification and Deglycosylation of α t Secreted from ldlD.Lec1 Transfectants—The α t secreted from ldlD.Lec1 stable transfectants was purified using an anti- α immunaffinity column prepared as follows: purified mAb 15-1 was covalently linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. Culture medium from ldlD.Lec1 was passed over the immunaffinity column at a constant flow rate of 8.5 ml/h at 4 $^{\circ}$ C. The column was sequentially washed with phosphate-buffered saline containing 0.05% Na $_3$ N and then 50 mM sodium phosphate, pH 7, until the absorbance at 280 nm was reduced to base-line level. The bound α t was eluted with a 5–7 M urea step gradient in 50 mM sodium phosphate, pH 8, 0.05% Na $_3$ N. The α t containing fractions were pooled, concentrated, and the buffer adjusted to 3 M urea, 50 mM sodium citrate, pH 5.5, using an Amicon ultrafiltration cell stirrer with a YM10 membrane (Amicon). The concentrate (about 1 mg/ml) was then incubated for 2 h at 37 $^{\circ}$ C with Endo H (100 units/20 μ g). Following this incubation, the buffer was changed to 3 M urea, 50 mM sodium phosphate, pH 8, and N-glycanase (Genzyme Corp., Cam-

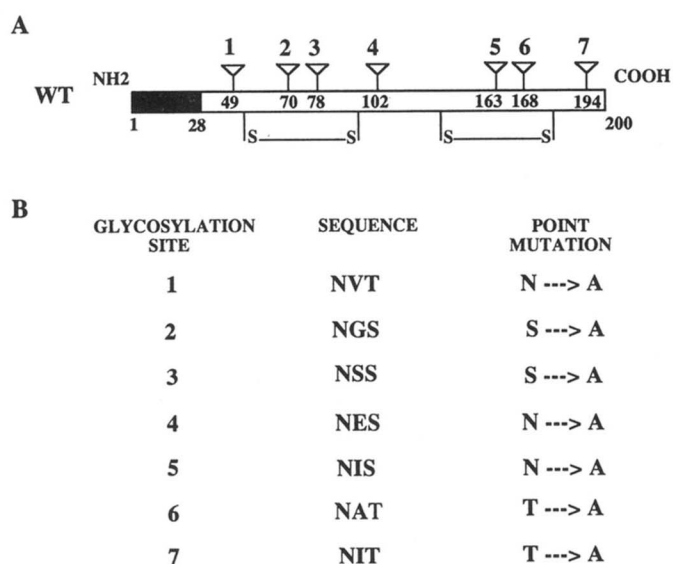


FIG. 1. Mutations of N-linked glycosylation sites. Panel A, schematic representation of FcεRI α chain indicating the positions of the N-linked glycosylation sites within the polypeptide sequence. The shaded bar represents the leader peptide, the open bar, the mature polypeptide. The N-linked glycosylation sites are numbered from 1 to 7 and their positions in the amino acid sequence is indicated. Panel B, amino acid changes for each N-glycosylation site mutation.

bridge, MA) was added at 0.2 unit/20 μg and then the sample was incubated overnight at 37 °C. Samples were analyzed after each step by SDS-PAGE and Coomassie Blue staining.

Detection of IgE Binding Activity of Secreted αt—The IgE-binding activity of αt was determined by calculating the capacity of αt to inhibit the binding of ¹²⁵I-IgE to FcεRI expressed on rat basophilic leukemia cells (RBL-2H3) as described previously (Blank *et al.*, 1991).

RESULTS

The Seven Putative N-Linked Glycosylation Sites on αt Are Glycosylated—We generated a panel of αt mutants in which the seven individual sequons (Asn-X-Thr/Ser) were mutated by substituting the Asn or Thr/Ser residue with Ala (Fig. 1) while trying to preserve as much as possible the consensus amino acid sequence among mouse, rat, and human species (Ra *et al.*, 1989). For instance, the first sequon in the human αt sequence (Asn⁴⁹-Val-Thr) corresponds to the sequence (Lys⁴⁹-Val-Thr) in mouse and rat. Therefore, we chose to mutate Asn⁴⁹ rather than the Thr residue which is conserved.

