Suppressors of α(1,3)fucosylation identified by expression cloning in the LEC11B gain-of-function CHO mutant

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Factors that regulate α(1,3)fucosyltransferase activity are important to identify because FUT genes are up-regulated during inflammation, cancer progression, and tumor metastasis. FUT gene activation increases the expression of cell surface oncofetal antigens such as Lewis X, sialyl-Le X and VIM-2. The LEC11B gain-of-function glycosylation mutant displays these antigens and binds E-selectin because it expresses the Fut6B gene that is shown here to lie immediately downstream of the Fut6A gene. A retroviral strategy for expression cloning factors that suppress α(1,3)fucosylation in LEC11B cells was developed, and several cDNAs that reverted the LEC11B glycosylation phenotype were isolated. cDNAs that arose most frequently and independently encoded SLC35C2, a putative GDP-fucose transporter (also termed CGI-15 or Ovcov1); Cd63, a tetraspanin membrane protein; and Hdac5, a histone deacetylase. When transfected into LEC11B cells the SLC35C2 cDNA reduced Le X expression with no concomitant suppression of Fut6B gene transcripts. Transfection of the Cd63 cDNA induced low levels of ricin A chain toxicity, and also did not suppress Fut6B gene transcripts in LEC11B. However, the Hdac5 cDNA induced ricin resistance, reduced fucosylated antigen expression, and essentially eliminated Fut6B gene transcripts. The Hdac5 cDNA isolated by expression cloning encoded the C-terminal region of hamster Hdac5. Overexpression of this partial Hdac5 cDNA or a full-length Hdac5 cDNA, suppressed Fut6B gene transcripts specifically. Thus the expression cloning strategy identified Hdac5 as a trans-acting repressor of the Chinese hamster ovary Fut6B gene and Cd63 and SLC35C2 as novel factors that suppress α(1,3)fucosylation by mechanisms unrelated to effects on Fut gene expression.

Key words: α(1,3)fucosyltransferase/dominant CHO mutant/expression cloning/Le X suppression

Introduction

The LEC11B gain-of-function Chinese hamster ovary (CHO) glycosylation mutant possesses an α(1,3)fucosyltransferase (Fuc-T) activity that is the product of the Chinese hamster Fut6B gene (Zhang et al., 1999). CHO cells have no detectable Fut6 gene transcripts by RNase protection analysis (Zhang et al., 1999) and no α(1,3)Fuc-T activity in cell extracts (Campbell and Stanley, 1983; Howard et al., 1987). Somatic cell hybrid analysis revealed a negative regulatory factor that suppresses Fut6B gene expression in parental CHO cells and is inactive in the LEC11B mutant (Zhang et al., 1999). The negative regulatory factor is also active in CHO mutants derived from this parent line, including LEC18 and LEC11A CHO mutants (Zhang et al., 1999).

Due to de novo Fut6B gene expression and consequent α(1,3)Fuc-T activity, LEC11B cells express α(1,3)fucosylated, cell surface, oncofetal antigens, including stage-specific embryonic antigen-1 (SSEA-1 or Lewis X), sialyl-Le X (SLe X), and VIM-2 (Campbell and Stanley, 1983; Howard et al., 1987). LEC11 cells were used to identify SLe X as a ligand for E-selectin (Phillips et al., 1990), one of the lectins that mediates leukocyte adhesion to vascular endothelium, initiating extravasation of leukocytes from the bloodstream (reviewed in Lowe, 2003). Increased expression of SLe X due to increased α(1,3)Fuc-T activity has been correlated with tumor progression and metastasis in mouse models (Fukuda et al., 2000; Fuster et al., 2003; Saitoh et al., 1992) and in humans (Hakomori, 2001). SSEA-1 is found on the surface of human lung cancer cell lines, human colon carcinoma, and human epidermal carcinoma (reviewed in Feizi, 1985). Moreover, the addition of α(1,3)fucose is the last step in the biosynthesis of SLe X (Campbell and Stanley, 1984; Holmes et al., 1985), so α(1,3)Fuc-Ts are crucial regulatory enzymes in the construction of selectin recognition determinants (see Lowe, 1997).

