Glycoconjugate Journal 15, 987–993 (1998)

Increased elongation of *N*-acetyllactosamine repeats in doubly glycosylated lysozyme with a particular spacing of the glycosylation sites

Ralph Melcher, Hans-Wilhelm Grosch, Oliver Grosse and Andrej Hasilik*

Institute of Physiological Chemistry, Philipps-Universität Marburg, Karl-von-Frisch-Straße 1, D-35033 Marburg, Germany

Lysozyme is an example of an extensively studied secretory enzyme. Glycosylated mutant human lysozyme has been used as a model in studies on the biosynthesis of *N*-acetyllactosamine repeats in *N*-linked oligosaccharides. We examined the biosynthesis of the repeats in two doubly glycosylated mutants and describe here a rapid purification and separation of singly and doubly glycosylated molecules. In one of the mutants, the elongation of the repeats is enhanced if the molecules are doubly glycosylated, but not if the carbohydrate is attached to either site individually. This enhancement is not seen in the other doubly glycosylated mutant. Since lysozyme is not structurally related to glycoproteins bearing carbohydrate with *N*-acetyllactosamine repeats, we propose that in multivalent substrates the synthesis of the repeats can be promoted by a proper spacing of the elongated carbohydrate antennae in addition to any role of the protein backbone.

Keywords: ***N*-acetyllactosamine, glycosylation, lysozyme, polylactosamine length**

Abbreviations: **CHO, chinese hamster ovary; dL, lysozyme bearing two complex oligosaccharides; eβ, *endo*-β-galactosidase; F2, *endo*-β-*N*-acetylglucosaminidase F2; GPF, glycopeptidase F; mL lysozyme bearing mannose-rich oligosaccharides; L, lysozyme; L', lysozyme bearing one or two possibly fucosylated *N*-acetylglucosamine residues; sL, lysozyme bearing one complex oligosaccharide.**

Introduction

It has been established that elongated *N*-acetyllactosamine repeats and their derivatives, such as the sialylated Lewis^x substance, play an important role in molecular and cellular recognitions [1–6]. Binding by selectins of sialylated Lewis^x and similar ligands initiates the attachment to and the rolling of white blood cells along the endothelial surface and probably also of certain metastasing tumor cells. Patel et al. have shown that among P-selectin homologues those with a high number of the internal homology provide a particularly high attachment activity [7]. Similarly, the length of the oligosaccharides in the ligands of selectin is likely to increase the chance of the intercellular attachment. Although the length of *N*-acetyllactosamine repeats is of a significant biological importance, little is known about the regulation of their synthesis and length. Nevertheless, the issue has been addressed in several reports. Briefly, the repeats occur in various glycoconjugates such as proteoglycans (keratan sulfate), glycoproteins, and glycosphingolipids as constituents of blood group substances [8] and of tumor-associated markers [9, 10]. Their synthesis depends on development and differentiation of cells. In O-linked oligosaccharides, *N*-acetyllactosamine repeats are associated with the core 2 structure [11]. The elongation of the repeats correlates with the synthesis of the β-1,6-linked branches and antennae [12, 13]. The length of the repeats also reflects the retention time of the glycoconjugates in the Golgi compartment, where the elongation takes place [14]. In cells examined so far, β-galactosyl transferase is present in an excess. Therefore, the elongation is enhanced when the activity of the β-1,3-*N*-acetylglucosaminyl transferase "i" is increased [15–17]. It may be speculated that proteins bearing *N*-acetyllactosamine repeats are endowed with structures directing the elongation of the repeats . In the primary structure of the lysosomal membrane proteins lamp-1 and lamp-2, the repeats are restricted to two groups among the 16 *N*-linked carbohydrate side chains present [18]. However, nothing is known about the signals responsible for this pattern of glycosylation. We have reported that in glycosylated mutants of human lysozyme *N*-acetyl-

*To whom correspondence should be addressed. Tel: +49-6421-285020; Fax: +49-6421-286957; E-mail: hasilik@mailer.uni-marburg.de

lactosamine repeats are found when expressed in Chinese hamster ovary (CHO) cells [19]. Further, we have shown that in glycosylated lysozyme the repeats are present nearly exclusively in biantennary complex oligosaccharides [20] and that the elongation pattern is similar in oligosaccharides attached to either residue 22 or 68 [19]. The present communication describes a novel mutation in human lysozyme that generates a glycosylation site at residue 49 and the occurrence of double glycosylations at residues 22 plus 49 and 49 plus 68. In the latter mutant, the elongation of *N*-acetyllactosamine repeats is more extensive than in the former. Interestingly, in lysozyme mutants bearing any of the three mutations separately, the elongation is rather sparse, and oligosaccharides without repeats represent the most prominent species. In contrast, in a mutant bearing the carbohydrate at residues 49 plus 68, the elongated oligosaccharides are the prevailing species. We hypothesize that the synthesis of the repeats is stimulated by a particular steric presentation of closely spaced oligosaccharide side chains.

