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MINI REVIEW

# CHO cells provide access to novel *N*-glycans and developmentally regulated glycosyltransferases

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**Chinese hamster ovary (CHO) cells express only a subset of the glycosyltransferase activities known to exist. They do not express several fucosyltransferases, galactosyltransferases, sialyltransferases or *N*-acetylglucosaminyltransferases. However, following mutagenesis or transfection with large amounts of DNA, rare mutants that express a transferase activity *de novo* have been obtained. The first CHO mutant of this type was LEC10, which expresses the *N*-acetylglucosaminyltransferase, GlcNAc-TIII, that adds the bisecting GlcNAc to complex *N*-glycans. Several analogous gain-of-function mutants have now been characterized and, all express a new glycosyltransferase activity. In several cases, expression is known to reflect gene activation at the transcriptional level. Thus, CHO cells contain quiescent glycosyltransferase genes that may be activated by mutational events. Several of these transferases have properties distinct from previously described enzymes. In fact, the most recently characterized dominant CHO mutants, LEC14 and LEC18, each express a GlcNAc-T activity that creates novel *N*-glycans never before observed in glycoproteins from any other source. In these and possibly other cases, it appears the CHO genome has provided access to new GlcNAc-Ts that may be difficult to identify by conventional methods.**

*Key words:* CHO/glycosyltransferase/mutation

## Introduction: defining glycosylation pathways

The multienzyme pathways of eukaryotic glycan biosynthesis have been revealed by classic biochemical, genetic and molecular biological approaches. Glycosyltransferase (Glyc-T) activities in mammalian tissues and secretions were originally identified by *in vitro* assays using exogenous carbohydrate acceptors (Beyer *et al.*, 1979; Schachter, 1986). Dr. Robert Hill, whose career was celebrated at the 'Hillfest', is a pioneer in these studies. The assays developed by Hill and colleagues led to the purification of several Glyc-Ts, but this was a difficult task due to the extremely low abundance and membrane association of these enzymes (Sadler *et al.*, 1982). In addition, the number of Glyc-Ts identified in this manner was limited by the availability of exogenous acceptors that mimicked partially completed structures of known glycans. The latter were purified mainly from serum glycoproteins and therefore reflected only the structures on secreted glycoproteins. While the straight biochemical approach revealed certain complexities (e.g., distinct Glyc-Ts that transfer the same sugar to an identical acceptor but in different linkage; or a single Glyc-T that transfers a sugar in more than one linkage and to different acceptors), it was severely limited by the inability of investigators to know the intermediates in glycan biosynthesis, that is, the true acceptor substrates of the activities being assayed. This limitation was alleviated by the introduction of genetics.

To isolate mammalian cell glycosylation mutants, toxic plant lectins were used as selective agents (Briles, 1982; Stanley, 1984). The vast majority of lectin-resistant mutants proved to survive selection by synthesizing truncated glycan structures that lack the sugars required for lectins to bind at the cell surface. Most of these mutants had lost a glycosylation activity and expressed an immature glycan intermediate, that is, the *in vivo* substrate for that activity. Therefore, in such mutants, a biosynthetic pathway is 'frozen' so that biochemical quantities of a glycosylation intermediate can be isolated and structurally characterized.

The very first glycosylation mutants revealed the value of a genetic strategy. Both mutants were from CHO cells and both lacked the same Glyc-T activity—*N*-acetylglucosaminyltransferase I (GlcNAc-TI; E. C. 2.4.1.101; Gottlieb *et al.*, 1975; Stanley *et al.*, 1975). Structural analysis of *N*-glycans synthesized by these mutants, Lec1 and clone 15B, showed that the endogenous acceptor of GlcNAc-TI is $Man_5GlcNAc_2Asn$ and not the trimannosyl acceptor $Man_3GlcNAc_2Asn$, which was the predicted acceptor because it forms the core of all complex *N*-glycans (Kornfeld and Kornfeld, 1985). In addition to revealing the real substrate of GlcNAc-TI, Lec1 and 15B cells allowed α-mannosidase II (Tabas and Kornfeld, 1978) and GlcNAc-TII (Narasimhan *et al.*, 1977) to be discovered. The former activity removes two Man residues to generate the acceptor $GlcNAcMan_3GlcNAc_2Asn$, to which GlcNAc-TII transfers a GlcNAc to give the biantennary complex *N*-glycan, $GlcNAc_2Man_3GlcNAc_2Asn$.

