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Control of Carbohydrate Processing: the *lec1A* CHO Mutation Results in Partial Loss of *N*-Acetylglucosaminyltransferase I Activity

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Lec1 CHO cell glycosylation mutants are defective in *N*-acetylglucosaminyltransferase I (GlcNAc-TI) activity and therefore cannot convert the oligomannosyl intermediate ($Man_5GlcNAc_2Asn$) into complex carbohydrates. Lec1A CHO cell mutants have been shown to belong to the same genetic complementation group but exhibit different phenotypic properties. Evidence is presented that *lec1A* represents a new mutation at the *lec1* locus resulting in partial loss of GlcNAc-TI activity. Structural studies of the carbohydrates associated with vesicular stomatitis virus grown in Lec1A cells (Lec1A/VSV) revealed the presence of biantennary and branched complex carbohydrates as well as the processing intermediate $Man_5GlcNAc_2Asn$. By contrast, the glycopeptides from virus grown in CHO cells (CHO/VSV) possessed only fully processed complex carbohydrates, whereas those from Lec1/VSV were almost solely of the $Man_5GlcNAc_2Asn$ intermediate type. Therefore, the Lec1A glycosylation phenotype appears to result from the partial processing of *N*-linked carbohydrates because of reduced GlcNAc-TI action on membrane glycoproteins. Genetic experiments provided evidence that *lec1A* is a single mutation affecting GlcNAc-TI activity. Lec1A mutants could be isolated at frequencies of 10^{-5} to 10^{-6} from unmutagenized CHO cell populations by single-step selection, a rate inconsistent with two mutations. In addition, segregants selected from Lec1A × parental cell hybrid populations expressed only Lec1A or related lectin-resistant phenotypes and did not include any with a Lec1 phenotype. The Lec1A mutant should be of interest for studies on the mechanisms that control carbohydrate processing in animal cells and the effects of reduced GlcNAc-TI activity on the glycosylation, translocation, and compartmentalization of cellular glycoproteins.

Studies from many laboratories have revealed considerable variation in the carbohydrates expressed on the glycoprotein(s) of certain viruses when they are grown in different host cells (5, 11, 17). However, since these experiments involve comparisons between cells of very different genetic backgrounds, it is unclear whether the observed variation reflects differential regulation of carbohydrate processing or the expression of different glycosylation or trafficking pathways in different cell types. Mutations that affect the processing pathway but are expressed in the same genetic background should be very helpful in identifying the controlling mechanisms that operate within the cell to regulate carbohydrate processing. In this paper, we provide evidence that Lec1A CHO cell mutants possess a glycosylation mutation in this category.

Lec1A mutants belong to the genetic complementation group defined by *lec1* mutations but exhibit different phenotypic properties than do Lec1 cells (20). Lec1 mutants are defective in UDP-*N*-acetylglucosamine:α1,3-mannoside-β2-*N*-acetylglucosaminyltransferase I (GlcNAc-TI) activity (23), the enzyme responsible for the initiation of complex *N*-linked carbohydrate formation by the addition of *N*-acetylglucosamine to the oligomannosyl intermediate ($Man_5GlcNAc_2Asn$) (9, 15, 26). Structural studies of the carbohydrates synthesized by Lec1A cells and the genetic properties of the *lec1A* mutation reported here suggest that *lec1A* is a new mutation at the *lec1* locus. The Lec1A phenotype correlates with reduced processing of $Man_5GlcNAc_2Asn$ to complex carbohydrates and is apparently due to a mutation that causes partial loss of GlcNAc-TI activity. The mutant cell line should be of interest in determining the structural and functional roles of specific carbohydrate groups at different glycosylation sites in glycoproteins and the effects of altered processing on glycoprotein trafficking within the cell.

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MATERIALS AND METHODS

Materials. The leukoagglutinin from *Phaseolus vulgaris* (L-PHA) was from Burroughs Wellcome Co., Poole, England; the agglutinins from *Triticum vulgaris* (WGA) were from Sigma Chemical Co., St. Louis, Mo.; concanavalin A (Con A) from *Canavalia ensifomis* and ConA-Sepharose were from Pharmacia, Uppsala, Sweden; the toxin from *Ricinus communis* (RIC), the agglutinins from *Lens culinaris* (LCA), and L-PHA-agarose were from Vector Laboratories, Burlingame, Calif.; alpha medium, serum, colcemid, and medium chemicals were from GIBCO Laboratories, Grand Island, N.Y.; polyethylene glycol 1000 was from Koch Light, Cainbrook, England; Bio-Gel P2, P4, and P6 and Chelex were from Bio-Rad Laboratories, Richmond, Calif.; pronase (grade B) was from Calbiochem-Behring, La Jolla, Calif.; endoglycosidase-H (endo-H) was from Health Research Inc., Albany, N.Y.; UDP-[1-^{14}C]glucosamine (49 mCi/mol), [6-^{3}H]glucosamine hydrochloride (30 Ci/mol), [6-^{3}H]]galactose (25 Ci/mol), [2-^{3}H]mannose (16 Ci/mol), ^{125}I-labeled Bolton Hunter reagent (13.2 mCi/μg), ACSII scintillation fluid, and Amplify were from Amersham Corp., Arlington Heights, Ill.; and protein standards (^{14}C-labeled) were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Chicken ovalbumin, (grade V; Sigma) was used to prepare

$Man_5GlcNAc_2Asn$ as described (12). Purity was determined by ^1H nuclear magnetic resonance (NMR) spectroscopy (3). D_2O (99.8 and 99.996%) was from Stohler, Waltham, Mass. All chemicals were reagent grade.

Cells. The CHO cell lines used in this study included Pro^-5 and Gat^-2 (parental cells [20]), the Lec1A glycosylation mutants Pro^-Lec1A.3B (isolated previously [20]), Pro^-Lec1A.2C, Pro^-Lec1A.2A, and Gat^-Lec1A.5J (isolated during this study), and Lec1 mutants Pro^-Lec1.3C and Gat^-Lec1.1N (isolated previously [22]). Cells were routinely cultured in suspension at 37°C in alpha medium containing 10% fetal calf serum.