Materials and methods

Cells and culturing

Wild type and Lec2 mutant CHO cells [21] were obtained from the American Type Culture collection (ATCC CCL61 and ATCC CRL1736, respectively). The cells were grown in α-minimum essential medium (Life Technologies, Eggenstein) supplemented with 10% (v/v) fetal bovine serum (Sigma Chemie, Deisenhofen), penicillin (100 U/ml) and streptomycin (100 μg/ml).

Modeling, mutagenesis, and plasmids

The design of mutations introducing glycosylation signals into the amino acis sequence of human lysozyme was supported by molecular modeling. This was based on the crystal structure of human lysozyme [22] and used the program Whatif, which was provided by Dr. G. Vriend, EMBL, Heidelberg. We examined three mutants of human lysozyme with single *N*-glycosylation signals and two mutants with two glycosylation signals each using the eucaryotic expression vector pBEHpac18 [19]. The preparations of mutant I with the replacement G22N and of mutant II with replacements G68N/P71S have been described previously [19]. Mutant IV (D49N) was prepared by site directed mutagenesis [23], using the oligonucleotide 5′-CAGTGGTTCTGTTTCCAGCATTGTA. Mutants I/IV and II/IV contained the corresponding combination of the glycosylation signals. Mutant II/IV cDNA was subjected to further mutagenesis to enhance the rate of the glycosylation by substituting a threonine for the serine residue [24] in the glycosylation sequon at site IV. This mutation was referred to as II/IVT. It was introduced into the II/IV mutant by primer-mediated mutagenesis [25, 26]. Briefly, the downstream portion of the cDNA was amplified using the mutagenesis primer 5′-GCTGGAAACAGAA**C**CACTGATTATGG (the mutation is indicated by the bold character) and a vector primer 5′-CTGCATTCTAGTTGTGGTTTGTCC. The upstream portion was amplified with the primers 5′-CCATAATCAGTG**G**TTCTGTTTCCAGC and 5′-GCTCCTCGAGGAACTGGAAAACCAG. The primers shown without bold characters corresponded to vector sequences and were also used in the final amplification. In five independent mutant clones, the expected sequence was confirmed by the fluorescent dideoxy chain termination method using an ABI377 DNA sequencer (Pharmacia, Freiburg) . One of the examined mutant II/IVT cDNAs was cloned into the EcoRI site of plasmid pMCI. This plasmid was derived from plasmid pBEHpac18 by one of us (R.M., unpublished). Briefly, the plasmid was altered by replacing its SV40 promoter with a cytomegalovirus promoter and inserting an intron from vector pMT2 [27] between the multiple cloning site and the polyadenylation signal.

Transfection and expression

Subconfluent cultures of CHO cells grown in 25 cm^2 flasks were transfected with vectors encoding mutant lysozyme cDNAs. The cells were transfected by lipofection using the method of Felgner et al. [28], as modified in the manual of Life Technologies (Eggenstein), with 7.5 μg circular DNA per 2.5×10^5 cells. After a selection of the transfected cells with puromycine (14 μg/ml), stable transfectants were isolated and examined for the secretion of lysozyme activity using the lysoplate method [29].

Characterization of glycosylated lysozyme forms

CHO cells expressing lysozyme were metabolically labeled with a mixture of radioactive methionine and cysteine (Tran^{35}S-label from ICN Biomedicals, Eschwege, specific activity 40 GBq/mmol). The cells were labeled for 18 h with 4 MBq in 3.4 cm diameter dishes. Labeled lysozyme was isolated by immunoprecipitation, separated by size in SDS-PAGE [30] and visualized by fluorography as described previously [20, 31]. For quantitative purposes, the radioactivity was detected by phosphor imaging Molecular Dynamics, Krefeld). Nonradioactive lysozyme was isolated from spent culture medium of a clone (referred to as 2A6) expressing the mutant II/IVT. In an immunoaffinity step, lysozyme was bound to a column with an immobilized rabbit polyclonal *anti*-human lysozyme antibody. The antibody itself was isolated from the antiserum with immobilized human lysozyme. The immobilization was performed with cyanogen bromide-activated Sepharose 4B-CL (Pharmacia). The bound proteins were eluted with 2 M $MgCl_2$. Lysozyme was enriched by FPLC chromatography using a hydroxylapatite column (CHT2-I, BioRad, München). At

this step, lysozyme bearing distinct numbers of carbohydrate side chains was separated into different fractions. The carbohydrate was examined for sensitivity to glycopeptidase F, *endo*-glycosidase F2, and *endo*-β-galactosidase [20]. After a separation in SDS-PAGE the nonradioactive protein bands were visualized by silver staining [32].