In order to assess the usage of each individual sequon, COS-7 cells were transfected with mutated or WT αt constructs and metabolically labeled with [³⁵S]methionine-cysteine for 3 h. The WT and mutated αt proteins, including both intracellular and secreted forms, were then immunoprecipitated with the anti-α antibody, mAb 15-1, and resolved on SDS-PAGE. The intracellular WT αt is composed of polypeptides with different degrees of glycosylation. This generates a ladder of bands with the two at the top, around 40 kDa, being most prominent (Fig. 2A). All mutants of αt show a similar pattern but with a 4-kDa increase in electrophoretic mobility. This systematic downward shift indicates that all the potential N-glycosylation sequons of WT αt are glycosylated (see also Fig. 9). The bands corresponding to the secreted forms of WT and mutated αt are much broader, indicating a heterogeneity most likely due to the Golgi processing of αt (Fig. 2B). These broad bands are not detected intracellularly, probably because of a rapid secretion after Golgi processing. The increase in electrophoretic mobility observed intracellularly with the mutated forms of αt is also seen on secreted proteins, except when glycosylation sites 1 and 5 are mutated. This could result from an influence of the local environment of the glycosylation site on the type of carbohy-

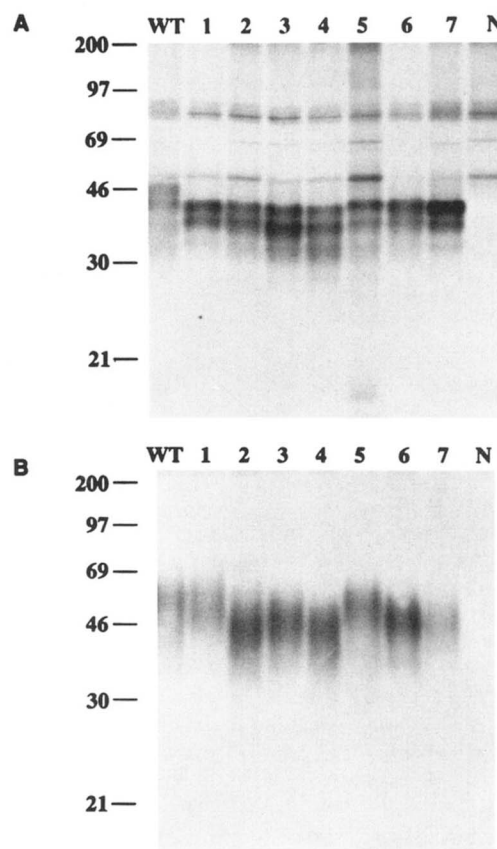


FIG. 2. Expression of WT αt and αt lacking single N-glycosylation sites in COS-7 cells. COS-7 cells transfected transiently with a plasmid encoding WT αt or single-site N-glycosylation mutants were metabolically labeled with [³⁵S]methionine-cysteine for 3 h at 37 °C. The αt proteins were immunoprecipitated with mAb 15-1 from cell lysates (A) or cell supernatants (B) before analysis by 14% SDS-PAGE in reducing conditions. The designation of the transfected construct is indicated above each line. The numbers correspond to the N-glycosylation sequons mutated. N, untransfected cells. The position of the *M_r* standards is indicated on the left.

drate processing to which this particular site is subjected.

Mutation of Several N-Linked Glycosylation Sites Decreases the Amount of Secreted Active αt from Eukaryotic Cells—To determine whether single or multiple mutations of the seven N-linked glycosylation sequons could affect the efficiency of secretion of active αt, we constructed mutants of αt in which two, three, four, five, six, or all seven N-oligosaccharide sites were inactivated. The various mutants were transfected into COS-7 cells and the IgE-binding capacity of the corresponding αt secreted in the supernatant was determined (Table I). There was no significant difference in the IgE-binding capacity of the single αt mutants when compared with the WT αt (data not shown). However, there was a cumulative effect on the IgE-binding capacity as the number of mutations increased. No activity was detected in cells transfected with the Δ1-7 αt construct. This effect could be attributed to either the lack of N-glycosylation or the changes in the amino acid sequence.

Mutation of the Seven N-linked Glycosylation Sites Does Not Affect the Production of Active αt from *E. coli*—To analyze whether the substitution of amino acids could affect the folding of αt we compared unglycosylated WT αt and Δ1-7 αt expressed in *E. coli*. The IgE-binding activity was determined for the respective proteins recovered from the culture supernatant and from the periplasmic space. IgE binding of Δ1-7 αt was 82 ± 15% (*n* = 2) of WT αt from the culture supernatant and 91 ± 17% (*n* = 2) of WT αt in samples isolated from periplasmic extracts. Periplasmic extracts from WT αt and Δ1-7 αt trans-

TABLE I
Effect of mutations of N-linked glycosylation sites on the secretion of active α

Mutated sites	No. of mutations	IgE-binding activity of secreted α by transfected COS-7 cells	No. of experiments
		% of wild type	
1 and 2	2	77	1
1, 2, and 3	3	57 ± 12	2
4, 5, and 6	3	49 ± 11	3
1-4	4	40 ± 10	3
1-5	5	18 ± 12	4
3-7	5	3 ± 1	3
1-6	6	1	1
1-7	7	0	2

formed bacteria were subjected to immunoprecipitation with mAb 15-1. The precipitates were analyzed by SDS-PAGE and immunoblotted with the polyclonal antibody 997. Similar amounts of α were immunoprecipitated from both WT α and Δ1-7 α (Fig. 3). Thus the mutation of the seven glycosylation sites does not decrease the secretion of active α in *E. coli*.