Most mammalian cell glycosylation mutants (Stanley, 1993) and all yeast glycosylation mutants described to date (Herscovics and Orlean, 1993) are loss-of-function. Such mutations are recessive and can be assigned to genetic complementation groups. Complementation analysis identifies mutants that are phenotypically different but are actually mutated in the same gene. For example, Lec1 and Lec1A CHO mutants have distinct phenotypes but belong to the same complementation group (Stanley and Chaney, 1985). Each exhibits reduced GlcNAc-TI activity, and it is predicted that both mutants will have a distinct mutation in the *Mgat1* gene that encodes GlcNAc-TI (Chaney and Stanley, 1986). In at least one Lec1 mutant, *Mgat1* RNA is of the expected size and is present at normal levels (Kumar *et al.*, 1990), indicating that a point mutation provides the basis of the GlcNAc-TI deficiency. This

**Table I.** Different α(1,3)Fuc-T activities of CHO cells

| CHO line | Specific activity (pmol/mg/min) | $Le^x$ | $SLe^x$ | VIM-2 |
|---|---|---|---|---|
| LEC11 | 50 | ++++ | ++++ | ++++ |
| LEC12 | 86 | ++++ | — | ++++ |
| LEC29 | 2.4 | ++++ | — | — |
| LEC30 | 1216 | ++++ | — | ++ |

The α(1,3)Fuc-T activity of each mutant was assayed using Galβ(1,4)GlcNAc as acceptor (Potvin and Stanley, 1991). Expression of α(1,3)fucosylated cell surface glycans with the determinants recognized by monoclonal antibodies SSEA-1 ($Le^x$; Galβ(1,4)[Fuc(α1,3)]GlcNAc); CSLEX-1 ($SLe^x$; NeuAcα(2,3)Galβ(1,4)[Fuc(α1,3)]GlcNAc; and VIM-2 (NeuAcα(2,3)Galβ(1,4)GlcNAcβ(1,3)Galβ(1,4)[Fucα(1,3)]GlcNAc). Relative binding of each monoclonal antibody is taken from data reported by Potvin and Stanley (1991).

is now known to be the case in the mutants Lec4 and Lec4A, which are both affected in GlcNAc-TV activity and which belong to the same genetic complementation group (Chaney *et al.*, 1989). Sequence analysis of GlcNAc-TV gene transcripts revealed that Lec4 cells encode a truncated transferase while Lec4A cells encode GlcNAc-TV with a single nucleotide change (Weinstein *et al.*, 1996). This change places an arginine at amino acid 188 rather than a leucine and has a significant hydrophobic effect on that region of the protein. This appears functionally important since GlcNAc-TV in Lec4A cells is mislocalized to the endoplasmic reticulum, where it cannot act because it does not encounter its glycan substrate.

It is now clear that recessive glycosylation mutants have enormous potential for revealing new aspects of glycosylation pathways, for providing access to glycosylation intermediates, and for obtaining insight into structure/function relationships by the analysis of glycosylation mutations. Finally, and very importantly, recessive mutants provide vehicles for expression cloning of glycosylation activities (Kumar *et al.*, 1990; Cummings *et al.*, 1993; Eckhardt *et al.*, 1995). Once a Glyc-T gene is isolated, related transferases can, in some instances, be obtained by cross hybridization (Lowe, 1991; Joziasse, 1992; Kleene and Berger, 1993).