Results and discussion

Choice of the glycosylation sites

Several signals for glycosylation were introduced into the cDNA of human lysozyme. To achieve this goal with a minimum of structural disturbance, the signals were introduced in loops or extended chain regions at the surface of the molecule as shown in Figure 1. Mutants I and II were described previously [19]. Near the C-terminus, the lysozyme molecule contains an extended stretch of residues that appears suitable to accommodate a glycosylation signal. However, the use of a site that has been created by the substitution D120S is negligible [19]. It is probable that the terminal portion of the molecule is subject to a rapid folding that precludes the glycosylation. To avoid similar problems, a glycosylation signal was introduced at another position that was homologous to the carbohydrate attachment site in β-lactoglobulin [33]. This was achieved by the substitution D49N and resulted in mutant IV. Similar to the previously prepared mutants I (G22N) and II (G68N/P71S) [19], mutant IV (D49N) was enzymatically active (not shown). The mutations were unlikely to affect grossly the structure of the protein, since neither the export of the mutant proteins from the endoplasmic reticulum nor their reactivities with antilysozyme antibody were impaired.

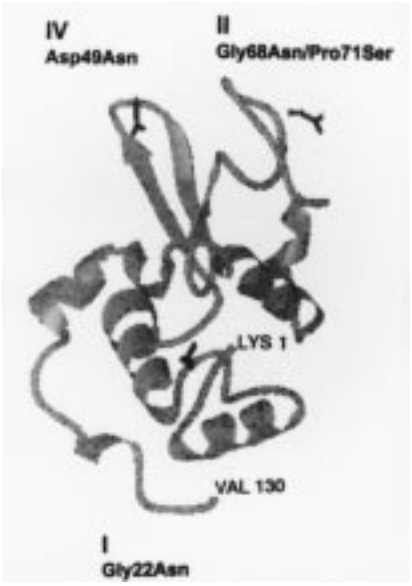

Figure 1. Model of human lysozyme indicating the positions of the glycosylation sites I, II, and IV. A ribbon structure is shown with the glycosylation sites indicated by the side chains of the accepting asparagine residues.

Comparisons between singly glycosylated lysozyme mutants

In two clones of CHO cells expressing the novel mutant IV biosynthesis and glycosylation were examined (Figure 2). Densitometric scanning of the secreted protein indicated a fractional glycosylation of approximately 0.2.

As can be judged from the pattern of radioactive bands shown in Figure 2, the carbohydrate processing in mutant IV was similar to that of the previously described mutants. In SDS-PAGE of metabolically labeled lysozyme mutants, the fastest band migrated like the nonglycosylated species (labeled L in Figure 2). A faint band (labeled mL) that migrated behind the L form has been shown previously to bear a mannose-rich oligosaccharide. A group of "slower" (sL) bands behaved like molecules with biantennary oligosaccharides without or with one or more *N*-acetyllactosamine repeats. This was concluded from the previous studies of mutants I and II [19, 20]. In mutants II and IV, the relative rate of the glycosylation was similar to each other. In both mutants, in several experiments the rate was estimated to be about three times less than in mutant I. In contrast to bands L and sL, the relative intensity of band mL in different mutants varied rather inconsistently between the experiments. In all three singly glycosylated mutants, within the sL group of the bands the fastest represented the major glycosylated species. The species with an increasing number of repeats were progressively less represented.

Synthesis of elongated oligosaccharides in doubly glycosylated lysozyme mutants

In the doubly glycosylated mutant I/IV, an additional group of glycosylated forms (dL) was observed that appeared to represent molecules bearing two complex oligosaccharides of various length. The abundance of the bands in the two groups was compatible with an independent glycosylation. It corresponded roughly to the product of the fractional glycosylation at the sites involved. In both groups, the fastest band showed the highest abundance. These bands contained biantennary oligosaccharides without *N*-acetyllactosamine repeats as judged from their sensitivity to *endo*-*N*-acetylglucosaminidase F2 and resistance to *endo*-β-galactosidase (an example will be shown below). Biantennary oligosaccharides containing *N*-acetyllactosamine repeats have been demonstrated in red cells [34]. In contributions from several laboratories, it has been established that the linkage region in asparagine-linked keratan sulfates from different species is represented by biantennary oligosaccharides [35].