GlcNAc-TI Assay. GlcNAc-TI activity was determined essentially as described previously (23). Cells were extracted with 2.5% Triton X-100, and samples (100 to 200 μg of protein) of the postnuclear (1,500 × *g* for 1 min) supernatant were incubated at 37°C for 1 or 1.5 h with 0.05 μmol of $Man_5GlcNAc_2Asn$, 2.5 μmol of 2-(*N*-morpholino)ethanesulfonate (MES) (pH 6.25), 0.5 μmol of $MnCl_2$, and UDP-*N*-[^{14}C]acetylglucosamine (0.024 μmol; 5×10^6 to 8×10^6 cpm/μmol) in a final volume of 40 μl. Reaction products were separated by high-voltage electrophoresis in borate buffer (23) or by ConA-Sepharose chromatography (13) as previously described. Activity was determined as the difference in incorporation between samples with and without added exogenous acceptor.

Preparation of radiolabeled virus. Exponentially growing cells from suspension cultures were pelleted, washed once with alpha medium, and suspended at 2.5×10^7 cells per ml in alpha medium containing vesicular stomatitis virus (VSV) Indiana strain (20 PFU per cell). After 1 h of incubation with occasional shaking at 37°C, the cells were diluted to 1.7×10^6 cells per ml with alpha medium containing 2% dialyzed fetal calf serum and 0.1 mg of glucose per ml. After 1.5 h at 37°C, radiolabeled sugar was added to a final concentration of 8 μCi/ml, and the incubation was continued for 16 h. Intact cells and nuclei were removed by centrifugation (1,000 × *g* for 20 min), and the VSV-containing supernatant was layered over 27-ml linear gradients of potassium tartrate (15 to 33%, wt/wt) in ET buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0). The gradients were centrifuged to equilibrium for 2 h at 25,000 rpm at 4°C in a Beckman SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Harvested virus bands were diluted with ET buffer and pelleted by centrifugation at 25,000 rpm for 1.5 h in the SW28 rotor at 4°C. Virus was suspended in 1.0 ml of 5 mM Tris hydrochloride (pH 8.5) and stored in −20°C. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that only one radiolabeled band corresponding to the G glycoprotein (molecular weight, ~67,000) was present in each preparation.

Purified Sindbis virus grown in chicken embryo fibroblasts in the presence of [^{14}C]mannose was a gift from Paul Atkinson (Albert Einstein College of Medicine).

SDS-PAGE. PAGE was performed in 0.1% SDS under reducing conditions in discontinuous Tris buffers using a 3% stacking gel and a 10% running gel. Gels were run at constant current (10 mA) until the bromophenol blue dye entered the running gel and subsequently at 15 mA until the dye reached the bottom of the gel. After treatment with Amplify, gels were dried onto filter paper and exposed to preflashed Kodak XAR5 film at −70°C. For elution of G glycoprotein from the gel, excised bands were soaked in 1 ml of deionized water. The separated gel pieces were subsequently macerated in 3 ml of 25 mM Tris–0.2 M glycine (pH 8.5) containing 0.1% SDS. After 24 h at 37°C gel pieces were removed by centrifugation (12,000 × *g* for 10 min). Approximately 30% recovery of radioactivity was routinely achieved.

Enzyme digestions. Radiolabeled VSV or G glycoprotein was incubated with 1 mg of pronase per ml in 5 mM Tris hydrochloride (pH 8.5)–3 mM $CaCl_2$ at 50°C under toluene. After 24 h, an equal amount of pronase was added, and the incubation was continued for an additional 24 h. The sample was boiled for 2 min and centrifuged (12,000 × *g* for 5 min) to remove insoluble material. Greater than 95% of the radiolabel was soluble in 10% trichloroacetic acid after pronase digestion.

For endo-H digestion, samples were incubated at 37°C in 0.1 ml of 0.05 M citrate-phosphate buffer (pH 5.25) containing 1 mU of endo-H. After 18 h, the mixtures were placed for 2 min in a boiling water bath and subsequently centrifuged (12,000 × *g* for 5 min) to remove insoluble material.

Lectin-affinity chromatography. ConA-Sepharose equilibrated with ConA buffer (0.1 M $NaCOOCH_3$, 1.0 M NaCl, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, 0.01 M $MnCl_2$, 0.02% NaN_3; pH 7.0) was packed into a column (0.6 by 16 cm) at room temperature. Radiolabeled glycopeptides were applied in 0.5 ml of ConA buffer, the column was washed with 20 ml of ConA buffer, followed by a 40-ml linear gradient of 0 to 10 mM α-methylmannoside in ConA buffer and then 20 ml of 200 mM α-methylmannoside in ConA buffer. The flow rate was 14 ml/h. L-PHA-Sepharose was equilibrated with phosphate-buffered saline (PBS; pH 7.2) and packed into a column (0.5 by 22 cm) at 4°C. Samples were applied in 1.0 ml of PBS and eluted with PBS at a flow rate of 5 ml/h.

Gel filtration chromatography. A Bio-Gel P2 column (1.5 by 43 cm) equilibrated in deionized water was used for desalting the glycopeptides. The column was eluted with deionized water under pressure at a flow rate of 34 ml/h. A

