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Chinese Hamster Ovary Cell Mutants with Multiple Glycosylation Defects for Production of Glycoproteins with Minimal Carbohydrate Heterogeneity

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The production of glycoproteins with carbohydrates of defined structure and minimal heterogeneity is important for functional studies of mammalian carbohydrates. To facilitate such studies, several Chinese hamster ovary mutants that carry between two and four glycosylation mutations were developed. All of the lines grew readily in culture despite the drastic simplification of their surface carbohydrates. Therefore, both endogenous glycoproteins and those introduced by transfection can be obtained with specifically tailored carbohydrates. The lectin resistance properties of the mutants showed that each line expresses a novel array of cell surface carbohydrates useful for identifying specific roles for carbohydrates in cellular interactions. In addition, they showed that the epistatic relationships among different glycosylation mutations are not entirely predictable, providing insight into the complexity of the carbohydrate structures at the Chinese hamster ovary cell surface.

Glycosylation mutants of mammalian cells provide novel biochemical environments for the production of glycoproteins with specific structural alterations in their carbohydrate moieties (14, 27, 28; M. Krieger, P. Reddy, K. F. Kozarsky, D. M. Kingsley, and M. Penman, Methods Cell Biol., in press). By using these mutants, the functions of carbohydrates on glycoproteins endogenous to the cell (12, 32) and glycoproteins introduced into the cell by viral infection (19) or transfection of cloned DNA (8a, 13, 13a, 17) have been investigated. Such mutants also provide insight into the consequences of expressing a dramatically altered array of carbohydrates at the cell surface and have been used to identify a role for carbohydrates in tumorigenicity (20) and metastasis (5, 10).

To date, most of the glycosylation mutants described in the literature possess a single glycosylation defect (14, 27, 28; Krieger et al., in press). Nevertheless, several of these mutations alter the synthesis of both N- and O-linked carbohydrates on glycoproteins, as well as the carbohydrates of glycolipids. For example, Lec2 and Lec8 Chinese hamster ovary (CHO) mutants are defective in transporting one of the nucleotide sugars into the Golgi compartment (6, 7); consequently, both glycoproteins and glycolipids are synthesized with truncated carbohydrates in these mutants (23, 31). Similarly, the epimerase defect of the ldlD mutant results in the synthesis of truncated N-linked carbohydrates and prohibits the initiation of O-linked structures altogether (11).

To expand the variety of structural modifications that might be expressed on glycoproteins and to develop cells with a highly simplified array of cell surface carbohydrates for functional studies, a series of CHO mutants carrying between two and four glycosylation mutations have been isolated. These cell lines should be particularly useful for producing biologically active glycoproteins with minimal carbohydrate heterogeneity, a concern of growing importance in the biotechnology industry (1, 8). In addition, the presence of several glycosylation mutations in combination has given cell lines with novel arrays of cell surface carbohydrates and defined epistatic relationships among the glycosylation mutations. Interestingly, the latter relationships are not entirely predictable, indicating the complex range of carbohydrate structures present at the CHO cell surface.

MATERIALS AND METHODS

Lectins. The lectins used in selections and in determining lectin resistance (Lec^R) phenotypes were as follows: L-phytohemagglutinin (L-PHA; lymphoagglutinin from *Phaseolus vulgaris*); wheat germ agglutinin (WGA; agglutinin from *Triticum vulgaris*); concanavalin A (ConA; agglutinin from *Canavalia ensiformis*); ricin (RIC; toxin from *R. communis*); and LCA (agglutinins from *Lens culinaris*). They were obtained from the same commercial sources, prepared in solution, and stored at 4°C, and their concentrations were measured as described previously (26).

Cells. Mutants carrying single glycosylation mutations were used as parental cells: Pro^-Lec3.6B (29), Pro^-Lec4.12-2 (29), and ldlD-14 (12). For comparative lectin resistance tests, and growth curves, the original parental lines Pro^-5 (29) and CHO-K1 (12) were used. Both CHO-K1 and ldlD-14 were kindly provided by Monty Krieger, Massachusetts Institute of Technology, Boston.