Doubly glycosylated lysozyme was synthesized also in cells expressing lysozyme mutant II/IV (Figure 2). Its apparent size and a lower abundance of the "fastest" species

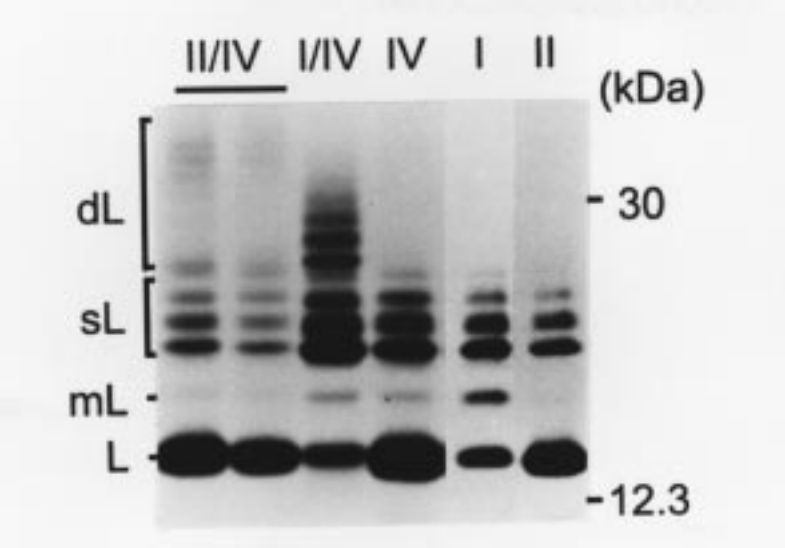

Figure 2. Glycosylation of lysozyme bearing glycosylation sequons at sites I, II, or IV or their combinations I/IV and II/IV. The mutations were expressed in CHO cells after selecting stable transfectants with the vector pBEHpac18. The cells were subjected to metabolic labelling with Tran^{35}S-label. The medium was collected after a 16 h labeling. Lysozyme was isolated by immunoprecipitation, and the different glycosylation forms were separated by SDS-PAGE and visualized by fluorography. Lysozyme forms bearing two complex oligosaccharides (dL), one complex oligosaccharide (sL), one mannose-rich oligosaccharide (mL), or no oligosaccharide (L) are indicated. Molecules bearing the complex oligosaccharides are heterogenous by size due to a varied content of *N*-acetyllactosamine repeats (see the explanation in the text).

within the doubly glycosylated group, that is, without the repeats, strikingly differed from those of the doubly glycosylated form of mutant I/IV. The low abundance of the doubly glycosylated forms indicated an independent glycosylation at the two sites. Attempts to obtain clones with a better expression failed. To obtain a higher expression of the doubly glycosylated species, the cDNA of the II/IV mutant was subject to an additional mutagenesis. In the mutant II/IVT, the serine codon within the glycosylation site IV was replaced by a threonine codon (S51T). In Figure 3, it is shown that this mutation strongly enhanced the chance of glycosylation of the mutant protein in CHO cells. This observation confirms the report by Kasturi et al. [24] on sequon NXT being glycosylated at a higher rate than sequon NXS. Correspondingly, the group of doubly glycosylated lysozyme forms could be better characterized in this mutant.

We have reported that in Lec2 CHO cells that are deficient in sialylation of the oligosaccharides in glycosylated lysozyme longer repeats are synthesized than in normal CHO cells [20]. It was of interest, therefore, to see if the elongation can be manifested in the doubly glycosylated molecules. The repeats were longer in molecules bearing single oligosaccharide side chains. Unexpectedly, the yield of the doubly glycosylated molecules was strongly decreased in Lec2 as compared with normal CHO cells. This precluded an accurate estimation of the elongation. The sialylation defect may have changed the yield or the secretion of the doubly glycosylated lysozyme.

Isolation of singly and doubly glycosylated lysozyme and characterization of lactosamine repeats in these glycoproteins

To further characterize the doubly glycosylated forms of lysozyme II/IVT, the corresponding cDNA was cloned into a vector with a high rate of protein expression in CHO cells. With vector pMCI that contained the cDNA of lysozyme II/IVT, several clones were obtained that expressed both singly and doubly glycosylated lysozyme at a rather high rate. The metabolic labeling of the glycosylated forms in the selected clones is demonstrated in Figure 4. This experiment showed that the synthesis of carbohydrate-rich forms of the doubly glycosylated lysozyme characteristic for mutants II/IV and II/IVT was observed at a high rate of expression using vector pMCI also.