LEC11 mutants provide models for studying the regulated expression of α(1,3)fucosylated glycans. Cloning of cDNAs that revert LEC11B should identify factors that directly or indirectly affect the generation of oncofetal antigens and selectin ligands. Transcripts of the Fut6B gene are suppressed in hybrids formed between CHO, LEC18, or LEC11A cells with LEC11B cells, whereas the expression of genes such as actin and GAPDH are not affected (Zhang et al., 1999). Therefore, expression of the Fut6B gene in LEC11B cells most likely occurs due to an effect on transcription or RNA stability. Activation of the silent Fut6B gene may be due to a change in local chromatin structure or to relief of transcriptional repression through a negative response element, or indirectly through a...
corepressor or a change in RNA turnover. To isolate factors that regulate the expression of the Fut6B gene, as well as factors that inhibit α(1,3)fucosylation by alternative mechanisms, a retroviral expression cloning strategy was developed to obtain cDNAs that revert the LEC11B phenotype. Three cDNAs that suppress the generation of α(1,3)fucosylated oncofetal antigens in LEC11B cells by different mechanisms were characterized.

Results

The Fut6A and Fut6B hamster genes are linked

The Chinese hamster genome encodes two Fut6 genes, termed Fut6A and Fut6B (Zhang et al., 1999). Previous studies isolated a 9-kb EcoRI genomic DNA fragment that contains the coding and 3' untranslated regions (UTRs) of the Fut6A gene and the 5' UTR of the Fut6B gene suggesting that the genes are linked. To identify upstream regulatory sequences that may control transcription of the Fut6B gene, it was important to know whether this relationship exists in the genome. Polymerase chain reaction (PCR) of genomic DNA from CHO and LEC11B cells showed that the 5' exon of the Fut6B gene is downstream of the Fut6A gene and that each Fut6 gene has coding and 3' UTR regions in a single exon (Figure 1A and 1B). Southern analyses of CHO and LEC11B genomic DNA with a probe (P4) that detects the 5' UTR exon of the Fut6B gene, gave a single band for each restriction enzyme (Figure 1C and 1D) indicating that the 5' exon of Fut6B is unique to the Fut6B locus. Southern blots probed with P4 and also P2 from the Fut6A and Fut6B coding regions (Figure 1C and 1D) showed that the Fut6B gene lies ~1.7 kb downstream of the 3' UTR of the Fut6A gene. This intergenic region was cloned and had promoter activity in a dual luciferase assay (Zhang, 1998). However, deletion analysis did not identify a sequence that might function as a negative regulatory element and thereby suppress transcription of the Fut6B gene.

Expression cloning of cDNAs that revert the LEC11B phenotype

Because of the expression of α(1,3)fucosylated glycans LEC11B cells are hypersensitive to the toxicity of ricin compared to parent CHO cells (Potvin and Stanley, 1991), allowing ricin to be used to select for LEC11B revertants. The lowest concentration of ricin that killed many LEC11B...
cells but preserved most CHO cells was determined in a
reconstruction experiment. CHO cells were used as mimics
of LEC11B revertants. At a ricin concentration of 2.5 ng/ml,
CHO cells formed colonies at high frequency and sponta-
naneous reversion of LEC11B mutants was only ~10^-4 per cell
per generation (Table I).

To isolate cDNAs that revert LEC11B, the retrovirus
vector pRex-IRES-green fluorescence protein (GFP) was
used, allowing stable, high-level expression of cDNA
inserts and selection of transductants by sorting for GFP
(Chatterton et al., 1999). To minimize spontaneous
revertants, LEC11B-Eco cells were sorted to remove Le
X-negative cells by flow cytometry before virus infection.
Nine library subpools were each transfected into Phoenix
packaging cells, and virus supernatant was used to infect
LEC11B-Eco cells formed colonies at high frequency and sponta-
naneous reversion of LEC11B revertants. At a ricin concentration of 2.5 ng/ml,
were subcloned into pCR3.1 or pREX-IRES-GFP and
expressed in LEC11B cells to directly test their ability to
revert the LEC11B phenotype. Transfectants were plated
in 2.5 ng/ml ricin and selected with G418. LEC11B cells
transfected with vector alone or SLC35C2 cDNA in reverse
orientation gave 3–8 ricin-resistant colonies per plate
(n = 3). By contrast, SLC35C2 and Hdac5 cDNAs in the
correct orientation gave an average of ~150 colonies and
Cd63 gave ~60 colonies per plate (n = 3 for each). Other
cDNAs that came through the selection including EF-1a,
RNA binding motif protein and Tmp21-I did not revert the
LEC11B phenotype consistently.