Nomenclature. The combination of several mutations in a single cell line could lead to an unwieldy nomenclature if the full name of each clone successively used in selection was used in the final name of the cell line. To simplify mutant names, therefore, only one Lec prefix is used for the multiple mutants. Each number following the prefix stands for a separate glycosylation mutation. Thus, Lec3.2.8.1 represents a mutant carrying the four glycosylation mutations Lec3, Lec2, Lec8, and Lec1. Isolates which were not cloned are referred to as colonies, while those cloned by limiting dilution are referred to as clones.

Cell culture. All cells were grown at 37°C in suspension or monolayer in alpha medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% horse serum and 2% fetal calf serum (FCS) unless otherwise stated. They were routinely shown to be negative for *Mycoplasma* contamination by staining with Hoeschst 33258.

Selections. Mutagenesis and selection procedures with

lectins applied sequentially or in combination were identical to those described previously (26).

Complementation analysis. Putative multiply marked Pro^- Lec^R clones were fused with polyethylene glycol with Lec1, Lec2, Lec3, or Lec8 CHO cells which carried the Gat^- auxothrophic marker (26, 29), and hybrids were selected in deficient medium as described previously (26). Spontaneous hybrid and reversion frequencies were determined for each cross. Resistance to WGA was determined immediately following fusion for the mass culture of hybrids or following isolation and expansion of hybrid colonies.

Karyotype determination. Modal chromosome numbers were determined from spreads obtained from cells treated with colcemid as described previously (26).

Determination of Lec^R phenotype (P-test). The sensitivity of cell populations to each lectin was determined by plating 2,000 cells in the presence of increasing lectin concentrations in 96-well microtiter dishes in alpha medium containing 10% FCS as described previously (26). When the wells containing no lectin were confluent, endpoints were scored microscopically and subsequently the plates were stained with methylene blue in 70% isopropanol. Endpoints were taken as the concentration of lectin which reduced confluency of the well to ≤10% compared with the control well.

Growth curves. To examine relative growth rates in monolayer culture, 24-well tissue culture dishes were seeded with 2×10^4 cells per well in 2 ml of alpha medium containing 5 or 10% dialyzed FCS. At each time point, the medium was removed from two wells and the cells were rinsed with citrate saline, trypsinized, and counted separately in a Coulter counter. Growth rates in suspension culture for Pro^-5 cells and their derivatives were also determined in duplicate 10-ml tubes from which 0.3 ml was removed for counting at each time point. Fresh medium (0.3 ml) was used to maintain the volume of suspension cultures at 10.5 ml.

RESULTS

Selection rationales and strategies. The aim of selecting multiple glycosylation mutants was to obtain mammalian cells capable of producing glycoproteins with carbohydrates of various types and reduced heterogeneity. By combining glycosylation mutations for which a biochemical basis has been identified, cells with novel arrays of more severely truncated carbohydrates than available from the single glycosylation mutants could theoretically be obtained. Mutations that affect N-linked as well as O-linked carbohydrates were therefore combined. The mutants sought were designed to obtain cells with severely reduced sialylation, galactosylation, or N-linked branching in combination with different O-linked truncations.

The six glycosylation mutations that were combined belong to complementation groups 1, 2, 3, 4, 8 and ldlD (14, 27, 28). To facilitate comparisons with the new mutants described in this paper, the lectin resistance (Lec^R) phenotypes of these mutants are given in Table 1. The truncated carbohydrates synthesized as a result of each glycosylation mutation can be deduced from Fig. 1. This diagram summarizes the carbohydrate structures expected on CHO glycoproteins and indicates which sugar addition is prohibited by the respective glycosylation mutation. Detailed structural studies of the truncated carbohydrates produced by several of the mutants, as well as evidence of a reduced enzyme or transport activity that correlates with each mutation, provide support for the conclusions of Fig. 1 (14, 27, 28): Lec1 mutants lack detectable *N*-acetylglucosaminyltransferase 1 (GlcNAc-T1) activity; Lec2 mutants exhibit reduced transport of CMP-sialic acid into the Golgi lumen; Lec3 mutants have a phenotype similar to that of Lec2 mutants but their biochemical defect is unknown; Lec4 mutants lack detectable GlcNAc-TV activity (W. Chaney, S. Sundaram, N. Friedman, and P. Stanley, manuscript in preparation); Lec8 mutants exhibit reduced transport of UDP-galactose into the Golgi lumen; and the ldlD mutant lacks detectable glucose-4-epimerase activity.