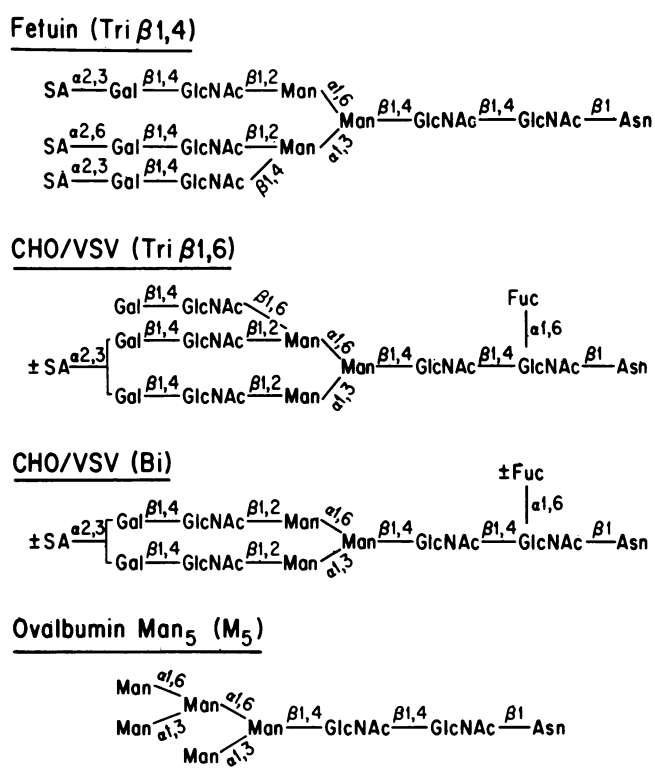

FIG. 1. Structures of authentic glycopeptides used to calibrate lectin-affinity and Bio-Gel columns (see the text).

Bio-Gel P4 column (1.5 by 190 cm) equilibrated in 0.1 M NH_4HCO_3 was used to analyze pronase- and endo-H-digested glycopeptides at a flow rate of 2 ml/h at 4°C.

Glycopeptides of known structure. Several glycopeptides of known structure (Fig. 1) were iodinated and used to calibrate the lectin-affinity and Bio-Gel columns. The fetuin glycopeptide was a gift from A. Adamany (Albert Einstein College of Medicine), the structure (Fig. 1) being taken from Nilsson et al. (14). The glycopeptides from VSV grown in CHO cells (CHO/VSV) were prepared in this laboratory, and their structures were determined previously by ^1H NMR spectroscopy (25). The $Man_5GlcNAc_2Asn$ glycopeptide was prepared from ovalbumin by the method of Huang et al. (12), and its structure was determined by ^1H NMR spectroscopy (3). Iodination was achieved by mixing 10 to 100 μg of dried glycopeptide with 1 mCi of ^{125}I-labeled Bolton Hunter reagent as described previously (1).

^1H NMR spectroscopy. Unlabeled carbohydrates were prepared from 150 mg of purified VSV grown in Pro^-Lec1A.2C cells (Lec1A/VSV). The Lec1A/VSV from different preparations was analyzed for purity by SDS-PAGE and silver staining, pooled, and digested with pronase for 5 days at 50°C. Enzyme was added to a final concentration of 1 mg/ml in 10 mM Tris hydrochloride–3 mM $CaCl_2$ (pH 8.5) every 24 h. After boiling for 10 min and removal of insoluble material by high-speed centrifugation, the supernatant was mixed with different amounts of each of the iodinated marker glycopeptides fetuin, CHO/VSV(Bi) and ovalbumin (Man_5) (Fig. 1) and chromatographed on Bio-Gel P6 (−400) at a flow rate of 5 ml/h in distilled water containing 0.02% NaN_3. The marker glycopeptides eluted in three major peaks beyond the void volume. They were pooled separately and fractionated on ConA-Sepharose as described previously (25), except that the bound biantennary glycopeptides were eluted with 3 mM α-methylmannoside in modified ConA buffer, and the bound oligomannosyl glycopeptides were eluted subsequently with 10 mM α-methylmannoside in modified ConA buffer. (These elution conditions were determined from the experiments presented in Fig. 3.) After several desalting steps on Bio-Gel P2 and passage over a 2-ml column of Chelex 100, the samples were exchanged three times in 99.8% D_2O and once in 99.996% D_2O and subjected to ^1H NMR spectroscopy in 0.5 ml of

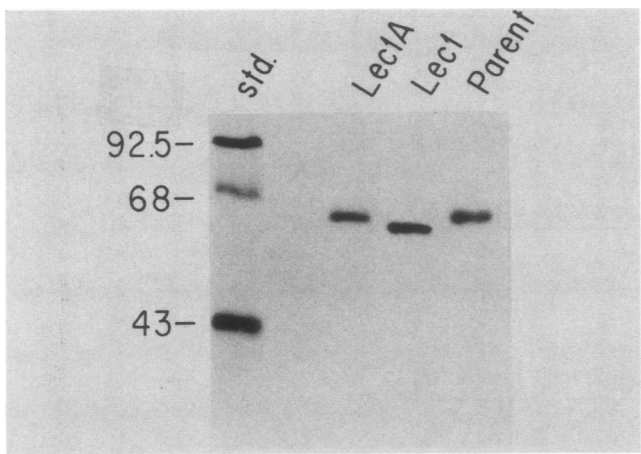

FIG. 2. SDS-PAGE of [^3H]glucosamine-labeled VSV grown in parental (Pro^-5), Lec1, and Lec1A CHO cells. Each sample contained 20,000 cpm. The standards (std.; 4,000 dpm per band) were (top to bottom) phosphorylase B, bovine serum albumin, and ovalbumin; their positions and molecular weights (in thousands) are indicated.