## Dominant mutations provide access to novel glycosyltransferases

Recessive glycosylation mutations interrupt pathways that are actually operating in a given cell, but a dominant mutation creates a glycosylation reaction that is missing from parental cells. The first mutant of this type was a lectin-resistant B16 melanoma line that exhibits a marked up-regulation of an α(1,3)Fuc-T activity (Finne *et al.*, 1982). The new activity competes with sialyl-T for terminal lactosamine units resulting in decreased sialylation of *N*-glycans (Finne *et al.*, 1980) and decreased metastasis of mutant cells in mice (Tao and Burger, 1977). Similar phenotypes were isolated from CHO cells (Campbell and Stanley, 1983), but in those cases, *de novo* expression rather than upregulation of α(1,3)Fuc-T activity was observed. Parental CHO cells do not have detectable α(1,3)Fuc-T activity, nor do they add α(1,3)fucose residues to lactosamine units (Howard *et al.*, 1987). A human Fuc-TIII gene probe identified transcripts in LEC11 CHO mutants but not in parental CHO cells (M.Bhaumik and P.Stanley, unpublished observations). Another Glyc-T gene activated by mutation in CHO cells is *Mgat3* (Ihara *et al.*, 1993; Bhaumik *et al.*, 1995), the gene encoding GlcNAc-TIII that adds the bisecting GlcNAc to *N*-glycans (Narasimhan, 1982). LEC10 CHO cells express GlcNAc-TIII and bisected *N*-glycans whereas CHO parental cells do not (Campbell and Stanley, 1984). A mouse probe for *Mgat3* gene transcripts detected RNA in LEC10 cells but not in parental CHO cells (J.Chang, M.Bhaumik, and P.Stanley, unpublished observations). Therefore, the CHO genome contains silent Glyc-T genes that may be expressed following an activating mutation-like event.

While many of the Glyc-Ts that are not normally expressed in CHO cells are established transferase activities, others appear novel. For example, four CHO mutants with distinct phenotypic properties express an α(1,3)Fuc-T activity (Potvin and Stanley, 1991; Table I and Figure 1). LEC11 mutants have an α(1,3)Fuc-T that adds fucose to both sialylated and to nonsialylated lactosamine units to create both sialyl-$Le^x$ ($SLe^x$) and $Le^x$ structures, respectively (Howard *et al.*, 1987; Stanley and Atkinson, 1988). The biochemical properties of the LEC11 α(1,3)Fuc-T are very similar to those of human Fuc-TV (Weston *et al.*, 1992b). LEC12, LEC29, and LEC30 are distinguished from LEC11 cells in that they do not synthesize $SLe^x$, but transfer α(1,3)fucose only to lactosamine. These α(1,3)Fuc-T activities appear most similar to human Fuc-TIV (Kumar *et al.*, 1991; Lowe *et al.*, 1991), but they each have distinctive properties. The LEC29 enzyme has very low transferase activity with lactosamine but expresses $Le^x$ levels comparable to those of LEC12 and LEC30. Also, in contrast to the LEC12 transferase, the LEC29 activity does not add fucose to internal lactosamine units. The LEC30 enzyme, though it has very high transferase activity with lactosamine as acceptor, also adds less fucose to internal lactosamines (Table I, Figure 1). Thus it seems likely that one or more of these mutants is expressing a novel α(1,3)Fuc-T activity. Only a single Fuc-TIV-type activity has been reported in humans, though faintly

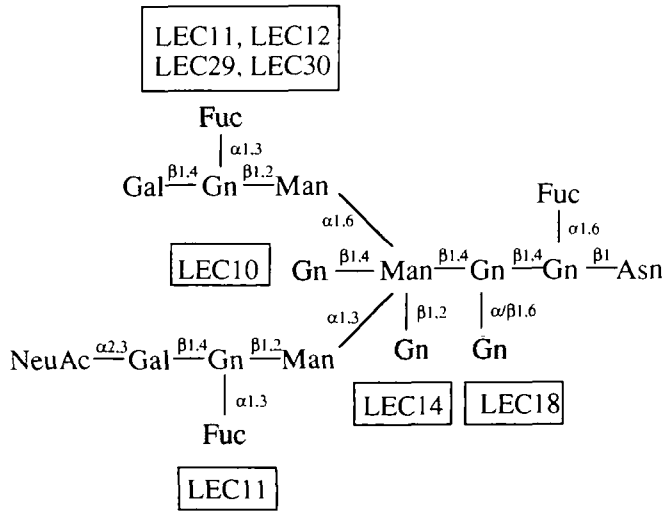


**Fig. 1.** Sugar residues associated with complex *N*-glycans in gain-of-function CHO glycosylation mutants. A simple biantennary complex *N*-glycan is shown with the sugar modification characteristic of the particular CHO mutant in which it is expressed highlighted. The diagram serves to illustrate where each residue is found; a molecule with all of these modifications in combination has not been shown to exist NeuNAc, sialic acid; Gal, galactose; Gn, *N*-acetylgluosamine, Man, mannose; Fuc, fucose.

hybridizing bands were observed on Southern analysis of human DNA with probes specific for the Fuc-TIV gene (Weston *et al.*, 1992a). Expression cloning of the CHO genes will reveal whether one or more of the CHO mutants express a novel α(1,3)Fuc-T.