The Polyclonal Anti-peptide Antibody 974 and mAb 15-1 Recognize Different Forms of α—To further analyze why the COS-7 cell product of the Δ1-7 α construct does not bind IgE, we generated a polyclonal anti-peptide antibody (974) against a sequence of 10 amino acids close to the amino terminus of α, a sequence which does not contain any N-linked glycosylation sites. We compared the reactivity of mAb 15-1 and 974 toward WT α and Δ1-7 α expressed in *E. coli*. Both antibodies immunoprecipitated proteins around 20 kDa (Fig. 4A). However, the polypeptide precipitated by 974 has a slightly faster electrophoretic mobility. To test whether the two antibodies reacted with different species of α, we performed a depletion experiment. After a first immunoprecipitation with mAb 15-1 or 974, the resulting supernatants were subjected to a second immunoprecipitation with the same antibody to insure complete depletion of the reactive α species (Fig. 4B). The α species remaining in the supernatant after two sequential mAb 15-1 or 974 immunoprecipitations were then immunoprecipitated with 974 or mAb 15-1, respectively (Fig. 4B). Depletion with mAb 15-1 has no effect on the form and the amount of α that is precipitated by 974, and vice versa. Therefore the antibodies 15-1 and 974 react with different species of α and do not display any cross-reactivity. In addition, these antibodies immunoprecipitate similar amounts and apparently similar species from both the WT and Δ1-7 α constructs. Thus, mAb 15-1 and antibody 974 do not detect any difference of reactivity between the WT α and the Δ1-7 α when expressed in *E. coli*.

The Polyclonal Antibody 974 Reacts Only with Denatured α Expressed in Eukaryotic Cells—We analyzed the reactivity of the antibody 974 toward the fully glycosylated WT α produced in COS-7 cells. When compared to IgE or mAb 15-1, 974 immunoprecipitates WT α very poorly (Fig. 5, compare lane 3 with lanes 1 and 2). Therefore, unlike IgE and mAb 15-1, 974 does not recognize the native WT α. By contrast, after denaturation in SDS, WT α can be precipitated by 974 but not by IgE or mAb 15-1 (compare lane 6 with lanes 4 and 5). Thus, the antibody 974 recognizes only denatured species of α incapable of binding IgE or mAb 15-1.

Mutation of Several N-Linked Glycosylation Sites Affects the Folding of α—The antibodies mAb 15-1 and 974 were used to study the effect of multiple mutations on the folding, stability, and secretion of α in eukaryotic cells. A pulse-chase metabolic labeling was conducted with COS-7 cells transfected with WT α (Fig. 6A). The labeled proteins from the cell lysates were immunoprecipitated with mAb 15-1 or 974, treated or not with Endo H, and resolved by SDS-PAGE. Secreted α was similarly

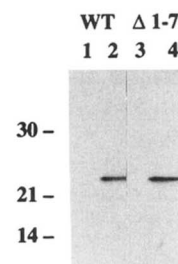


FIG. 3. Expression of WT α and mutant Δ1-7 α in *E. coli*. Periplasmic extracts from *E. coli* cells transformed with either WT α (lane 2) or mutant Δ1-7 α (lane 4) DNA were immunoprecipitated with mAb 15-1. Control immunoprecipitates using an isotype matched antibody are in lanes 1 (WT α) and 3 (Δ1-7). The immunoprecipitates were subjected to SDS-PAGE, and immunoblotted with antibody 997. The position of the M_r standards is indicated on the left.

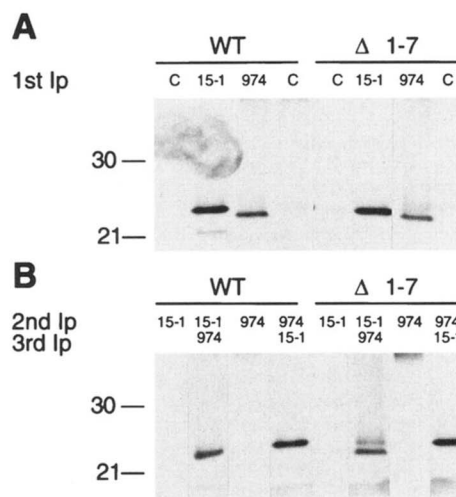


FIG. 4. Reactivity of WT α and Δ1-7 α produced in *E. coli* with mAb 15-1 and antibody 974. Panel A, periplasmic extracts from *E. coli* cells transformed with either WT α or mutant Δ1-7 α DNA were immunoprecipitated with mAb 15-1, antibody 974, or control antibodies. Panel B, the resulting supernatants were subjected to a second immunoprecipitation with the same antibody, mAb 15-1 or 974. The supernatants depleted with mAb 15-1 or 974 were then immunoprecipitated with 974 or mAb 15-1, respectively. All immunoprecipitates were resolved on SDS-PAGE and immunoblotted with antibody 997. The position of the M_r standards is indicated on the left.