TABLE 1. Lectin resistance phenotypes of single glycosylation mutants[a]

Mutant	Resistance (R) or hypersensitivity (S) (fold)[b]					Reference
	L-PHA	WGA	ConA	RIC	LCA	
Lec1	$R_{>1,000}$	R_{30}	S_6	R_{100}	$R_{>300}$	29
Lec2	S_2	R_{11}	—	S_{100}	S_2	29
Lec3	(S)	R_5	—	S_{10}	S_2	29
Lec4	$R_{>1,000}$	(R)	(S)	(S)	(S)	29
Lec8	R_{10}	R_{100}	(S)	(R)	S_{10}	24
ldlD	R_{16}	R_{10}	S_{2-3}	R_2	S_7	12

[a] The Lec^R phenotypes of CHO mutants with single glycosylation mutations are summarized to facilitate comparison with mutants carrying two to four glycosylation mutations in Table 3. The truncated carbohydrate structures expressed on the glycoproteins of these mutants can be deduced from Fig. 1.

[b] Property compared with parental CHO cells. —, Unchanged.

To select mutants with combined glycosylation lesions, the relative lectin sensitivities of the single mutants were exploited (Table 1). Selection strategies used lectins applied sequentially or in combination to select against unwanted phenotypes and for mutants with the desired genotype (Table 2).

Selection of mutants with multiple glycosylation defects. The first selection in Table 2 was aimed at introducing the *lec2* mutation onto a cell line already carrying the *lec3* mutation. Since an attempt to obtain a double mutant from an unmutagenized Lec3 population was unsuccessful, Lec3 cells treated with ethylmethane sulfonate were subjected to the sequential lectin selection shown in Table 2. The concentration of WGA was designed to select for Lec2 mutants (D_{10} = 25 μg/ml [29]), while the addition of ConA and LCA after 6 days was designed to inhibit the growth of mutants carrying the *lec1* (sensitive to ConA) or the *lec8* (sensitive to LCA) mutation. Of 22 isolates tested, only 2 exhibited the 100-fold-increased sensitivity to RIC typical of Lec2 CHO cells (24, 29). The Lec^R phenotype of a clonal derivative (Table 3) was indistinguishable from that expressed by Lec2 CHO mutants (24, 29), and the new isolate exhibited noncomplementation with both Lec3 and Lec2 CHO cells (Table 4). It therefore carries both *lec3* and *lec2* mutations.

To obtain a cell line with the *lec3*, *lec2*, and *lec8* mutations, the double mutant Lec3.2 was subjected, without prior mutagenesis, to a sequential selection, using 80 μg of WGA per ml, which was replaced after 6 days with ConA at 7.5 μg/ml (Table 2). Twenty-six of the largest survivors were P-tested, and several phenotypes, typical of different combinations of glycosylation mutations, were obtained (Table 2). Ten colonies expressed a new phenotype typical of cells that had acquired the *lec8* mutation (24, 29; Table 1) except for an increased sensitivity to RIC (Table 3). The latter isolates were cloned, and complementation analyses showed that one of the clones (designated Lec3.2.8) exhibited noncomplementation with mutants from complementation groups 3, 2, and 8 (Table 4).