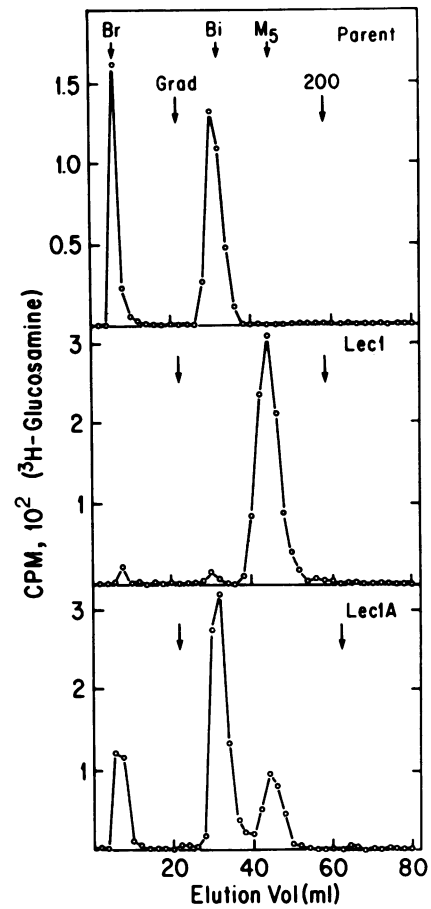

FIG. 3. ConA-Sepharose chromatography of [^3H]glucosamine-labeled glycopeptides from pronase-digested VSV grown in parental, Lec1, or Lec1A CHO cells. Samples (0.5 ml) were loaded in ConA buffer. The column was washed with 20 ml of ConA buffer before a 40-ml gradient of 0 to 10 mM α-methylmannoside in ConA buffer (Grad) was applied. The column was subsequently eluted with 20 ml of 200 mM α-methylmannoside in ConA buffer (200 point on figure). The elution positions of iodinated glycopeptide standards are shown by the arrows (see Fig. 1). Br, Branched carbohydrate standards, Tri(β1,4) and Tri(β1,6); Bi, biantennary standard; M_5, $Man_5GlcNAc_2Asn$ standard.

99.996% D_2O containing approximately equimolar acetone. Spectra were obtained at 23 and 70°C with the 500-MHz spectrometer at the Yale University Northeastern Regional NMR Facility with the help of Paul Atkinson (Albert Einstein College of Medicine). A sweep width of 4,000 Hz, pulses of 90°, and a cycle delay time of 3 s were used to collect spectra in 16K data blocks. The chemical shift of acetone at 2.225 ppm compared with that of 4,4-dimethyl-4-silapentane was used as an internal reference standard.

Selection of hybrids. Equal numbers of the two cell lines to be fused (carrying Pro^- and Gat^- recessive markers) were mixed in monolayer culture and treated with polyethylene glycol as described previously (21). Viable hybrids were selected in deficient alpha medium lethal to diploid auxotrophs. Controls for auxotrophic revertants and spontaneous hybrid formation were performed in each experiment. The frequency of both was usually $\leq 10^{-5}$.

Selection of lectin-resistant colonies. Cells were incubated at 10^6 cells per 100-mm tissue culture dish in alpha medium containing 10% fetal calf serum and selective lectin(s) for 8 days without disturbance at 37°C in a humidified atmosphere

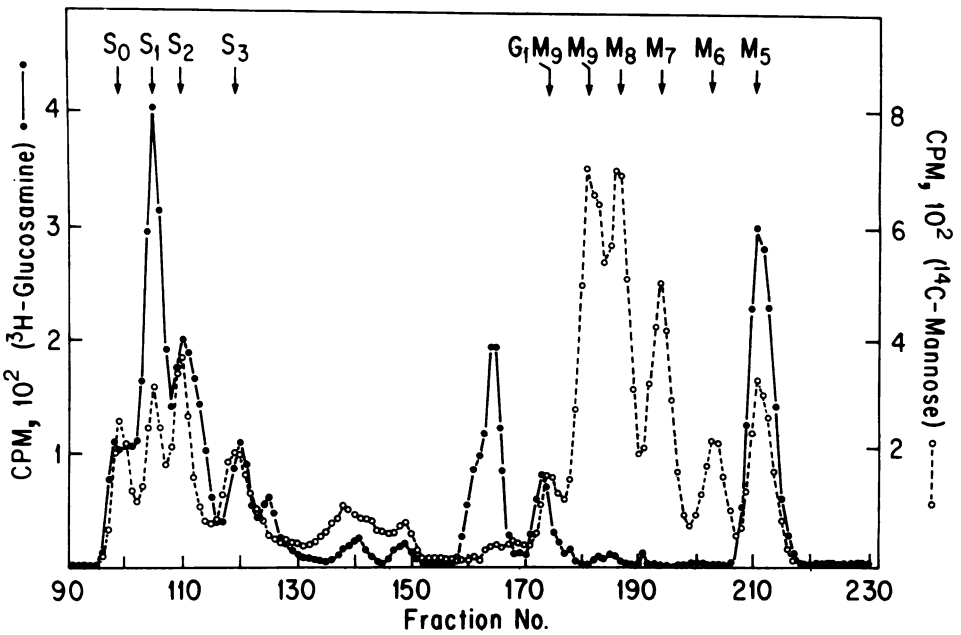

FIG. 4. Analysis of [^{3}H]glucosamine-labeled Lec1A/VSV on Bio-Gel P4. Sindbis virus ([^{14}C]mannose; ~20,000 cpm) was added to Lec1A/VSV ([^{3}H]glucosamine; ~8,000 cpm), and the sample was subjected to pronase and endo-H digestions. It was subsequently applied to a Bio-Gel P4 column and eluted with 0.1 M NH_4HCO_3. Samples (1 ml) were collected, and ^{3}H and ^{14}C were determined by liquid scintillation spectrometry. The elution positions of the Sindbis glycopeptides (S_0, S_1, S_2, S_3) and oligosaccharides (M_9, M_8, M_7, M_6, M_5) are indicated. G_1M_9, M_9 intermediate with one glucose residue.

of 5% CO_2. When colonies of the desired size were visible, the plates were washed and colonies were picked into nonselective medium. As soon as possible they were tested for lectin resistance and, if appropriate, cloned by limiting dilution.

Determination of lectin resistance. The toxicity of lectins for different isolates was determined by the Lec^R P-test as previously described (21). Briefly, lectins were titrated in 96-well dishes to include many concentrations near the likely endpoint. Cells (2,000) were added to each well, and the plate was incubated at 37°C in a CO_2 incubator. When control wells were confluent, the plate was stained, and the endpoint was taken as the concentration of lectin that caused 90% cell death.