Using plasmid pMCI, sufficient material was obtained in culture flasks for a structural characterization of the recombinant protein without radioactive labeling of the cells. The protein was separated in SDS-PAGE and visualized by silver staining. Singly and doubly glycosylated forms of lysozyme II/IVT were enriched from the spent medium of transfected CHO cells by immune affinity chromatography. A separation of these forms was achieved using hydroxylapatite column chromatography. In Figure 5, a silver stained gel with the proteins that have been eluted from the column is shown. Most of the singly glycosylated lysozyme was concentrated in fraction 13. In fractions 9–11, the doubly glycosylated forms were found such that the species containing

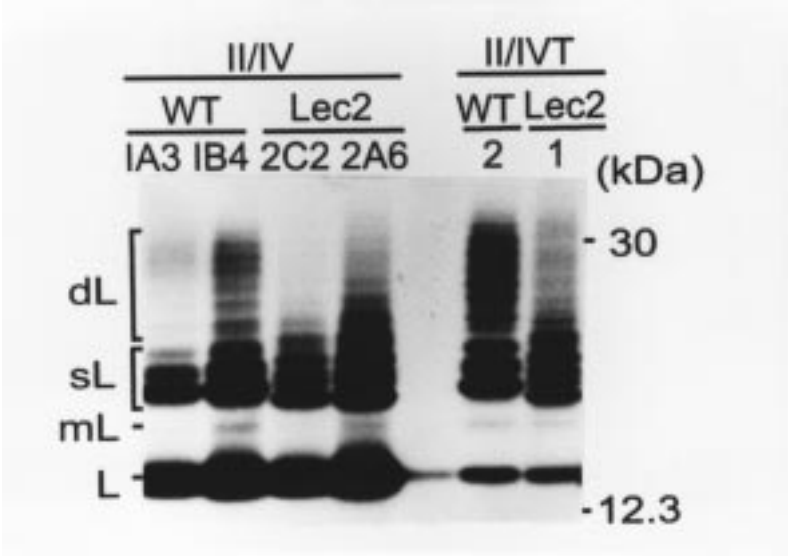

Figure 3. Enhanced glycosylation in lysozyme mutant II/IVT with threonine in the glycosylation sequon at site IV and low efficiency of double glycosylation in both II/IV and II/IVT mutants in the absence of sialylation in CHO cells. Stable expression of the mutants was examined in Lec 2 and wild type (WT) CHO cells. The labeling and the analysis of secreted lysozyme was performed as described in the legend of Figure 2 where the symbols referring to various lysozyme forms are also explained. Several independent clones of mutant II/IV were isolated, and two of them were characterized as indicated by numbers above the lanes.

the highest number of *N*-acetyllactosamine repeats eluted earlier, and the form considered to contain oligosaccharides without repeats was rather enriched in fraction 11. Thus, the more carbohydrate is attached to lysozyme, the earlier it is eluted from the hydroxylapatite column.

The fractions separated in the hydroxylapatite column chromatography were of sufficient purity to confirm the presence of one and two oligosaccharides in the two groups of glycosylated lysozyme species. These groups appeared as ladders of bands in SDS-PAGE and to demonstrate the presence of *N*-acetyllactosamine repeats in both the singly and doubly glycosylated forms. Deglycosylation with glycopeptidase F resulted in a band that migrated like normal lysozyme (Figure 6). Treatment of either form with endoglycosidase F2 resulted in a large reduction in the apparent size. The products of both reactions appeared to be slightly