Some LEC11B SLC35C2 cDNA transfectants were
~8-fold ricin-resistant compared to vector control,
approaching the wild-type level of ~10-fold resistant
compared to LEC11B (Figure 2A). Le X antigen expression
for these ricin-resistant colonies was reduced by up to 50%,
compared to LEC11B (Figure 2A). Le X antigen expression
was suppressed in SLC35C2 and Hdac5 cDNAs transfectants
expressed in LEC11B cells (Figure 2B). However, all the
SLC35C2 transfectants expressed Fut6B gene transcripts at
a similar level to LEC11B cells (Figure 2C). Therefore
SLC35C2 overexpression did not suppress Fut6B gene
transcripts and SLC35C2 is therefore not a candidate for
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By contrast, transductants expressing the Hdac5 cDNA
suppressed Fut6B gene transcripts in LEC11B. The ~2.1-kb
insert from each of four Hdac5 transductants had the same
RsaI digestion pattern (Figure 3A). Sequencing revealed a
partial Hdac5 sequence encoding 600 amino acids that con-
tain the Hdac activity domain in the C-terminal portion but
lacking 511 amino acids of the N-terminus (Figure 3D).
Northern analysis was performed using the Fut6B coding
region probe P2 (see Figure 1). Clones C1, C28, and C4.1

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**Table I. Selection conditions for isolating LEC11B revertants**

<table>
<thead>
<tr>
<th>Ricin (ng/ml)</th>
<th>Mixed cells plated</th>
<th>No. resistant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LEC11B</td>
<td>CHO</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>2 × 10^5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2 × 10^5</td>
<td>50</td>
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<tr>
<td></td>
<td>2 × 10^5</td>
<td>100</td>
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<tr>
<td></td>
<td>5 × 10^5</td>
<td>0</td>
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<tr>
<td></td>
<td>5 × 10^5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>5 × 10^5</td>
<td>100</td>
</tr>
</tbody>
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NA, not applicable.

aCHO cells (LEC11B revertant mimics) were mixed with LEC11B cells
and colony formation determined after 8 days incubation.

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**Identification of cDNAs that revert LEC11B cells**

To identify the cDNAs present in LEC11B revertants,
retroviral inserts were amplified by PCR of genomic DNA
using primers that hybridized to the upstream sequence of
the 5' multiple cloning site and the IRES sequences that
flanked the cDNA. PCR products were grouped according
to size and those ≥500 bp were digested with RsaI and
analyzed by agarose gel electrophoresis. Clones with PCR
products of the same size and RsaI digestion pattern were
sequenced. Among the cDNAs identified, SLC35C2, Cd63,
and histone deacetylase 5 (Hdac5) arose frequently (11, 11,
and 4, respectively) from independent pools and best
reverted the LEC11B phenotype. Other cDNAs that were
obtained more than once were integral membrane protein
(AJ004912), ribosomal protein L8 (NM012053), RNA
binding motif (NM009033), Tmp21-I (AJ004912), and
EF-1a (M22432).

The SLC35C2 cDNA encoded a multimembrane-
spanning protein homologous to human CGI-15
(AF132949) originally identified by bioinformatics methods
(Lai et al., 2000). The human gene was subsequently named
OVCOV1 (Leach et al., 2001, 2002). A recent analysis
places this gene in the SLC35 gene family that includes
nucleotide-sugar transporters. SLC35C2 is most homo-
logous to the Golgi GDP-fucose transporter termed
SLC35C1 (Ishida and Kawakita, 2004). Cd63 (NM_007653) is a lysosome-associated membrane glycoprotein
that is also found on other cellular membranes, including
the plasma membrane (Rous et al., 2002). Cd63 is involved
in cell activation (Skubitz et al., 1996) and cytokine media-
tor release (Mahmudi-Azer et al., 2002; Smith et al., 1995).
Hdac5 (NM_010412) belongs to the group 2 histone deace-
ytases that deacetylate nucleic acids and proteins (reviewed
in Bertos et al., 2001).