RESULTS

Biochemical characterization of the Lec1A phenotype. Lec1 CHO cell mutants lack detectable GlcNAc-TI activity (6, 23), and as a consequence, they synthesize truncated *N*-linked carbohydrates blocked at the processing intermediate $Man_5GlcNAc_2Asn$ (6, 18). Standard GlcNAc-TI assays giving 4 to 6 nmol of GlcNAc transfer to $Man_5GlcNAc_2Asn$ per mg of protein per h with parental cell extracts and no detectable transfer with Lec1 cell extracts also failed to reveal significant GlcNAc-TI activity in Lec1A cell extracts. Surprisingly, however, electrophoresis of Lec1A/VSV suggested that GlcNAc-TI was active in intact cells. The electrophoretic mobility of the G glycoprotein from Lec1A/VSV was similar to that of G from CHO/VSV rather than that of G from Lec1/VSV (Fig. 2). The latter migrates significantly faster in SDS gels because of its altered carbohydrate complement (26). The fact that G from Lec1A/VSV comigrated with G from CHO/VSV suggested that Lec1A cells synthesize complex carbohydrates that require the action of GlcNAc-TI for their formation.

Indirect evidence that G from Lec1A/VSV possesses complex carbohydrates was obtained by lectin-affinity and Bio-Gel chromatography of pronase-derived glycopeptides. The G bands were excised from SDS gels of glucosamine-labeled Lec1/VSV and Lec1A/VSV, exhaustively digested with pronase, and analyzed on ConA-Sepharose in parallel with pronase glycopeptides derived from CHO/VSV (Fig. 3). CHO/VSV exhibited only fully processed, complex, *N*-linked carbohydrates which coeluted from ConA-Sepharose with branched and biantennary standard glycopeptides. By contrast, Lec1/VSV exhibited mainly glycopeptides that coeluted with authentic $Man_5GlcNAc_2Asn$. The glycopeptide profile from Lec1A/VSV was a combination of the profiles of Lec1/VSV and CHO/VSV. The Lec1A/VSV glycopeptides eluted from ConA-Sepharose with the characteristics of branched and biantennary complex carbohydrates and, in addition, contained glycopeptides that coeluted with the unprocessed intermediate $Man_5GlcNAc_2Asn$. Treatment of intact Lec1A/VSV with endo-H released only the latter species, as would be predicted for authentic $Man_5GlcNAc_2Asn$. In other experiments, independent Lec1A isolates gave similar glycopeptide profiles on ConA-Sepharose.

The structural characteristics of the carbohydrates synthesized by Lec1A cells and expressed on the G glycoprotein were further investigated by chromatography on Bio-Gel P4 (Fig. 4). The major carbohydrates from Lec1A/VSV coeluted with the sialylated complex carbohydrates of Sindbis virus and with the $Man_5GlcNAc$ endo-H-released Sindbis virus oligosaccharide, as expected from their behavior on ConA-Sepharose (Fig. 3). The Sindbis glycopeptides released by pronase and endo-H have been shown by ^{1}H NMR spectroscopy (7) to comprise differentially sialylated biantennary carbohydrates (S_1, S_2, S_3) and oligomannosyl oligosaccharides ($Man_{5-9}GlcNAc_2Asn$). The glycopeptides denoted S_0 in Fig. 4 are branched, complex structures and correspond to the fraction that passed unretarded through ConA-Sepharose (Fig. 3). Investigation of this fraction from Lec1A/VSV showed that the glycopeptides were structur-

ally similar to the branched glycopeptides from CHO/VSV (Fig. 5). Both preparations were retarded by L-PHA-agarose in a manner typical of triantennary carbohydrates that contain a β1,6-linked branch GlcNAc residue (4, 8).

Although the indirect structural data provided clear evidence for the synthesis of complex carbohydrates by Lec1A cells, more direct information was required to determine whether the structures of the Lec1A cell carbohydrates were actually identical to those synthesized by parental CHO cells. For example, a second mutation in Lec1A cells might convert the Man_5 intermediate to a novel, complex structure. Alternatively, the putative Man_5 intermediate of Lec1A/VSV might be a novel structure that would not serve as a GlcNAc-TI substrate. To address these questions, ^1H NMR spectroscopy of the ConA-bound glycopeptides from Lec1A/VSV was performed.

The spectra obtained showed directly that the complex carbohydrates of Lec1A/VSV were essentially identical to those previously characterized from CHO/VSV (25). The Lec1A/VSV pronase glycopeptides that eluted from ConA-Sepharose with the ^{125}I-CHO/VSV(Bi) marker (see above) were typical of biantennary, complex carbohydrates with variation in the presence of α2,3-linked sialic acid residues and α1,6-linked fucose residues. The only significant difference from the spectra obtained previously with the biantennary carbohydrates of CHO/VSV was that the Lec1A derived biantennaries possessed an increased proportion of sialic acid residues. This is consistent with the increased proportion of S_1 glycopeptides observed on Bio-Gel P4 (Fig. 4) compared with that in the previous profile reported for

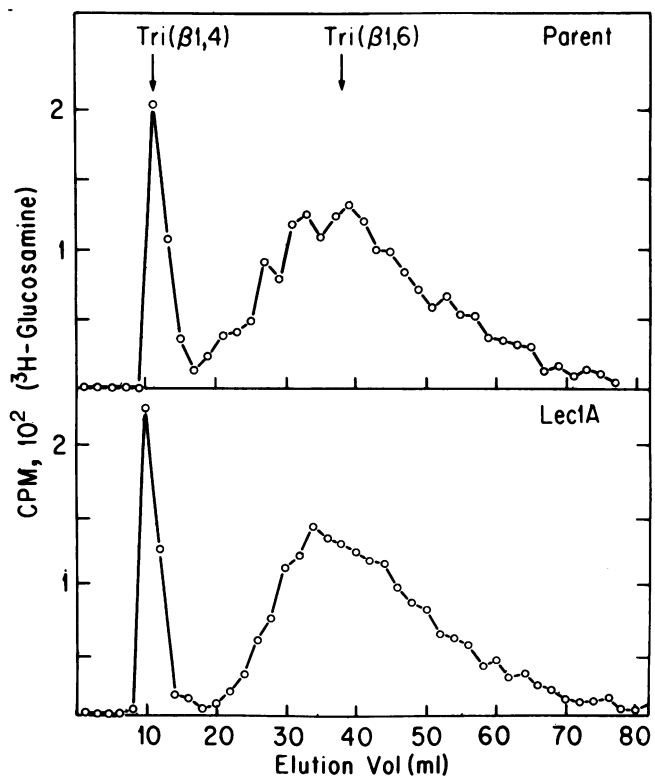

FIG. 5. L-PHA-agarose chromatography of glycopeptides which passed unretarded through ConA-Sepharose. After ConA-Sepharose chromatography (Fig. 3), pooled glycopeptides from the voided volume were desalted on Bio-Gel P2 and lyophilized. The samples were subsequently dissolved in 1 ml of PBS (pH 7.2), applied to L-PHA-agarose, and eluted with PBS.