analyzed except for the omission of Endo H treatment. After a 20-min pulse, nearly all of the intracellular WT α is Endo H sensitive (Fig. 6A), indicating a pre-Golgi localization. As expected, no material is secreted at that time. In addition, very little material is precipitated with 974 when compared with mAb 15-1. After a 2-h chase, about one-third of the labeled WT α is secreted in the medium; after 6 h of chase, most is secreted. The fraction remaining intracellularly is still Endo H sensitive and is not precipitated by 974. The absence of Endo H-resistant α species in the intracellular compartments confirms that the processed WT α is rapidly secreted after carbohydrate processing in the Golgi.

The mutant with the first five sequons inactivated, Δ1-5 α, shows an increased electrophoretic mobility when compared with WT α (Fig. 6B). The amount of secreted Δ1-5 α which can be immunoprecipitated with mAb 15-1 is much less than that of secreted WT α and corresponds well with the drop in IgE-binding activity seen in the supernatant of the Δ1-5 α mutant (see above). The amount of intracellular Δ1-5 α immunoprecipitated by mAb 15-1 also decreases. In contrast, 974 immunoprecipitates more intracellular Δ1-5 α than WT α (compare Fig. 6, B and A). A large amount of Δ1-5 α remains stable and undegraded intracellularly throughout the chase.

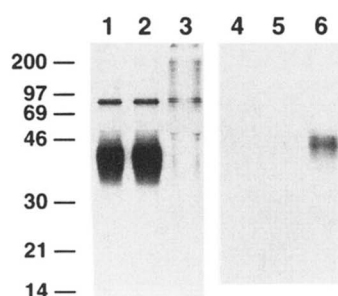


FIG. 5. Demonstration of the specificities of mAb 15-1, 974, and IgE. COS-7 cells transiently expressing WT α chain were metabolically labeled with [^{35} S]methionine-cysteine for 3 h at 37 °C. Cell lysates were divided into 3 portions: four-sixths was immunoprecipitated using IgE, one-sixth with mAb 15-1, and one-sixth with 974. Three-fourths of the washed IgE precipitate was then denatured and divided into 3 equal portions which were immunoprecipitated with either IgE, or mAb 15-1 or 974. Immunoprecipitates were analyzed by 12% SDS-PAGE under reducing conditions. Lanes 1-3, immunoprecipitation in native conditions; lanes 4-6, immunoprecipitation after denaturation; lanes 1 and 4, immunoprecipitation using IgE; lanes 2 and 5, using the mAb 15-1; lanes 3 and 6, using 974. The position of the M_r standards is indicated on the left.

Virtually no 974 reactive Δ 1-5 α is secreted. Therefore, the mutation of sequons 1-5 affects the intracellular folding and hence the secretion of Δ 1-5 α , but causes little change in its stability.

The effect of multiple mutations is even clearer when all the sequons are mutated. Under these conditions, mAb 15-1 does not precipitate any material around 22 kDa from intracellular or secreted Δ 1-7 α (Fig. 6C). However, intracellular Δ 1-7 α is clearly seen after 974 immunoprecipitation. Thus, unlike in *E. coli*, mAb 15-1 and antibody 974 detect differences in reactivity between WT α and Δ 1-7 α expressed in eukaryotic cells. The amount of α detected by mAb 15-1 decreases with the number of mutations. In striking contrast, the amount of α detected by antibody 974 increases with the number of mutations (compare panels A, B, and C in Fig. 6). Therefore, increasing the number of mutations and thereby decreasing the glycosylation, induces misfolding of α in the pre-Golgi compartment. Taken together, these data indicate that glycosylation of α is necessary for its efficient folding in the endoplasmic reticulum of eukaryotic cells.