The rescued hamster SLC35C2, Cd63, and Hdac5 cDNAs
were subcloned into pCR3.1 or pRex-IRES-GFP and
expressed in LEC11B cells to directly test their ability to
revert the LEC11B phenotype. Transfectants were plated
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Genes that inhibit α(1,3)fucosylation

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lacked *Fut6B* gene transcripts, whereas clone C30 had trace amounts (Figure 3B). All clones expressed similar levels of endogenous Hdac5 transcripts of ~4.4 kb compared to CHO and LEC11B cells (Figure 3C). However, only the Hdac5 transductants expressed higher-molecular-weight transcripts of ~8 kb, consistent with a partial Hdac5/GFP bicistronic transcript. Northern analysis using the hamster Hdac5 cDNA as a probe and RNA from all 123 colonies isolated from the library screen revealed that only the four revertants shown in Figure 3 overexpressed Hdac5.

**Hdac5 suppresses Fut6B gene expression and α(1,3)Fuc-TVI activity**

To further examine the ability of the rescued Hdac5 cDNA to revert the LEC11B phenotype, the partial cDNA and a full-length Hdac5 cDNA cloned from CHO cells were subcloned into pCR3.1 and pREX-IRE-GFP. After transfection of LEC11B cells with the partial Hdac5 in pCR3.1, only 2 of 15 G418-resistant transfectants had reduced Le X expression. In a larger experiment with full-length Hdac5 in pCR3.1 LEC11B cells were also not significantly reverted. However, all LEC11B-Eco colonies selected on the basis of GFP expression from Hdac5 in pREX-IRE-GFP had reduced Le X expression. Figure 4A shows that LEC11B cells expressing the partial Hdac5 from this construct were GFP-positive and Le X-negative. Sixteen other transductants expressing the partial Hdac5 cDNA and 10 transductants expressing a full-length hamster Hdac5 cDNA gave the same results with mean fluorescence index (MFI) for anti-SSEA-1 antibody binding ranging from 4.4 to 9.7. Nontransduced LEC11B cells were GFP-negative and Le X-positive (Figure 4B), whereas nontransduced CHO cells were GFP- and Le X-negative (Figure 4C). LEC11B cells with pREX-IRE-GFP vector alone were GFP-positive and Le X-positive (Figure 4D), showing that neither the pREX-IRES-GFP vector nor the expression of GFP affected Le X expression levels.

Northern analysis showed that suppressed Le X expression correlated with reduced *Fut6B* gene transcripts in all Hdac5 transductants. Vector control had the same level of *Fut6B* transcripts as LEC11B cells, and transductants expressing the partial Hdac5 cDNA had undetectable *Fut6B* transcripts (Figure 5A). However, the levels of both GAPDH and actin transcripts were unaffected (Figure 5B and 5C) as observed previously for the trans-acting negative regulator in CHO cells (Zhang et al., 1999). The same results were obtained for the full-length Hdac5 cDNA in pREX-IRE-GFP (data not shown). The spectrum of...
Hdac5 transcripts expressed was the same as that of the four clones shown in Figure 3C.

Western analysis was performed on transductants expressing full-length Hdac5. The level of the ~120-kDa endogenous CHO Hdac5 protein was ~1.6-fold greater compared to vector control (Figure 6A). This increase was less than expected from the high amounts of Hdac5 transcripts present in transductants. However, a higher-molecular-weight Hdac5 species (~150 kDa) was detected at variable levels in all transductants (Figure 6A), making the increase in Hdac5 ~ three- to five-fold. This species was not apparent in pCR3.1 Hdac5 LEC11B transfectants that only rarely reverted LEC11B. Although the amount of the ~150 kDa species did not correlate directly with revertant phenotype, the overall level of Hdac5 protein was higher in retroviral transductants.

Because northern analysis revealed no differences in Hdac5 transcripts between CHO and LEC11B (Figure 3C) and endogenous Hdac5 protein levels were the same (Figure 6A), sequencing of full-length Hdac5 from two parent CHO lines (Pro5 and Gat2) and LEC11B was performed to see if LEC11B Hdac5 carries a mutation. The CHO Hdac5 sequence was found to be 97% identical to mouse Hdac5 and 95.9% identical to human HDAC5 (Figure 6B). Sequencing did not reveal a single nucleotide difference in the 3336-nucleotide coding region of both the CHO and the LEC11B Hdac5 cDNAs. Therefore, Hdac5 is not the negative regulator previously identified by somatic hybridization to be inactive in LEC11B cells (Zhang et al., 1999). Nevertheless, it behaves as a trans-acting suppressor of CHO Fut6B gene expression.