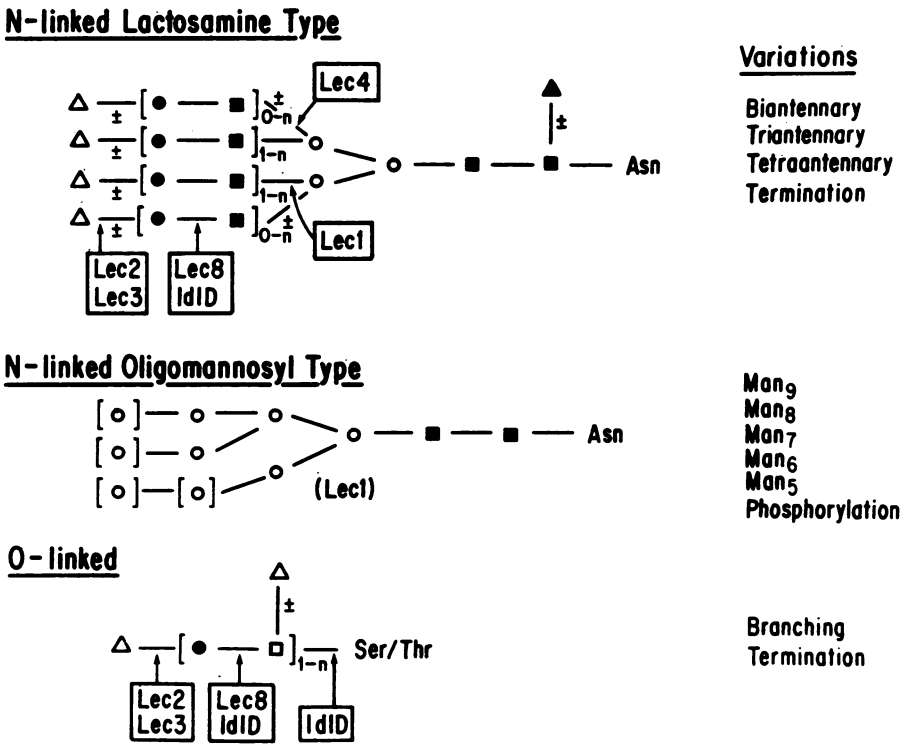


FIG. 1. General carbohydrate structures associated with CHO glycoproteins. On the basis of structural studies performed on pronase-digested CHO membrane glycoproteins (15, 16) and the carbohydrates of individual glycoproteins synthesized in CHO cells (12, 22, 33), it is possible to predict that the most simple array of N- and O-linked carbohydrates expressed at the CHO cell surface (and thus available for lectin binding) would include the general structures described above in various forms of completion and unknown abundance. Symbols: △, NeuAc; ●, Gal; ■, GlcNAc; ○, mannose; □, GalNAc; ▲, fucose. The oligomannosyl moieties may contain phosphate residues and lactosamine units may terminate with sugars such as α-1,3-Gal and β-1,4-GalNAc. In addition, it is likely that O-linked GlcNAc residues occur at the CHO cell surface (34). The sugar linkage affected by a particular glycosylation mutation is indicated by an arrow (14, 27, 28; Krieger et al., in press). The truncated carbohydrate structures synthesized by each mutant can thereby be deduced.

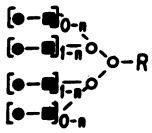
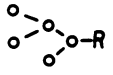
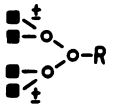
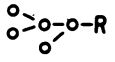
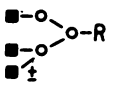
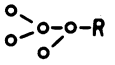
To obtain a mutant with no N-linked branched carbohydrates and O-linked moieties lacking sialic acid, the double mutant Lec3.2 was subjected, without prior mutagenesis, to selection with 50 μg of WGA per ml (Table 2). Eighteen of the largest surviving colonies were tested for lectin resistance (Table 2). All of the colonies were mixtures of different mutants (see reference 26), but, following cloning, two isolates with the sensitivity to ConA typical of cells carrying a *lec1* mutation (26, 29) were obtained (Table 3). The presence of the *lec1* genetic marker was confirmed by

TABLE 2. Selection of multiple glycosylation mutants[a]

Cells subjected to selection	Lectin selections (μg/ml)		No. of colonies per 10^6 colony-forming cells		Lec^R phenotypes (no./colonies tested)
	1	2	1	2	
Lec3	WGA → (30)	ConA + LCA (25) (10)	428	320	Lec3 (1/22), novel (19/22), Lec3.2 (2/22)
Lec3.2	WGA → (80)	ConA (7.5)	200	30	Lec3.2 (7/26), Lec3.2B (6/26), Lec3.2.1 (3/26), Lec3.2.8 (10/26)
Lec3.2	WGA (50)		20	NA	Mixed (18/18), Lec3.2.1 (2 clones)
Lec4	WGA → (20)	ConA + RIC (5) (0.005)	ND	1	Lec4.1 (7/11), Lec4.1A (1/11), Lec4.8 (3/11)
Lec3.2.8	LCA (10)		65	NA	Lec3.2.8.1 (5/5)
IdlD	RIC (0.1)		74	NA	Novel (3/6), IdlD.Lec1 (3/6)

[a] Pro^-Lec3.6B cells were mutagenized with ethylmethane sulfonate (200 μg/ml) for 18 h, cultured for 3 days, and subjected to selection with WGA at 30 μg/ml (selection 1). After 6 days, the plates were washed and incubated with ConA (25 μg/ml) and LCA (10 μg/ml) (selection 2). The Lec^R phenotypes of 22 large colonies were determined for WGA and RIC. A clone obtained from a Lec3.2 phenotype was used to obtain Lec3.2.1 and Lec3.2.8 mutants. Similar protocols gave rise to Lec4.8, Lec3.2.8.1, and IdlD.Lec1 mutant clones. NA, Not applicable; ND, not determined.