Prevention of Glycosylation of α by Tunicamycin or Mutation Has Similar Effects on Folding and Secretion—We previously published that stable CHO transfectants treated with tunicamycin could secrete deglycosylated α capable of binding IgE (Blank *et al.*, 1991). However, the amount of active α secreted in that experiment was very small when compared with fully glycosylated α . To test whether tunicamycin-induced deglycosylation could affect the folding of α in a manner similar to the mutations, we compared the folding of Δ 1-7 α with the folding of unglycosylated WT α obtained from cells treated with tunicamycin. COS-7 cells transfected with WT α were treated, or not, with tunicamycin and labeled with [^{35} S]methionine-cysteine for 3 h. Both intracellular (Fig. 7A, lane 1) and secreted WT α (Fig. 7A, lane 2) were precipitated with mAb 15-1, but much less material was seen after treatment of the cell with tunicamycin (Fig. 7A, lanes 3 and 4). Nevertheless, after longer exposure, small amounts of WT α deglycosylated with tunicamycin were detected with mAb 15-1, both as intracellular and secreted material (Fig. 7B, lanes 3 and 4). Similar results to those obtained with tunicamycin-treated cells were obtained with COS-7 cells transfected with Δ 1-7 α (Fig. 7, A and B, lanes 5 and 6). Thus deglycosylation whether by using tunicamycin or by mutations affects dramatically the amount of intracellular and secreted α detected by mAb 15-1. The higher

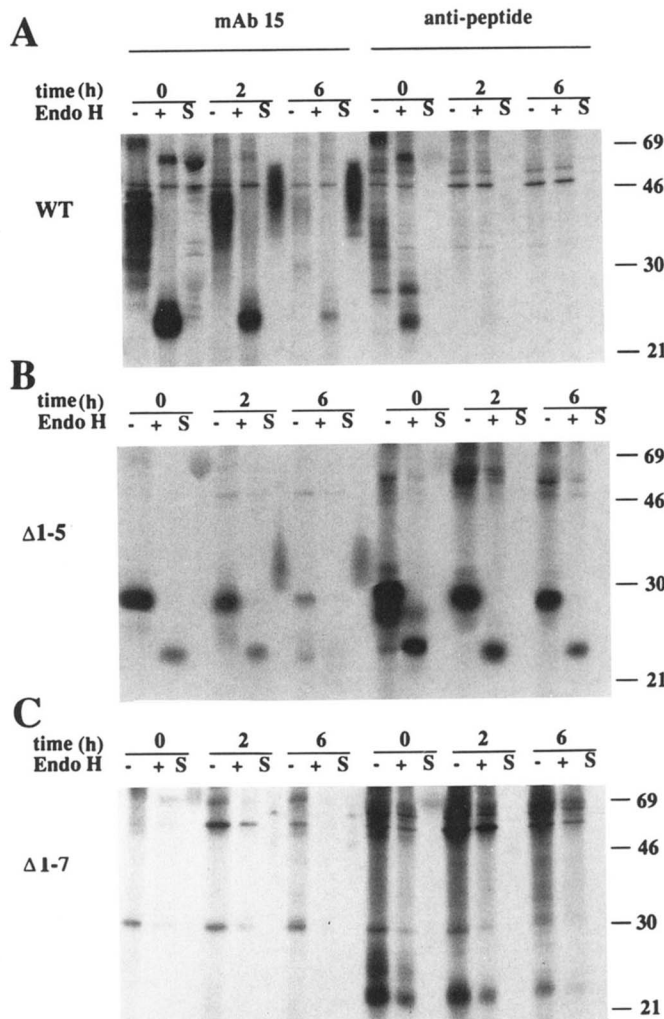


FIG. 6. Pulse-chase analysis of WT α , mutant Δ 1-5 α , and mutant Δ 1-7 α in transfected COS-7 cells. COS-7 cells transfected transiently with plasmids encoding WT- α (A), Δ 1-5 α (B), or Δ 1-7 α (C) constructs were pulse-labeled with [^{35} S]methionine-cysteine for 20 min at 37 °C and chased for 0, 2, or 6 h. The α proteins were immunoprecipitated either with mAb 15-1 (left) or antibody 974 (right). Immunoprecipitates from cell extracts were either treated with Endo H (+) or not (-), while immunoprecipitates from supernatants (S) were untreated before analysis on 14% SDS-PAGE under reducing conditions. Note that panels A and B correspond to an overnight exposure of the gels while panel C corresponds to a 4-day exposure. The position of the M_r standards is indicated on the right.

amount of α from tunicamycin-treated WT α cells (Fig. 7B, lane 3) compared to that of α from Δ 1-7 α cells (lane 5) probably reflects different levels of transcription/translation by the two transfectants. In agreement with the results shown in Fig. 6C, the antibody 974 immunoprecipitates more Δ 1-7 α material than does mAb 15-1 (compare lanes 5 and 6 in Fig. 7, B and C) and only from the intracellular compartment. In addition, antibody 974 immunoprecipitates similar amounts of material from the intracellular compartment of tunicamycin-treated cells and from cells transfected with Δ 1-7 α (compare lanes 3 and 5 in Fig. 7C). Thus misfolded forms of α detected by 974 are seen in equivalent amounts in tunicamycin-treated cells or in cells transfected with Δ 1-7 and very little folded material is detected by mAb 15-1 in both types of cells. These data confirm that glycosylation is important for the proper folding and secretion of α in eukaryotic cells.