**Discussion**

LEC11B mutants express the hamster Fut6B gene due to the loss of a negative regulatory factor that is present in parent CHO cells (Zhang et al., 1999). The retrovirus expression cloning strategy developed here was aimed at isolating this factor as well as other extragenic suppressors of the LEC11B phenotype, including factors that regulate Fut6B gene expression, the turnover and translation of Fut6B gene transcripts, the activity and half-life of α(1,3)FucT protein, and the stability and half-life of α(1,3)fucose residues transferred to cellular glycoproteins. In fact, only extragenic suppressors were obtained and the molecular basis of the LEC11B mutation remains unknown. The mechanisms that control the expression of α(1,3)fucose-containing glycans are complex (Kannagi, 2001) and understanding them is important because their expression correlates with poor prognosis and metastasis of human tumors (Hakomori, 2001). The three extragenic suppressors of LEC11B partially characterized here each identify a new mechanism by which...
α(1,3)fucosylation may be regulated. Understanding how each suppressor achieves this regulation is a challenge for the future as will be discussed.

Of the cDNAs that reverted the ricin resistance and Le X expression of LEC11B, only Hdac5 consistently suppressed the future as will be discussed. 

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Fig. 6. Hdac5 is the same in CHO and LEC11B cells. (A) Cell lysates from CHO, LEC11B, and LEC11B E.coli cells expressing pREX-IRES-GFP (Vector) or full-length CHO Hdac5 cDNA (transductants 1–5) were subjected to western analysis using antibodies to HDAC5 (upper panel) and subsequently to actin (lower panel). (B) cDNAs encoded as sequence of Actin (dark line) and the core region for new activity is underscored (thick line). The core region for Hdac5 catalytic activity is underscoring (thick line), and the critical amino acids for Hdac activity (Hassig et al., 1998) are marked with an asterisk. These four amino acids are conserved in human, mouse, and CHO Hdac5 proteins and all Hdac catalytic domains. The partial Hdac5 cDNA sequence starts from aa 511.
and control the accessibility of transcription factors to gene promoters. Hyperacetylation of histones is usually correlated with transcriptional activity and hypoacetylation of histones is often correlated with transcriptional repression (reviewed in Cress and Seto, 2000). Hdacs are found in large complexes containing other transcription repressors involved in gene-specific repression. Hdac5 contains two distinct N- and C-terminal domains connected by a glutamic acid (E)-rich linker (Verdel and Knochb, 1999). The C-terminal domain contains the core domain for deacetylase activity. Hdac5 is associated with myocyte enhancer factor-2A (MEF2A) and represses MEF2A transcriptional activity (Lemercier et al., 2000). It is also directly recruited by Bel6 to form the repressor complex that controls the POZ/zinc finger (POK) transcription factors activity (Lemercier et al., 2000). Hdac5 has been shown to interact directly with Gata1 during cell differentiation (Watanote et al., 2003).

The partial Hdac5 cDNA recovered from expression cloning contains part of the N-terminus, the E-rich linker, and the entire C-terminal deacetylase domain (Figure 6B). There are several in-frame methionines in the N-terminal portion of the C-terminal domain. Partial or full-length Hdac5 from CHO cells introduced into LEC11B-Eco cells by the retroviral vector completely suppressed Le X expression and Fut6B gene transcripts. Based on these results, overexpression of human HDAC5 may lead to inhibition of human FUT6 gene expression in pancreatic tumors in which this gene is up-regulated and thought to aid in metastasis (Hiller et al., 2000; Mas et al., 1998). However, the mechanism by which Hdac5 suppresses Fut6B transcripts in CHO cells is not known. A mutation in the Hdac5 coding region was shown not to be the basis of the LEC11B mutation. In addition, reversion of LEC11B was rarely achieved with Hdac5 expressed in pCR3.1. These transfectants had increased levels of Hdac5 ~ two-fold but did not make the ~150-kDa species seen in Hdac5 retrovirus transductants. Hdac5 in the retrovirus vector always reverted LEC11B. It is possible that the larger amount of Hdac5 produced by the retrovirus is the basis of reversion. Alternatively, sense or antisense or small interfering RNAs generated from the Hdac5 retrovirus constructs may be indirectly responsible for inhibiting the transcription or degradation of Fut6B gene transcripts. The mechanisms by which Hdac5 affects gene expression are complex and may be mediated by a non-catalytic region and independently of histone deacetylase activity (see Castet et al., 2004). In fact, treatment of parent CHO cells with various concentrations of the histone deacetylase inhibitor trichostatin A did not lead to the synthesis of SLe X that would indicate relief of the suppression of the Fut6B gene (Chen and Stanley, unpublished data), indicating that histone deacetylase activity may not be involved in Fut6B gene silencing.