TABLE 3. Lec^R phenotype of the multiple mutants and the predicted carbohydrates of their glycoproteins[a]

Mutant	Carbohydrates		Lec^R Phenotype				
	N-Linked	O-Linked	L-PHA	WGA	CON A	RIC	LCA
Lec3.2		●–□–S/T	(S)	R_{20}	(S)	S_{100}	S_2
Lec3.2.1		●–□–S/T	$R_{\geq 100}$	$R_{\geq 125}$	S_6	S_{10}	$R_{>10}$
Lec3.2.8		□–S/T	R_8	$R_{\geq 100}$	(S)	S_8	S_8
Lec3.2.8.1		□–S/T	$R_{\geq 100}$	$R_{\sim 150}$	S_6	-	$R_{>20}$
Lec4.8		□–S/T	$R_{\geq 100}$	$R_{\geq 100}$	S_2	S_{10}	S_{10}
ldlD.Lec1		S/T	$R_{\geq 50}$	$R_{\geq 60}$	S_6	$R_{\sim 30}$	$R_{>32}$

[a] The relative fold resistances and hypersensitivities of multiple glycosylation mutant clones compared with Pro$^-$5 or CHO-K1 parental cells were determined as described in Materials and Methods. R, Resistant; S, sensitive; –, similar to parental; ( ), ≤2-fold sensitive or resistant. The symbols for the sugars in the carbohydrate structures predicted to occur on glycopoteins of the mutants are the same as in Fig. 1. R, GlcNAc-GlcNAc(Fuc)-Asn; S/T, Ser/Thr. None of the mutations would be expected to affect the occurrence or abundance of Man_9, Man_8, Man_7, or Man_6 oligomannosyl carbohydrates.

complementation analysis (Table 4). Therefore, mutant Lec3.2.1 had been obtained.

To obtain a cell line with more moderately truncated N-linked moieties and severely truncated O-linked carbohydrates, cells carrying the *lec4* mutation were grown in the presence of ConA (5 μg/ml) and RIC (0.5 ng/ml) for 2 days (to select against Lec1 and Lec2 phenotypes) and plated in 20 μg of WGA per ml for 6 days and subsequently in ConA (5 μg/ml) and RIC (0.5 ng/ml) for 2 days. Of 11 survivors tested, 7 had Lec^R phenotypes expected for a Lec4.1 double mutant (29), 1 was like a Lec4.1A double mutant (25), and 3 were hypersensitive to LCA and highly resistant to WGA, as would be expected for cells carrying the *lec8* mutation (24; Table 1). Except for a marked hypersensitivity to RIC, the Lec^R phenotype of Lec4.8 double mutants (Table 3) is consistent with that predicted by the combination of the *lec4* and *lec8* genotypes (26, 29; Table 1). The presence of the *lec8* mutation was confirmed by complementation analysis (Table 4).

To obtain a mutant carrying four glycosylation mutations that would give rise to severely truncated N- and O-linked carbohydrates, Lec3.2.8 cells were subjected, without prior mutagenesis, to selection with LCA (Table 2). Only one type of colony survived the selection, and a clonal derivative exhibited a Lec^R phenotype typical of Lec1 cells except for an increased resistance to WGA and lack of resistance to RIC (Table 3). Complementation analyses showed that the new cell line did not complement mutants of complementation groups 1, 2, 3, and 8 (Table 4) and therefore carried these four glycosylation mutations.