TABLE 1. Chemical shifts from ^1H NMR spectra of ConA-bound Lec1A/VSV glycopeptides[a]

Sugar residues[b]	Chemical shifts (ppm) of reporter proton			
	Biantennary glycopeptides[c]		Oligomannosyl glycopeptides[c]	
Mannose	C-1	C-2[d]	C-1	C-2
α1,3$_i$	5.117	4.192	5.092	4.074
α1,3$_t$			5.092	4.064
α1,6$_i$	4.926	4.113	4.870	4.147
α1,6$_t$			4.906	3.982
β1,4	4.766	4.250	4.783	4.257
GlcNAc	C-1	*N*-Acetyl	C-1	*N*-Acetyl[e]
β1,2 (sialic acid)	4.573	2.050[f]		
β1,2	4.581	2.043[f]		
Core β1,4 (fucose)	4.697	2.094		
Core β1,4	4.615	2.081	4.603	2.061
Asparagine	5.068	2.007[g]	5.069	2.012
Galactose	C-1			
β1,4 (sialic acid)	4.551			
β1,4	4.477			
	4.462			
Fucose	C-1	CH_3		
α1,6	4.877	1.202		
Sialic acid	C-3	*N*-Acetyl		
α2,3	2.758 (eq)	2.030		
	1.800 (ax)[h]			

[a] ConA-bound glycopeptides were prepared from purified pronase-digested Lec1A/VSV and subjected to ^1H NMR spectroscopy (see the text). The chemical shifts from spectra obtained at 23°C are given. The $J_{1,2}$ coupling constants of the C-1 protons from α-linked mannose residues were ~2 Hz, and those from β-linked GlcNAc and galactose residues were ~7 to 8 Hz. Assignments are based on previous spectra obtained by this laboratory (25) as well as on published data from several laboratories which describe the spectral properties of identical and related compounds (2, 3, 27).

[b] Abbreviations for the sugar residues are based on those previously published for these compounds, where *i* is the internal residue and *t* is the terminal residue (2, 3).

[c] Biantennary glycopeptides were coeluted with ^{125}I-CHO/VSV(Bi), and oligomannosyl glycopeptides were coeluted with ^{125}I-$Man_5GlcNAc_2Asn$ from Con A-Sepharose (Fig. 3).

[d] A small, nonequimolar resonance at 4.156 ppm was observed in these spectra as reported previously for the biantennary carbohydrates of CHO/VSV (25). This resonance has not been assigned.

[e] Small, nonequimolar resonances at 2.075 and 2.007 ppm were observed that did not correspond to resonances in the anomeric region and might reflect the effects of different amino acids on the peptide portions of these glycopeptides.

[f] These assignments are interchangeable.

[g] There was a small resonance at 2.010 ppm which might be due to amino acid variation on the peptide portion.

[h] eq, Equatorial; ax, axial.

CHO/VSV (19). The chemical shifts obtained for each of the reporter protons of the biantennary fraction are presented in Table 1. These shifts, as well as observed $J_{1,2}$ coupling constants and the molar proportion of each resonance, agree within experimental error with the spectral properties of the biantennary carbohydrates of CHO/VSV shown in Fig. 1 (25) and with those of many related biantennary compounds reported in the literature (2, 27).

Also given in Table 1 are the chemical shifts for the reporter proton(s) of the Lec1A/VSV pronase glycopeptides that eluted with the ^{125}I-$Man_5GlcNAc_2Asn$ ovalbumin marker from ConA-Sepharose (see above). These data, the molar proportions of each resonance, and the corresponding $J_{1,2}$ coupling constants were completely consistent with the results of previously published ^1H NMR spectra and the

TABLE 2. Selection of independent Lec1A mutants[a]

Parental cells	Frequency of smallest colonies	Lectin resistance (μg/ml)		No. of Lec^R colonies	Lec^R type[b]
		RIC	ConA		
Pro^-5	$\sim10^{-5}$	>0.025–0.01	>7.5<10	8	Lec1A
		>0.25	>10	2	Ric^R
		>0.01≤0.025	>10	5	Ric^R
		>0.025–0.25	5–10	6	Lec1A/Lec1
Gat^-2	$\sim10^{-6}$	>0.025<0.05	>5<7.5	1	Lec1A
		>0.05<0.25	5	3	Lec1A/Lec1
		>0.25	>3<5	5	Lec1/Lec1A
		>0.25	>1<3	12	Lec1

[a] Parental cells were subjected independently to selection with L-PHA (15 μg/ml) and LCA (10 μg/ml). Small colonies (0.1 to 0.5 mm) were picked into nonselective medium, cultured, and tested for resistance to RIC and ConA in parallel with authentic parental, Lec1, and Lec1A cells.

[b] Many of the colonies exhibited mixed endpoints typical of the presence of both Lec1A and Lec1 cells in the cell population (21). Such mixtures are indicated by a slash, with the predominant cell type given first.

chemical shifts for the ovalbumin $Man_5GlcNAc_2Asn$ depicted in Fig. 1 (2, 3, 27). Therefore, the endo-H-sensitive oligomannosyl carbohydrate from Lec1A/VSV has the correct structure to be the authentic processing intermediate proposed to act as the in vivo substrate for GlcNAc-TI (16, 26).