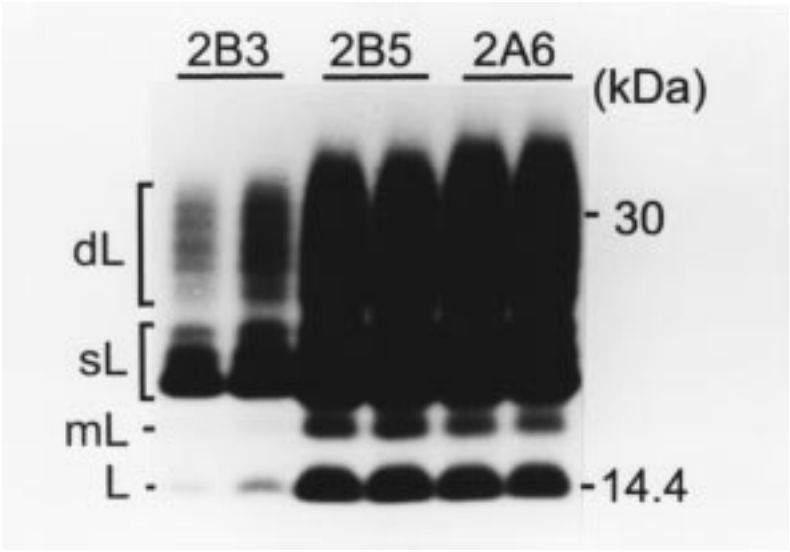

Figure 4. High efficiency of the metabolic labeling of lysozyme mutant II/IVT in CHO cells stably transfected with the vector pMCl. The labeling of each transfectant (indicated by numbers above the lane s) was performed in two dishes in parallel. The symbols referring to the varried forms of lysozyme are explained in the legend of Figure 2.

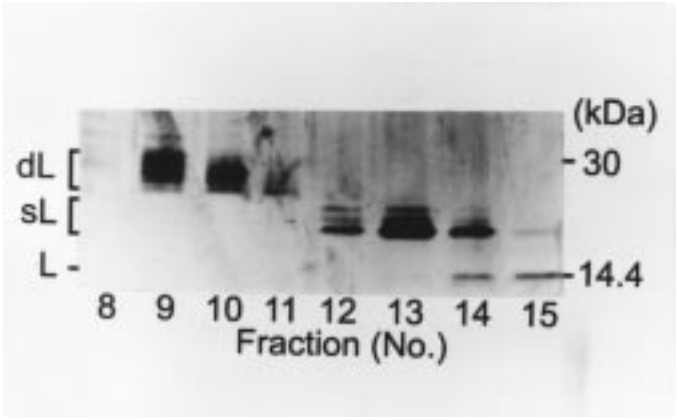

Figure 5. Separation of the different glycosylation forms of lysozyme II/IVT in a hydroxyl apatite column. Aliquots of fractions eluted with increasing concentrations of sodium phosphate were analyzed by SDS-PAGE and stained by the $AgNO_3$ method. The symbols referring to the varried forms of lysozyme are explained in the legend of Figure 2.

larger than normal human lysozyme, as expected after an endoglycosidic cleavage of the *di-N*-acetylchitobiose unit. Treatment with *endo*-β-gal actosidase of both the singly and the doubly glycosylated lysozyme resulted in a digestion of all except for the fastest band in the respective group. A separate control showed that the double band that was seen in the digested samples (Figure 6, lanes 2 and 6)

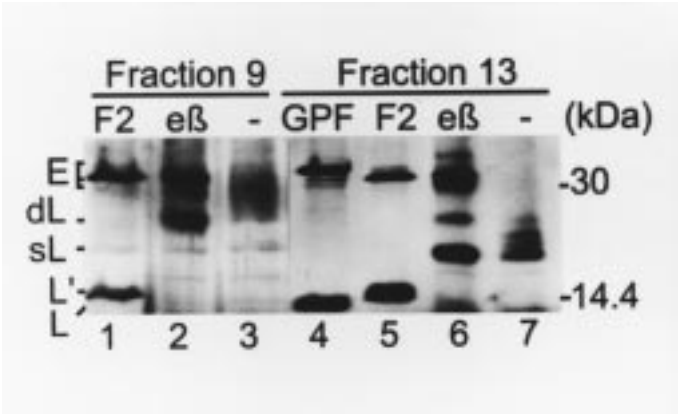

Figure 6. Characterization of singly and doubly glycosylated lysozyme using *endo*-glycosidase F2, *endo*-β-galactosidase and glycopeptidase F (GPF). GPF is an amidohydrolase and forms a fully deglycosylated deamido lysozyme (L). *Endo*-glycosidase F2 (F2) cleaves the outer oligosaccharide off the asparagine-bound *N*-acetylglucosamine. The apparent similarity of the products of hydrolysis of singly and doubly glycosylated lysozyme forms (fractions 13 and 9, respectively) may be due to a resolution limit of the separation method. Cleavage of the singly and doubly glycosylated lysozyme forms with *endo*-β-galactosidase (eβ results in insufficiently resolved bands of lysozyme bearing intact nonelongated biantennary oligosaccharides or oligosaccharides missing galactose and the peripheral carbohydrate residues if the parental structures contained *N*-acetyllactosamine repeats. The positions of the bands corresponding to the enzymes used in the digestion are indicated (E). The products were visualized by silver staining of the gel.