Mutant Lec3.2.8.1 should initiate O-linked carbohydrates with *N*-acetylgalactoseamine (GalNAc), whereas in ldlD mutants GalNAc-containing O-linked structures should be missing altogether (Fig. 1). Therefore, the double mutant ldlD.Lec1 should possess a more simple array of carbohydrates than all of the preceding multiple mutants. To obtain a ldlD.Lec1 double mutant, ldlD-14 cells were subjected to selection, without prior mutagenesis, with 0.1 μg of RIC per

TABLE 4. Complementation analysis of mutants carrying two to four mutations to lectin resistance[a]

Cell lines hybridized	No. of colonies per 6×10^5 cells plated in WGA		Complementation
	None	2 μg/ml	
Lec3.2			
× Lec3	208	128	−
× Lec2	431	332	−
Lec3.2.1.			
× Lec1	216	229	−
× Lec2	193	192	−
× Lec3	277	213	−
× Lec8	356	38	+
Lec3.2.8			
× Lec1	1036	34	+
× Lec2	464	460	−
× Lec3	502	220	−
× Lec8	254	270	−
Lec3.2.8.1			
× Lec1	549	522	−
× Lec2	183	180	−
× Lec3	207	111	−
× Lec8	206	185	−
× Parental	302	2	+
Lec4.8			
× Lec8	300	288	−
× Parental	501	6	+
ldlD.Lec1 × Lec1[b]	792	648	−

[a] The multiply marked clones in Table 2 carry the Pro⁻ auxotrophic marker. They were crossed with Gat⁻ lines from complementation groups 1, 2, 3, and 8 as described previously (26). The hybrids were plated in 0, 1, 2, and 3 μg of WGA per ml, and after 8 to 10 days at 37°C the plates were stained and counted. In a number of cases, the results obtained from the mass population were confirmed by determining the WGA resistance of individual hybrids formed in the absence of WGA. Many of these isolates were shown to be pseudotetraploid by karyotype analysis. In each hybridization experiment, spontaneous hybrid formation was ≤10% the number of hybrids induced by polyethylene glycol. Reversion frequencies were determined for each line to be $\leq 10^{-5}$.

[b] In this cross, resistance to RIC at 0.025 μg/ml instead of resistance to WGA was determined.

ml. Approximately one-third of the survivors were large, dense colonies and, from six colonies of this type tested, three were hypersensitive to ConA and highly resistant to RIC as expected for Lec1 cells. A clonal derivative of one of these isolates was hybridized to Lec1 CHO cells and shown by complementation analysis to carry the *lec1* mutation (Table 4).

Truncated carbohydrates synthesized by the multiple glycosylation mutants. The Lec^R phenotypes of the single glycosylation mutants are consistent with the predicted consequences of their biochemical defects (Table 1 and Fig. 1). Thus, Lec1 mutants are very resistant to lectins that bind terminal sugars (L-PHA, WGA, and RIC) and hypersensitive to ConA due to their increased proportion of oligomannosyl residues; Lec2 and Lec3 mutants are resistant to WGA due to reduced sialylation of glycoconjugates and hypersensitive to RIC due to increased exposure of Gal residues; Lec4 cells are highly resistant to L-PHA, which binds to Gal residues in β-1,6-branched N-linked carbohydrates, and barely altered in resistance to other lectins, consistent with the continued synthesis of biantennary and β-1,4-branched, triantennary, complex N-linked carbohydrates; Lec8 cells are highly resistant to WGA, consistent with their reduced galactosylation (and therefore sialylation) of glycoproteins, and hypersensitive to LCA due to the exposure of GlcNAc (and underlying Man) residues in N-linked carbohydrates; and ldlD cells are similar to Lec8 cells due to their reduced ability to synthesize UDP-Gal and UDP-GalNAc (11), although, presumably because of their ability to scavenge Gal from serum glycoproteins (12; Krieger et al., in press), they exhibit somewhat lower levels of lectin resistance and hypersensitivity compared with Lec8 mutants (24).