The combined structural studies showed that Lec1A cells were able to synthesize branched and biantennary complex carbohydrates. However, unlike parental CHO cells, they did not convert all the $Man_5GlcNAc_2Asn$ associated with the G glycoprotein in infected Lec1A cells to complex carbohydrates. This suggests that Lec1A cells possess a partially inactivated GlcNAc-TI.

Genetic characterization of the *lec1A* mutation. The biochemical phenotype of Lec1A cells may have arisen from a single genetic alteration at the *lec1* locus or from mutations affecting a gene(s) in addition to the one that defines complementation group 1. For example, a mutation modifying Lec1 cells to a Lec1A phenotype might allow the conversion of $Man_5GlcNAc_2Asn$ to normal complex carbohydrates by a novel pathway. In this case, further biochemical characterization of the *lec1A* mutation would require different approaches than if the *lec1A* mutation affected the gene that already defines the Lec1 mutant group. Thus, it was important to seek genetic evidence to determine whether the Lec1A phenotype was the result of one or more mutations.

Single-step selection of Lec1A cells. The Lec1A mutants previously described in the literature (10, 20) were both obtained by protocols that did not preclude the isolation of a rare mutant carrying more than one mutation. If, however, the *lec1A* lesion is due to a single genetic event, it might be expected to arise at a frequency close to 10^{-5} or 10^{-6}, as observed for the *lec1* mutation (24). A reconstruction experiment showed that, like Lec1 (18), the Lec1A phenotype survived selection with 15 μg of L-PHA and 10 μg of LCA per ml, with a relative plating efficiency of approximately 100%, whereas parental CHO cells were effectively killed. Moreover, the Lec1A colonies were very small after 8 days in this combination of lectins and could be distinguished from Lec1 cells partly on this basis. Therefore, to select Lec1A mutants in a single step, unmutagenized Pro^-5 and Gat^-2 cells were incubated separately with medium containing both L-PHA (15 μg/ml) and LCA (10 μg/ml). After 10 days at 37°C, stained plates showed a heterogeneous array of colonies including 1 to 10 small (0.1- to 0.5-mm) colonies per plate. The overall survival frequency was $\sim10^{-4}$ for Pro^-5 cells and $\sim10^{-5}$ for Gat^-2 cells, but only about 10% of the survivors were small colonies. Of the smallest colonies, 21 were picked from each selection, cultured, and tested for resistance to RIC and ConA.

All except seven of the Pro^-5 isolates were hypersensitive to ConA and therefore were putative Lec1 or Lec1A phenotypes (Table 2). Of these, six cultures appeared to be a mixture of Lec1 and Lec1A cells because they gave characteristically mixed endpoints in the Lec^R P-test (21). The remaining eight isolates exhibited phenotypes typical of Lec1A cells—more resistant to ConA (about 2-fold) and more sensitive to RIC (about 10-fold [11]) than Lec1 cells. Therefore, the frequency of Lec1A colonies in the Pro^-5 population was $\sim5 \times 10^{-5}$. It was somewhat lower in the Gat^-2 population, being closer to 2×10^{-6}. Both of these frequencies were significantly higher (≥5,000-fold) than would be predicted for the single-step selection of a small colony arising from two independent mutations in these experiments (10^{-10} to 10^{-12}).

Three of the Lec1A colonies from the Pro^-5 and Gat^-2 selections were tested against the five lectins L-PHA, WGA, ConA, RIC, and LCA and found to exhibit characteristic Lec1A phenotypes, as did clones derived from each of them. In addition, complementation analyses between the new Lec1A clones and Lec1 cells showed that they all belonged to complementation group 1. The frequencies with which Lec1A mutants were obtained from the two independent populations were most consistent with the likelihood that the *lec1A* mutation is a single genetic event.

Segregation analysis of hybrids. Previous studies have shown that the Lec1A phenotype behaves recessively in hybrids formed with parental CHO cells and that it fails to complement the Lec1 phenotype (20). A more extensive investigation of the lectin resistance properties of Lec1A × Lec1 hybrids revealed that the Lec1A phenotype is dominant over Lec1 (Table 3). The properties of the hybrids suggested a second genetic approach to determining whether Lec1A cells carry a mutation in addition to the one that falls into complementation group 1, i.e., to characterize segregants selected from hybrid cell populations for the loss of dominant alleles. If the Lec1A phenotype represents a double mutation, segregants expressing a Lec1 phenotype should be obtained among WGA- or RIC-resistant survivors of a selection from Lec1A × parental cell hybrids. On the other hand, segregants with the characteristics of Lec1A cells should not be obtained in this experiment, since the putative second mutation only gives rise to a Lec1A phenotype when expressed in a Lec1 genetic background (Table 3). By contrast, if Lec1A represents a single mutation affecting an alternative site in the gene that defines complementation group 1, segregants should include those with a Lec1A phenotype but should not include any survivors with a Lec1 phenotype.

The predictions outlined above were tested by subjecting

TABLE 3. Dominance of Lec1A over Lec1 phenotype[a]

Cell lines	No. of colonies tested	Lectin resistance (μg/ml)			Lec^R type
		WGA	ConA	RIC	
Hybrids					
Lec1A × parental	6–12	<3	ND[b]	0.01–0.025	Parental
Lec1A × Lec1	10	25–30	>5<7.5	0.05	Lec1A
Controls					
Parental		<2.5	>15<20	0.0075	
Lec1		>100	>3<5	0.5	
Lec1A		10	>5<7.5	0.05	

[a] Pro⁻Lec1A.3B cells were fused with Gat⁻2 (parental) or Gat⁻Lec1.1N cells. Hybrid colonies were picked, shown to be pseudotetraploid, and tested for lectin resistance by the Lec^R P-test in conjunction with pseudodiploid parental, Lec1, and Lec1A cells.
[b] ND, Not determined.

pseudotetraploid hybrids from crosses between Lec1A and parental CHO cells to selection for resistance to WGA or RIC. Large, resistant colonies (arising at frequencies of ~5 × 10^{-3} to ~10^{-5}) were picked, and those that maintained pseudotetraploid properties were subjected to P-test analysis (Table 4). From Lec1A × parental cell hybrids, each selection gave several segregants with a typical Lec1A phenotype or a phenotype similar to that of Lec1A cells. None of the survivors of either lectin selection expressed a characteristic Lec1 phenotype. However, several isolates exhibited a phenotype intermediate between Lec1A and Lec1. This result might reflect variation in the Lec1A GlcNAc-TI activity in a hybrid cell environment. Apparently it did not reflect the modified expression of a *lec1* defect on a pseudotetraploid background, because 20 RIC-selected segregants from a Lec1A × Lec1 hybrid population expressed a completely normal Lec1 phenotype (Table 4).