Processing of N-Linked Oligosaccharides and O-Linked Glycosylations Are Not Required for Transport and Secretion of an Active α —The trimming of the N-linked oligosaccharides is an

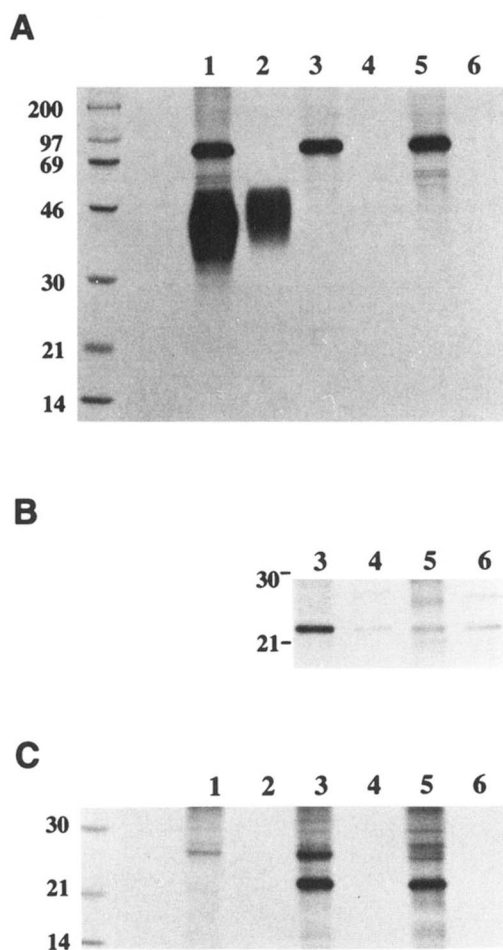


FIG. 7. Comparison of WT α from cells grown in the presence of tunicamycin with mutant Δ 1-7 α . COS-7 cells transiently expressing WT α grown without (lanes 1 and 2) or with tunicamycin (lanes 3 and 4) or expressing the mutant Δ 1-7 α (lanes 5 and 6) were metabolically labeled with [^{35} S]methionine-cysteine for 3 h at 37 °C. Cell lysates (lanes 1, 3, and 5) or supernatants (lanes 2, 4, and 6) were divided in two portions and immunoprecipitated using the mAb 15-1 (A and B) or 974 (C) before analysis on 12% SDS-PAGE under reducing conditions. Note that Panel B is a longer exposure of the gel in Panel A. The position of the M_r standards is indicated on the left.

essential step in the folding and transport of many proteins (Helenius, 1994). To study whether this is the case for α , we took advantage of two CHO cell lines which have defects in the glycosylation pathways. The Lec3.2.8.1 cell line produces proteins with all *N*-linked carbohydrates in the oligomannosyl form and *O*-linked carbohydrates initiating with *N*-acetylgalactosamine. The ldlD.Lec1 cell line gives rise to proteins with the same *N*-linked carbohydrate as Lec3.2.8.1 but with *O*-linked oligosaccharides completely absent (Stanley, 1989).

The α -containing pEE14 vector was transfected into the wild type CHO (CHOK1) and the two mutant Lec3.2.8.1 and ldlD.Lec1 cell lines. The α secreted from stable clones was metabolically labeled with [^{35} S]methionine-cysteine for 16 h, precipitated with IgE, and analyzed by SDS-PAGE (Fig. 8). As expected, the fully glycosylated α secreted by CHOK1 migrates as a broad heterogeneous band around 50 kDa. The electrophoretic mobility of α secreted by the mutant ldlD.Lec1 and Lec3.2.8.1 cell lines shows a 10–15 kDa reduction. In the three cell lines, secreted α can be precipitated with IgE and is therefore properly folded. Furthermore, after 16 h of metabolic labeling, α is detected almost exclusively in the cell supernatant with virtually no intracellular α remaining (data not shown). This indicates an efficient secretion of α . Therefore