at approximately 30 kDa was due to the digesting enzyme. After the treatment of the singly glycosylated lysozyme II/IVT (lane 6), the band corresponding to lysozyme bearing a single oligosaccharide without *N*-acetyllactosamine repeats became more prominent at the expense of the apparently larger forms of lysozyme in the untreated fraction 13 (lane 7). In the treated sample (lane 6), an additional band was detected that resembled a doubly glycosylated lysozyme lacking the repeats. Probably, this band origined from a small amount of doubly glycosylated lysozyme bearing various numbers of the repeats that might have accompanied the singly glycosylated lysozyme in fraction 13. The doubly glycosylated lysozyme in fraction 9 was converted to a prominent band without *N*-acetyllactosamine repeats, resembling the fastest band among the forms bearing two oligosaccharides. Using immunoprecipitates from radioactively labeled cells, we have confirmed the finding that in both singly and doubly glycosylated lysozyme the apparently larger forms were degraded by endo-β-galactosidase and that a great majority of the glycosylated lysozyme forms were sensitive to endo-glycosidase F2 (not shown). Thus, the increase in the apparent size of the doubly glycosylated lysozyme forms in mutant II/IVT is explained by an elongation of *N*-acetyllactosamine repeats in biantennary rather than synthesis of triantennary oligosaccharides.

Possible role of the spacing of the oligosaccharides in doubly glycosylated lysozyme mutants

In earlier studies, it has been shown that in various glycoproteins in complex oligosaccharides *N*-acetyllactosamine repeats are preferentially added to β-1,6-linked antennae [12, 13]. An increased synthesis of the corresponding triantennary oligosaccharides has been observed in tumor cells and was associated with an increase in the elongation of the repeats [12]. It is possible that in the doubly glycosylated lysozyme mutant II/IVT the elongation of the repeats is promoted if its two oligosaccharides are closely spaced and mimic a triantennary oligosaccharide.

Owing to the relatively high activity of β-galactosyl transferase, most of the nonreducing termini of the antenna bear galactose residues, unless the elongation is terminated by sialylation. Indeed, in transfected Lec2 CHO cells, in which the sialylation is impaired, the oligosaccharides in glycosylated lysozyme bear β-galactosyl residues at their termini [20]. Therefore, doubly glycosylated lysozyme might be considered as a bivalent substrate of the elongating β-N-acetylglucosaminyl transferase "i" or alike. The enzyme could glycosylate the termini of two oligosaccharides at an increased efficiency if it had more than one active site, and the oligosaccharide acceptors were properly spaced on the surface of the substrate. Alternatively, a transferase with one catalytic center could bind successively to the termini of the bivalent substrate with a high probability if they were properly spaced. Recently, the primary structure of the human

elongating enzyme has been deduced from cloned cDNA [36]. Further characterization of the enzyme might help to decide which hypothesis is more likely.

To consider possible differences in spacing of the oligosaccharides on the surface of the doubly glycosylated mutants, the molecules were modeled according to the known crystal structure of human lysozyme [22]. The models reveal that there is a considerable difference in the distance between the glycosylated residues in the two doubly glycosylated mutants (Figure 1). The direct distance between the α-C atoms of the glycosylated residues 49 and 68 in the available crystal structure is 14.2 Å. The direct distance between α-C atoms of residues 22 and 49 is 31.3 Å. It is obvious that the modeling comes short of showing the orientation of the oligosaccharides. Further studies are needed to determine whether the shorter distance of the glycosylated asparagines as observed between residues 49 and 68 is important for the elongation of the repeats in other substrates. In lysosomal membrane glycoproteins lamp-1 and lamp-2 that bear 18 and 16 oligosaccharides, respectively, *N*-acetyllactosamine repeats are restricted to a few oligosaccharides that are attached to distinct sites [18]. It had been speculated that a distinct apposition of the oligosaccharides may be crucial for their elongation [18]. This was based on the fact that lamp proteins do not contain a common amino acid sequence motif adjacent to the attachment sites of the elongated oligosaccharides. This consideration could not exclude the existence of a signal at a higher level of protein structure. The structure of lysozyme is not related to that of lamp proteins. Therefore, the lysozyme model of the synthesis and elongation of *N*-acetyllactosamine repeats provides an experimental basis for the suggestion that the synthesis of lactosamine repeats is directed by the carbohydrate moiety of the substrate glycoprotein. In the future, desialylated forms of the substrates of the elongating enzyme such as lamp-1, lamp-2, and mutant lysozyme might be available for a study of the elongation *in vitro*.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 286 and the Fonds der Chemischen Industrie. We thank Drs. H.-G. Löffler, this institute, and G. Vriend, EMBL, Heidelberg, for performing 3D-modeling of human lysozyme and sharing the modeling program Whatif, respectively, Mrs. R. Sauer for tissue culture work, Mrs. T. Becker for art work, and Dr. D. Jones for critically reading the manuscript.