The mechanisms by which C6d3 and SLC35C2 cDNAs revert LEC11B are distinct from Hdac5 because neither cDNA reduced the level of Fut6B gene transcripts. C6d3 is a member of the family of tetraspanin membrane proteins found in lysosomes, melanocytes, platelet dense granules, and at the cell surface (reviewed by Hemler, 2003). The tetraspanin proteins function in forming membrane microdomains that affect the expression of membrane molecules, such as the major histocompatibility complex (reviewed in Vogt et al., 2002). Another function for C6d3 is in enhancing the internalization of plasma membrane H,K-ATPase (Duffield et al., 2003). Thus overexpression of C6d3 in LEC11B cells may reduce the cell surface expression of Le X and increase ricin resistance by enhancing the endocytosis of membrane glycoproteins that carry Le X. Alternatively, C6d3 may interact directly with (1,3)Fuc-TVIB in the Golgi membrane of LEC11B to reduce its activity, or C6d3 may alter the microdomain structure of Golgi membranes and thereby indirectly reduce (1,3)Fuc-TVIB activity. The mechanism by which C6d3 reverts the LEC11B phenotype is of interest for understanding factors that regulate glycosylation in general and (1,3)Fuc-TVIB activity in particular.

The third suppressor of the LEC11B phenotype SLC35C2 was originally identified as CGI-15 by comparative proteomics (Lai et al., 2000) and subsequently as OVC0V1 by differential cloning of genes expressed in ovarian cancer (Leach et al., 2001, 2002). Based on homology comparisons, human SLC35C2 protein is related to membrane spanning transporters. It has ~32% amino acid identity to a vanadate resistance protein Gog5 from Saccharomyces cerevisiae (NP_013674) and a related protein from Caenorhabditis elegans (CAA94748). Vanadate resistance protein is a membrane transporter involved in drug resistance. Most interestingly, the human GDP-Fuc transporter 1 (XP_051033) sequence is 25.5% identical and 39.9% similar to the N-terminal 153 amino acids of human SLC35C2. Hydrophobicity analysis revealed a structural similarity between SLC35C2 and GDP-Fuc transporter 1 (SLC35C1) that are both predicted to contain nine transmembrane domains. Sequence comparisons place SLC35C1 and SLC35C2 in the same family of nucleotide-sugar transporters (Ishida and Kawakita, 2004), strongly suggesting that SLC35C2 is a GDP-fucose transporter. If SLC35C2 transports GDP-Fuc it may function in a compartment other than the Golgi and thereby reduce the Golgi concentration of GDP-fucose, resulting in a decrease in cell surface Le X glycans and ricin resistance in LEC11B revertants. The fact that patients with a defective Golgi GDP-Fuc transporter (SLC35C1) transfer O-fucose to Notch receptors (Sturla et al., 2003) and do not have developmental problems as would be predicted if they could not transfer O-fucose to Notch (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003) suggests that Notch receptors receive their O-fucose in a pre-Golgi or early Golgi compartment. Experiments are in progress to determine if SLC35C2 levels affect O-fucosylation of Notch receptors. In conclusion, both C6d3 and SLC35C2 reduce the expression of Le X by novel mechanisms that provide insight into factors that affect the functioning of an (1,3)fucosyl transferase and thereby the cell surface expression of Le X and SLeX oncofetal antigens and selectin ligands.