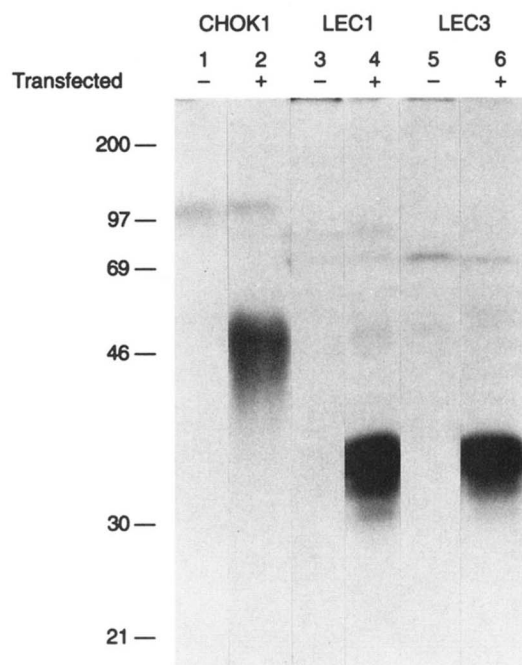


FIG. 8. Secretion of α by stable CHO transfectants. CHOK1, ldlD.Lec1 (*Lec1*), and Lec3.2.8.1 (*Lec3*), either untransfected (lanes 1, 3, and 5) or stably expressing α (lanes 2, 4, and 6), were metabolically labeled with [^{35} S]methionine-cysteine for 16 h at 37 °C. The α proteins were precipitated from the cell supernatants with IgE before analysis on 11% SDS-PAGE under reducing conditions. The position of the M_r standards is indicated on the left.

the processing of high mannose-type to complex-type oligosaccharides and the addition of *O*-linked oligosaccharides are not required for transport and secretion of efficiently folded α .

N-Linked Oligosaccharide Are Not Necessary for the Activity of α —We took advantage of the Endo H sensitivity of α secreted by ldlD.Lec1 to visualize the utilization of the various *N*-glycosylation sites. Truncated α secreted by stably transfected ldlD.Lec1 cells was purified on mAb 15-1 affinity column and then sequentially deglycosylated with Endo H and *N*-glycanase. After each step, the deglycosylated products were analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 9). The incomplete digestion with Endo H allows to visualize a ladder of bands most likely generated by glycosylation at the seven *N*-linked glycosylation sites (see above). Complete digestion was achieved with a further treatment of the sample with *N*-glycanase. The apparent M_r of 23,000 observed thereafter corresponds well with the M_r of 19,275 calculated for α .

The IgE-binding activity of glycosylated and deglycosylated α was compared quantitatively. No difference was detected (data not shown). Therefore once folded properly, α remained active and was still capable of binding IgE.

DISCUSSION

Analyzing the role of carbohydrates in the folding and stability of glycoproteins is an area of intensive investigation. At present, our understanding of carbohydrate function is still fragmentary. Recent works indicate that glycoprotein glycans may play multiple roles and that different proteins have different requirements for carbohydrates. Glycoprotein glycans have been shown to be involved in several functions which include regulation of intracellular trafficking, modulation of enzyme and hormone activities, and participation in cell-cell interactions (Lis and Sharon, 1993). It has been proposed that *N*-linked oligosaccharides could play several roles during the conformational maturation of glycoproteins (Helenius, 1994). Although for many glycoproteins it has been reported that

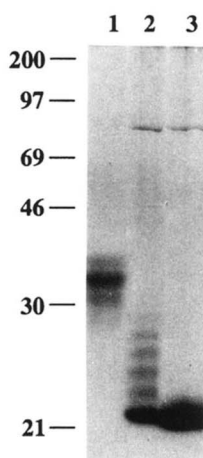


FIG. 9. Enzymatic deglycosylation of α secreted by IdD.Lec1. Secreted α was purified on an agarose-bound mAb 15-1 column (lane 1). The affinity purified product was digested with Endo H (lane 2), followed by further deglycosylation with *N*-glycanase (lane 3). Proteins were resolved on SDS-PAGE under reducing conditions and stained with Coomassie Blue. The position of the M_r standards are indicated on the left.

N-linked oligosaccharides are needed for folding, some, such as IgM or major histocompatibility complex molecules fold efficiently without any. Carbohydrate trimming may also be essential for the folding of many proteins (reviewed in Helenius (1994)).

Our first question was whether the core *N*-glycosylation which occurs in the pre-Golgi compartment has any relevance to the folding of α . We first generated a series of single glycosylation site mutants of α (Fig. 1). Transient expression of the single site mutants in COS-7 cells shows that all 7 potential *N*-glycosylation sites of the α chain are glycosylated in the mature native molecule (Fig. 2). The individual elimination of any one site has no significant effect on the secretion of active α (data not shown).

Once we had established that all 7 glycosylation sites were modified *in vivo*, and that no one site was particularly critical for the folding process, we constructed a panel of multiple mutants to test the effect of the cumulative loss of core glycosylation on the intracellular transport of α . As illustrated in Table I, there is a strong cumulative effect of the number of mutated glycosylation sites on the amount of active α secreted, raising the question of the cause of the decreased secretion. Possible mechanisms include a primary effect of the amino acid substitutions, misfolding due to the loss of *N*-glycosylation, and either increased degradation or intracellular retention of unglycosylated α . While single replacements of serine or threonine with alanine rarely induce conformational changes (Argos, 1987), the effect of multiple substitutions could have resulted in the misfolding of α .