References

1 Dunon D, Piali L, Imhof B A (1996) *Curr Opin Cell Biol* 8: 714–23.
2 Ley K (1996) *Cardiovas Res* 32: 733–42.
3 Etzioni A (1996) *Pediatr Res* 39: 191–8.
4 Lasky LA (1995) *Annu Rev Biochem* 64: 113–39.
5 McEver RP, Moore KL and Cummings RD (1995) *J Biol Chem* 270: 11025–8.
6 Tedder TF, Steeber DA, Chen A, Engel P (1995) *FASEB J* 10: 866–73.
7 Patel KD, Nollert MU, McEver RP (1995) *J Cell Biol* 131: 1893–902.
8 Fukuda M, Hakomori S (1982) *J Biol Chem* 257: 446–55.
9 Singhal A, Hakomori S (1990) *BioEssays* 12: 223–30.
10 Capon C, Laboisse CL, Wieruszenski JN, Maoret JJ, Augeron C, Fournet B (1992) *J Biol Chem* 267: 19248–57.
11 Fukuda M, Carlsson SR, Klock JC, Dell A (1986) *J Biol Chem* 251: 12796–806.
12 Yousefi S, Higgins E, Daoling Z, Pollex-Kruger A, Hindsgaul O, Dennis J W (1991) *J Biol Chem* 166: 1772–82.
13 Koenderman HL, Koppen PL, van den Eijnden DH (1987) *Eur J Biochem* 166: 199–208.
14 Wang WC, Lee N, Aoki D, Fukuda MN, Fukuda M (1991) *J Biol Chem* 266: 23185–90.
15 Lee N, Wang WC, Fukuda M (1990) *J Biol Chem* 265: 20476–87.
16 Radons J, Faber V, Buhrmester H, Volker W, Horejsi V, Hasilik A (1992) *Eur J Cell Biol* 57: 184–92.
17 Cho SK, Yeh J, Cho M, Cummings RD (1996) *J Biol Chem* 271: 3238– 46.
18 Carlsson SR, Fukuda M (1990) *J Biol Chem* 265: 20488–95.
19 Horst M, Harth N, Hasilik A (1991) *J Biol Chem* 266: 13914–9.
20 Hummel M, Hedrich HC, Hasilik A (1997) *Eur J Biochem* 245: 428–33.
21 Deutscher SL, Nuwayhid N, Stanley P, Briles EIB, Hirschberg CB (1984) *Cell* 39: 295–9.
22 Artymiuk PJ, Blake CCF (1981) *J Mol Biol* 152: 737–62.
23 Kunkel TA (1985) *Proc Natl Acad Sci U S A* 82: 488–92.
24 Kasturi L, Eshleman JR, Wunner WH, Shakin-Eshleman SH (1995) *J Biol Chem* 270: 14756–61.
25 Higuchi R, Krummel B, Saiki RK (1988) *Nuleic Acids Res* 16: 7351–67.
26 Vallette F, Mege E, Reiss A, Adesnik M (1989) *Nucleic Acids Res* 17: 723– 33.
27 Kaufman RJ, Davies MV, Pathak VK, Hershey JWB (1988) *Mol Cell Biol* 9: 946–58.
28 Felgner PL, Gadek TR, Holms M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987) *Proc Natl Acad Sci U S A* 84: 7413–7.
29 Gupta DK, von Figura K, Hasilik A (1987) *Clin Chim Acta* 165: 73–82.
30 Laemmli UK (1970) *Nature* 227: 680–5.
31 Laskey RA, Mills AD (1975) *Eur J Biochem* 56: 335–41.
32 Heukeshoven J, Dernick R (1988) *Electrophoresis* 9: 28–32.
33 Yoshimura K, Toibana A, Nakahama K (1988) *Biochem Biophys Res Commun* 150: 794–801.
34 Fukuda M, Dell A, Fukuda MN (1984) *J Biol Chem* 259: 4782–91.
35 Tai G, Nieduszynski IA, Fullwood NJ, Huckerby T (1997) *J Biol Chem* 272: 28227–31.
36 Sasaki K, Kurata-Miura K, Ujita M, Angata K, Nakagawa S, Sekine S, Nishi T, Fukuda M (1997) *Proc Natl Acad Sci U S A* 94: 14294–9.

Received 1 April 1998, revised 11 June 1998 and accepted 12 June 1998