The Lec^R phenotypes of the multiple mutants were also largely consistent with the predicted consequences of combining different glycosylation mutations in a single cell (Table 3). However, the epistatic relationships of the combined glycosylation mutations were not exactly as might have been predicted. Thus, Lec3.2 mutants were significantly more resistant to WGA (~20-fold) than either Lec3 or Lec2 mutants alone (Table 1). Therefore, the *lec2* mutation is not classically epistatic to the *lec3* mutation, since the Lec3.2 double mutant is not identical in phenotype to a Lec2 mutant. The combination appears to reveal a subset of carbohydrates affected in Lec3 though not in Lec2 cells. Similarly, the Lec3.2.8 phenotype is not identical to that of Lec8 mutants (24) since the triple mutant was eightfold hypersensitive to RIC (Table 3) whereas Lec8 mutants are slightly resistant to RIC (Table 1). This is interesting because *lec2*, *lec3*, and *lec8* mutations all affect both N-linked and O-linked carbohydrates (Fig. 1). That the *lec8* mutation is not epistatic, however, suggests that the *lec3* or *lec2* mutation prohibits the addition of sialic acid to structures which are not affected by the *lec8* mutation, thus exposing terminal Gal residues and causing hypersensitivity to RIC. The latter structures appear to be N linked since the addition of a *lec1* mutation reverses the RIC sensitivity to a level similar to that of Lec8 cells in the Lec3.2.8.1 mutant (Table 3). Further evidence for a subset of N-linked carbohydrates not affected by the *lec8* mutation is suggested from the RIC hypersensitivity of Lec4.8 double mutants (Table 3).

As expected, the *lec1* mutation appears to be epistatic to the *lec3*, *lec2*, and *lec8* mutations insofar as N-linked carbohydrates are concerned because all lines with a *lec1* mutation are sixfold hypersensitive to ConA and highly resistant to L-PHA and LCA (Table 3). However, the combination of different mutations affecting O-linked structures on a *lec1* genetic background gives rise to somewhat different levels of resistance to WGA and markedly different degrees of resistance to RIC (Table 3). Only in the case of ldlD.Lec1 is the phenotype of the double mutant indistinguishable from that of a single Lec1 mutant (Table 1). The Lec1 phenotype appears, therefore, to be epistatic to the ldlD phenotype.

The truncated carbohydrate structures predicted to be present on glycoproteins synthesized by the multiple mutants are also given in Table 3. These predictions are based on the known biochemical defect that correlates with each glycosylation mutation (14, 27, 28). Methods for determining, in a timely fashion, the actual range of carbohydrate structures synthesized by each mutant are not currently available. Even if they were, an individual glycoprotein might not possess a typical array. However, it would be expected that most glycoproteins synthesized by the multiple mutants would express most of their carbohydrates in the forms shown in Table 3.

Growth rates and morphology of multiple glycosylation mutants. If mutants synthesizing simplified carbohydrates are to be useful for producing glycoproteins with tailored

carbohydrates, they must be able to grow well in culture. Growth rates were determined, therefore, for the mutants expressing the most drastically modified carbohydrate structures: Lec3.2.8.1 and ldlD.Lec1. In medium containing 10% dialyzed FCS, the Pro$^-$ mutant Lec3.2.8.1 grew similarly to Pro$^-$5 parental cells (doubling time, 14 h compared with 12 h). It also took only 2 to 3 h longer to double than Pro$^-$5 cells in 5% dialyzed FCS and in limiting glucose. Thus, severely truncated carbohydrates do not cause a drastic reduction in growth rate. However, all of the Pro$^-$5 glycosylation mutants, including Lec3.2.8.1, were altered in morphology. When grown on plastic, they were very rounded and tended to pile on each other rather than to form compact monolayers like Pro$^-$5 cells. This phenotype is typical of Lec4 mutants for which phase-contrast micrographs have previously been published (30).

In contrast, both the ldlD and ldlD.Lec1 mutants grew somewhat more slowly than CHO-K1 cells (doubling time, 28 h compared to 21 h), and the ldlD.Lec1 mutant was strongly affected by limiting glucose (doubling time, 21 h longer than CHO-K1 cells). However, in 10% FCS the doubling time of the ldlD.Lec1 mutant was not dramatically longer than that of CHO-K1 cells, and thus it should be a useful line for preparing glycoproteins with altered carbohydrates. Interestingly, the morphology of ldlD.Lec1 cells was indistinguishable from that of CHO-K1 cells. Unlike the mutants from Pro$^-$5 CHO cells, the CHO-K1 mutants were not noticeably more rounded or more aggregated with each other.

DISCUSSION

The multiple glycosylation mutants described in this paper provide novel cell lines with which to study mammalian carbohydrate functions and to produce glycoproteins with tailored carbohydrates. Despite a dramatic truncation of N- and O-linked carbohydrates, even the most severely affected mutants (Lec3.2.8.1 and ldlD.Lec1) grow well in tissue culture. This is consistent with the dual role of carbohydrates (28, 35): maintenance of the correct physicochemical properties of glycoconjugates and ability to vary in branching and terminal glycosylation when appropriate (e.g., during development, differentiation, and transformation). Small carbohydrate units appear to fulfill the basic requirements for growth and division in CHO cells, although certain specialized functions of individual glycoproteins may be lost (e.g., the LDL receptor in ldlD mutants does not function) (12; Krieger et al., in press).