The fact that none of the segregants from Lec1A × parental cell hybrids expressed a Lec1 phenotype but a significant proportion expressed a phenotype indistinguishable from that of Lec1A cells is most consistent with the hypothesis that the Lec1A phenotype arises from a single genetic alteration affecting the gene that defines complementation group 1.

DISCUSSION

The *lec1A* CHO mutation results in an interesting new glycosylation-defective phenotype. Investigation of Lec1A carbohydrates has shown that the complex carbohydrates of Lec1A/VSV are identical (except for somewhat increased sialylation) to those of CHO/VSV by lectin-affinity and Bio-Gel chromatography and by ^1H NMR spectroscopy. The oligomannosyl Lec1A/VSV carbohydrate was shown by the same criteria (and by its sensitivity to endo-H) to be $Man_5GlcNAc_2Asn$. This structure is the major carbohydrate of Lec1/VSV and on this basis was proposed as the in vivo substrate for GlcNAc-TI (16, 26). Thus, it appears that GlcNAc-TI, although active in Lec1A cells, is considerably less active than it is in parental cells, resulting in the slow processing of $Man_5GlcNAc_2Asn$ to complex carbohydrates. Consistent with this interpretation is the observation that the biantennary carbohydrates of Lec1A/VSV possess more sialic acid than do those from CHO/VSV. The availability of a cell line that processes *N*-linked carbohydrates at a reduced rate should be extremely useful for studies of the effects of altered glycosylation kinetics on the secretory pathway for glycoproteins and the determination of transferase preference for different glycosylation sites in glycoproteins. In addition, the effect of this mutation on the array of carbohydrate structures expressed at the cell surface might be important to our understanding of the mechanisms that control this array during development and differentiation.

The inability to detect significant GlcNAc-TI activity in Lec1A cell extracts under standard assay conditions is surprising. However, this finding could well be explained by a structural change in the affected gene product so that the conditions for extracting GlcNAc-TI activity, its substrate and ionic requirements, or its pH optimum might differ in Lec1A and parental cell extracts. This possibility is being examined by searching for conditions under which the Lec1A cell GlcNAc-TI might be active in vitro. The reason for focusing on this approach is that the genetic properties of the *lec1A* mutation suggest that it arises from a single genetic event at the *lec1* locus. The frequencies (~10^{-5} to ~10^{-6}) with which independent Lec1A mutants may be selected in a single step are inconsistent with two separate genetic events. In addition, the fact that the Lec1 phenotype was never observed among more than 40 segregants from Lec1A × parental cell hybrids is a strong indication that the Lec1A phenotype does not arise from mutations at two loci. However, genetic arguments are not definitive, and therefore it remains possible that *lec1* and *lec1A* identify tightly linked genes which are not resolved by segregation analysis. On the other hand, this possibility seems remote because *lec1* and *lec1A* clearly belong to a single genetic complementation group (20) (Table 3).

Determination of the molecular mechanisms responsible for the Lec1 and Lec1A phenotypes must await the isolation

TABLE 4. Segregation analysis[a]

Hybrid population	Selective lectin (μg/ml)	Lectin resistance (μg/ml)			No. of Lec^R segregants	Lec^R type[b]
		WGA	ConA	RIC		
Lec1A × parental	WGA (10)	20–30	>5–10	0.05–0.075	4	Lec1A
		>30–50	>3–5	>0.1–0.2	10	Lec1A/Lec1
Lec1A × parental	RIC (0.02)	5–10	>5<7.5	0.05–0.1	3	Lec1A
		>5–20	>5–7.5	0.1–0.4	9	Lec1A/Ric^R
		<5	<15	0.1	7	Ric^R
Lec1A × Lec1	RIC (0.2)	≥100	≥3<5	0.4–0.5	20	Lec1

[a] Pro⁻Lec1A.3B cells were fused with Gat⁻2 or Gat⁻Lec1.1N cells, and viable hybrids were selected. After expansion in nonselective medium, they were subjected to selection with WGA or RIC. RIC selection was performed on five hybrid populations known to have arisen independently. Survivors forming large colonies were picked, cultured, and tested for lectin resistance. Control pseudodiploid lines were included in the Lec^R P-tests and gave values similar to those shown in Table 3.
[b] Some segregant populations exhibited intermediate Lec^R phenotypes (designated by shill, with predominant phenotype given first).

of the gene that corresponds to the *lec1* locus. One point of considerable interest is why, if Lec1A results from partial gene disruption (presumably by a conservative structural change in the genome that allows the retention of GlcNAc-TI activity), the same phenotype arises in independently derived Lec1A mutants. One might expect instead a spectrum of phenotypes reflecting variable inactivation of GlcNAc-TI by different mutations. That this did not occur in several independent isolates is suggestive of a hot spot for mutation, conversion, or deletion at the *lec1* locus. This, in turn, might reflect a regulatory mechanism at the level of the genome by which a cell may control the expression of GlcNAc-TI activity.

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ADDENDUM IN PROOF

Lec1A cell extracts have recently been found to exhibit GlcNAc-T1 activity under assay conditions different from those giving optimal activity with parental CHO cells (W. Chaney and P. Stanley, unpublished data).

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