To determine whether the multiple substitutions in the primary sequence could affect the folding, we expressed WT α and mutant Δ 1-7 α in *E. coli*. As shown in Fig. 3, the mutation of the seven *N*-linked glycosylation sites does not influence the production and the folding of α . This was also confirmed with IgE-binding activity data where no significant difference was noticed between the WT α and the mutant Δ 1-7 α . The above data demonstrate that the amino acid substitution *per se* does not interfere with the folding of α .

To compare the fate of the various forms of α , WT α , Δ 1-5 α , and Δ 1-7 α , we used a monoclonal antibody that precipitates only efficiently folded α molecules (mAb 15-1) and an anti-peptide antibody that precipitates unfolded α molecules (974) (Figs. 4 and 5). In transfected COS-7 cells, immediately

after biosynthetic labeling, a mixture of properly folded and misfolded α is detected intracellularly in all 3 cases. The ratio of misfolded over properly folded α increases with the extent of loss of glycosylation (Fig. 6). The Endo H sensitivity of misfolded WT and Δ 1-5 α indicates retention in a pre-Golgi compartment. The low levels of secretion of Δ 1-5 and Δ 1-7 α are most likely due to retention of misfolded α .

The above data seem to contradict our previously published work on tunicamycin-treated cells where glycosylation was demonstrated not to be required for the secretion of active α (Blank *et al.*, 1991). However, in this previous study no attempt was made to determine the efficiency of secretion. To clarify this apparent contradiction we used the antibodies mAb 15-1 and 947 to compare α produced by the Δ 1-7 multiple mutant to that produced by WT in cells treated with tunicamycin. Tunicamycin treatment of COS-7 cells transiently transfected with WT α results in misfolding and intracellular trapping of approximately 95% of the unglycosylated proteins, nearly identical to the results with the Δ 1-7 (see Fig. 7, A and C). Despite the virtually complete absence of glycosylation in both cases, small amounts of efficiently folded α are formed and secreted (see Fig. 7B). This confirms the previous work demonstrating that glycosylation is not absolutely required for secretion (Blank *et al.*, 1991), but indicates that the loss of *N*-glycosylation results in misfolding of α and a substantial decrease in secretion of α . Taken together, these results demonstrate that core *N*-glycosylation facilitates correct folding, with the individual contributions of the carbohydrate chains being additive in promoting folding and, as a consequence, transport of α through the secretory pathway.

Our next question was whether the maturation of core *N*- or *O*-glycosylation which occurs in the Golgi compartment is involved in the intracellular transport and in the folding of α . We chose to address this question by stably expressing α in two glycosylation deficient cell lines, Lec3.2.8.1 and IdD.Lec1. Both cell lines produce α which binds IgE (Fig. 8) and mAb 15-1 (data not shown). In addition, equilibrium labeling shows that the α produced is completely secreted with essentially no retention in either the Golgi or the endoplasmic reticulum. Retention would be expected if the missing saccharide residues were necessary for proper folding. This result is identical to that obtained after transfection of α into CHOK1 cells, the parental cell line for both Lec3.2.8.1 and IdD.Lec1. These results demonstrate that the Golgi compartment mediated maturation of *N*-glycosylation and the addition of *O*-glycosylation are dispensable for the proper folding and efficient intracellular transport of α .

Our data indicate that α belongs to a group of molecules such as human transferrin receptor, human glucuronidase, and influenza A virus hemagglutinin which are also retained in the endoplasmic reticulum as a consequence of a loss of oligosaccharides (Helenius, 1994). However, once folded, these molecules remain stable. Therefore, the oligosaccharides seem to be necessary only in the endoplasmic reticulum and pre-Golgi compartment. These striking features have been the basis for the theory of specific interactions between the carbohydrate moieties of glycoprotein and the folding machinery. These interactions would be necessary for efficient folding and would control the retention of the non-folded or partially folded forms in the endoplasmic reticulum. Our results are consistent with this concept.

Interactions of proteins with molecular chaperones such as BiP have been well documented. However, these chaperones interact directly with the polypeptide moiety (reviewed in Hartl *et al.* (1994)). Interactions with chaperones that are dependent on the presence of carbohydrates have been investigated only

recently. One example is the folding of vesicular stomatitis virus G protein which interacts sequentially with BiP and calnexin (Hammond and Helenius, 1994). The latter interaction is necessary for proper folding and is prevented by removal of the sugars or by inhibition of the endoplasmic reticulum glucosidases. It will be interesting to investigate whether α t could also interact with calnexin or a similar, carbohydrate-dependent, chaperone.

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