The combination of several glycosylation mutations in a single cell line has generated novel cell surface carbohydrate arrays not present in the single glycosylation mutants described previously. This is apparent from the unexpected Lec^R phenotypes of the multiple mutants for certain lectins (Table 2) as discussed in Results. That the epistatic relationships of the mutants are complicated provides evidence for the existence of distinct classes of N- and O-linked carbohydrates that are differentially affected by different mutations. Because a wider range of glycoproteins is altered in the multiple mutants, they extend the variety of glycosylation phenotypes that may be used to define roles for carbohydrates in cellular interactions (such as tumorigenicity, metastasis, and binding to lectinlike proteins), membrane mobility, endocytosis, and receptor recycling. They also extend the range of modified glycoproteins that may be produced with tailored carbohydrates for functional studies as well as for practical reasons.

One of the major aims of the biotechnology industry is to produce biologically active glycoproteins such as growth factors (erythropoietin and colony-stimulating factors), hormones (insulin and growth hormone), and enzymes (tissue plasminogen activator) carrying carbohydrates that are nonimmunogenic and that allow for maximal biological activity, stability, and half-life, as well as appropriate tissue targeting. The multiple glycosylation mutants extend the possibilities for producing glycoproteins of the desired type. The Lec3.2.8.1 mutant can provide glycoproteins with all N-linked carbohydrates in the Man_5 oligomannosyl form and O-linked carbohydrates truncated to a single GalNAc (Table 3). The presence of the GalNAc might be important for maximizing the activity of certain glycoproteins (11, 13a). Alternatively, if an even more simple carbohydrate complement is required, the ldlD.Lec1 mutant would be the cell line of choice (Table 3). However, to achieve a maximal effect on O-linked carbohydrates in this mutant, care must be taken to minimize production of UDP-GalNAc and UDP-Gal from sugars provided by the degradation of serum glycoproteins. The ldlD phenotype is reversible due to the existence of scavenger pathways that circumvent the epimerase route for producing UDP-Gal and UDP-GalNAc (11). Growth conditions that essentially eliminate the scavenger pathways have recently been devised (Krieger et al., in press) and should be used to maximize the effects of the ldlD mutation.

In addition to providing glycoproteins with a homogeneous complement of carbohydrates (a consideration that may be important for patenting reasons; see reference 8), the multiple glycosylation mutants can provide carbohydrates that are useful for targeting glycoproteins to specific tissues. This is because carbohydrate-binding receptors of different sugar specificities are found in the plasma membrane of many cells in the body (reviewed in reference 18). Glycoproteins produced in the Lec3.2 mutant will have terminal Gal residues and should be targeted to liver hepatocytes via the asialogycoprotein receptor. Glycoproteins from Lec3.2.8.1 or ldlD.Lec1 mutants may be targeted to the mannose receptor of Kupfer cells or macrophages because of their Man_5 oligomannosyl carbohydrates. However, if this were not desirable, it might be avoided by adding a *lec6* mutation to mutants with the *lec1* mutation. The combined phenotype Lec1.6 produces Man_4 oligomannosyl carbohydrates (21) with distinct lectin recognition properties (9).

The multiple mutants described in Table 3 are clearly only a subset of the mutants that might be readily isolated (Table 2). All manner of novel carbohydrate structures might be obtained for particular uses. In addition, it is important to remember that dominant glycosylation mutants previously described by this laboratory may be used to add specific sugar residues to glycoproteins if desired. For example, LEC11 and LEC12 CHO mutants add α1,3-fucose residues to lactosamine units on glycoconjugates (3), and this may be useful for targeting to the fucose receptor; LEC10 CHO mutants add the bisecting GlcNAc to N-linked carbohydrates (4), and this residue also profoundly affects the lectin recognition properties of complex carbohydrates (2) and could influence targeting properties. Therefore, the availability of both single and multiple glycosylation mutants broadens the possibilities for obtaining homogeneous carbohydrate structures on introduced glycoproteins.

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