The first CHO mutant shown to have a dominant, gain-of-function phenotype was LEC10 (Campbell and Stanley, 1984). The GlcNAc-TIII activity expressed in LEC10, but not in parental CHO cells, was initially identified in hen oviduct membranes (Narasimhan, 1982). It is a developmentally regulated activity as revealed by northern analysis of rat and mouse tissues with probes from the *Mgat3* gene (Ihara *et al.*, 1993; Bhaumik *et al.*, 1995). The α(1,3)Fuc-Ts described above are also expressed in a tissue specific fashion. The gain-of-function transferases are therefore the products of developmentally regulated genes that are differentially expressed in the animal. CHO cells, which were derived from hamster ovarian cells (Puck *et al.*, 1958), contain perhaps the entire repertoire of Glyc-T genes that are normally silenced in hamster during development and differentiation. In theory, therefore, additional activation mutations have the potential to reveal new Glyc-Ts that may synthesize novel glycans. In fact, the discovery of completely new activities by this approach has recently occurred (Raju *et al.*, 1995; Raju and Stanley, 1996; Figure 1).

Two CHO glycosylation mutants have now been shown to synthesize *N*-glycans with novel core regions not observed previously in glycoproteins from any source. They are LEC14 and LEC18, and both were isolated as mutants resistant to pea lectin (Ripka and Stanley, 1986). They are also resistant to *L.culinaris* (LCA), but the overall lectin resistant phenotype of each is different (Table II). The subtle but significant differences in lectin resistance were predicted to signify different biochemical phenotypes for these mutants. As shown in Figure 1, LEC14 cells add a GlcNAc residue in β(1,2)-linkage to the core Manβ(1,4) residue in complex *N*-glycans (Raju and Stanley, 1996), while LEC18 cells add a GlcNAc residue to the 6-position of the core GlcNAc residue in complex *N*-glycans (Raju *et al.*, 1995). A GlcNAc-T activity that can transfer GlcNAc to a biantennary *N*-glycan terminating in GlcNAc is present in LEC18 and in LEC14 cell extracts, but not in parental CHO extracts (T.S.Raju and P.Stanley, unpublished observations). Once these activities have been cloned, it will be clear whether they represent the products of new genes. However, it seems extremely likely that they will do so because, to date, all seven GlcNAc-Ts that have been cloned are encoded by separate, unique genes (Kleene and Berger, 1993; Bierhuizen *et al.*, 1995).

## Potential of CHO cells to identify additional novel glycans

Assuming that the new GlcNAc-T activity expressed by LEC14, and that in LEC18, are generated from previously undiscovered GlcNAc-Ts, these mutants provide ready access to the corresponding cDNAs. If the sequences of these genes are unique, they would not be identified by cross hybridization. If they are expressed during only a particular window of development, or only in a certain cell type in the adult animal, they would not be easily detected. And, in either of the latter cases, or if the GlcNAc transferred by each is added only to certain membrane glycoproteins, the novel *N*-glycans would not be easily purified for structural characterization. Thus, without the chance activation of these genes in CHO cells, their encoded activities and the new structures they generate may have remained unknown for many years to come. The question now arises as to whether other novel transferases can be discovered in this way and how this might be expeditiously accomplished.

The likelihood of there being other silent, but potentially active, glycosyltransferase genes in CHO cells is high. The challenge is in devising selections that will give mutants that express a new transferase gene rather than one that is already known. An approach that has been successful previously is selection utilizing several lectins designed to give rise to a novel lectin-resistance phenotype (Stanley, 1983). Another approach would be to transfect several copies of all the Glyc-T genes that CHO cells are known *not* to express, and then select for a gain-of-function glycosylation phenotype. However, the latter is a formidable task, and we will probably know all Glyc-T gene sequences from the human genome project before the requisite CHO genome could be generated!

## Biological relevance of individual residues in mammalian glycans

The cloning of Glyc-T genes has revealed the enormous stake that mammals place in maintaining their glycosylating abilities. Most Glyc-T genes, including those that appear to encode ubiquitously expressed Glyc-Ts such as GlcNAc-TI (Kumar *et al.*, 1992; Yang *et al.*, 1994) and β(1,4)Gal-T (Russo *et al.*, 1990; Shaper *et al.*, 1990; Harduin-Lepers *et al.*, 1993), are regulated in their expression and produce more than one gene

**Table II.** Lectin resistance patterns of gain-of-function CHO glycosylation mutants

| CHO mutant | Glyc-T newly expressed | Relative lectin-resistance | | | | | |
|---|---|---|---|---|---|---|---|
| | | RIC | E-PHA | PSA | L-PHA | LCA | WGA |
| LEC10 | GlcNAc-TIII | $R_{20}$ | $S_{15}$ | — | $R_{(2)}$ | — | — |
| LEC14 | GlcNAc-T | — | ND | $R_3$ | (R) | $R_3$ | — |
| LEC18 | GlcNAc-T | (S) | ND | $R_{39}$ | — | $R_{16}$ | — |
| LEC11 | α(1,3)Fuc-TI | $S_{25}$ | (R) | — | $R_4$ | $R_3$ | $R_8$ |
| LEC12 | α(1,3)Fuc-TII | $S_4$ | ND | — | $R_3$ | $R_2$ | $R_{50}$ |
| LEC29 | α(1,3)Fuc-T? | $S_{10}$ | ND | ND | (R) | — | (R) |
| LEC30 | α(1,3)Fuc-T? | (S) | ND | ND | $R_{10}$ | $R_4$ | $R_{50}$ |

Lectin resistance was determined by the lectin concentration that results in 10% survival ($D_{10}$). Relative fold-resistance (R) or hypersensitivity (S) compared to parental CHO cells is shown. This summary is based on previously published data (Stanley, 1983; Campbell and Stanley, 1984; Ripka and Stanley, 1986; Potvin and Stanley, 1991). RIC, ricin; E-PHA, erythroagglutinin from *P vulgaris;* PSA, pea lectin; L-PHA, lymphoagglutinin from *P. vulgaris;* LCA, agglutinin from *L. culinaris;* WGA, wheat germ agglutinin. ND, Not determined

product. The extent of this regulation suggests that controlling both the nature and level of glycosylation is of widespread functional importance. Some key recognition functions of cell surface glycans have been established, such as the determinants recognized by the selectins (Lasky, 1995), and it seems likely that more remain to be discovered. This prediction is based on the drastic consequences to cell surface glycan recognition by plant lectins that follow the activation of a single Glyc-T gene in CHO cells (Table II). It can be seen from the summary of lectin resistance properties that LEC10 cells with a bisecting GlcNAc residue on a subset of *N*-glycans, become highly resistant to ricin, even though all Gal residues that are primarily responsible for ricin binding remain in place. It is known from $^1$H-NMR spectroscopy that the bisecting GlcNAc changes the conformation of a biantennary *N*-glycan (Brisson and Carver, 1983). Thus, by controlling the expression of only the *Mgat3* gene, a cell may control the recognition potential of cell surface glycans. The same is true for α(1,3)Fuc-T genes; expression of a single one of them generates one or more glycan determinants recognized by a variety of monoclonal antibodies (Hakomori, 1984) and/or by selectins (Lasky, 1995). Based on these, and other examples of the consequences of expressing a particular glycan array at the cell surface, it is predicted that the GlcNAc-T characteristic of LEC14, and that of LEC18, create functionally important glycan determinants. The generation of mice that lack these Glyc-Ts will shed light on the functions of the *N*-glycans they synthesize (Stanley and Ioffe, 1995). Important insights have already been gained from the properties of mice lacking GlcNAc-TI (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). Mice with a disrupted *Mgat3* gene have been produced (Bhaumik and Stanley, 1995) and provide an ideal model with which to assay for the functions of bisected, complex *N*-glycans.

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