Materials and methods

Materials

Restriction enzymes were from Boehringer Mannheim, New England Biolabs (Beverly, MA), Promega (Madison, WI),
and Invitrogen (Carlsbad, CA). T4 DNA ligase, protease inhibitor tablets, RNaseH, DnaseI, and RNase inhibitor, shrimp alkaline phosphatase, and Expand Long Template PCR System were from Boehringer Mannheim. RNasein and Klenow fragment were from Promega. The TRlzel reagent, Superscript II reverse transcriptase, terminal deoxynucleotidyl transferase, DNA molecular weight markers, ELONGASE Enzyme mix, Lipofect-AMINE Reagent, Opti-MEM I Reduced Serum Medium, α medium, Dulbecco’s modified Eagle medium, and synthetic oligonucleotides were from GIBCO/Invitrogen. (α-32P) dCTP (6000 Ci/mmol) were from Du Pont–New England Nuclear (Boston, MA). Taq polymerase and dNTPs were from Perkin Elmer (Wellesley, MA) or Boehringer Mannheim. Hybond nylon membrane and Rapid-hyb buffer were from Amersham (Piscataway, NJ). Nonidet P-40, MOPS, bovine serum albumin (BSA), and Polybrene were from Sigma (St. Louis, MO). G418 and hygromycin were from Invitrogen. Wheat germ agglutinin and ricin were from Vector Laboratories (Calabas, CA). Hygromycin was from Invitrogen. Wheat germ agglutinin and ricin were from Vector Laboratories (Burlingame, CA). DNA gel extraction kit was from Qiagen (Valencia, CA). Other chemicals and reagents were usually obtained from either Sigma or Fisher Scientific (Silver Spring, MD).

Monoclonal antibody anti-SSEA-1 was prepared by 40% ammonium sulfate precipitation of ascites produced by CAF/J mice injected with the hybridoma cell line 480 obtained from Dr. Barbara Knowles. This antibody was conjugated directly to phycoerythrin by Dr. Olga Blumenfeld. Human anti-HDAC5 antibody sc-5252, anti-goat, and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody was a gift of Dr. E. R. Stanley. Phoenix ecotropic packaging cells (ATCC SD 3444) were a generous gift from Dr. G. Nolan (Stanford University Medical Center).

**Cell lines and cell culture**

Parental CHO cells Pro–5 and Gat–2 (Stanley et al., 1975), LEC11B (Gat–LECl1.F2; Zhang et al., 1999) and LEC18 (Pro–5LEC18.21B; Raju et al., 1995) were cultured in suspension or monolayer at 37°C in a medium containing 10% FBS. Phoenix ecotropic packaging cells (ATCC SD 3444) were cultured in Dulbecco’s modified Eagle medium containing penicillin, streptomycin, glutamine (2 mM), and 10% FBS at 37°C in a 5% CO2 incubator. Transfectants expressing antibioic resistance genes were grown in a medium containing G418 (1.0 mg/ml active weight) or hygromycin (700 μg/ml) respectively.

**Mapping the CHO Fut6 Gene Locus**

Southern analysis of genomic DNA from CHO and LEC11B cells digested with a variety of restriction enzymes was performed with the expression region and gene specific UTR probes described previously (Zhang et al., 1999) to map the region of the CHO genome that contains the Fut6A and Fut6B genes. Restriction mapping was confirmed by PCR of genomic DNA with the following primers: primer 1 specific for Fut6A coding region (5’ CAGGCCATGGA 3’); primer 2 specific for Fut6A 3’ UTR (5’ CTGAGAATAAGGTTACATGCTGG 3’); primer 3 specific for Fut6B coding region (5’ GTACCCCA- CAGGGCATGGA 3’); primer 4 specific for Fut6B 3’ UTR (5’ AGCTATATTTGCTAACCTCC 3’); primer 5 specific for 3’ UTR of Fut6A (5’ GTGCTAGAC- CACTCCCTGATGACG 3’); primer 6 specific for Fut6B 5’ UTR (5’ TTCTGAGGCAAGCTACTGC 3’); primer 7 specific for Fut6B 5’ UTR (5’ TCCTTGCGGCTG- GCAGTGCTTCG 3’).

**Construction of CHO cells expressing an ectopic retroviral receptor**

LEC11B cells were transfected with pcB7-Eco (a gift from Drs. Xudong Liu and Harvey Lodish, Massachusetts Institute of Technology) using LipofectAMINE and selected in a 10% FBS medium and 700 μg/ml hygromycin. Ten colonies were tested for Eco receptor expression by their ability to be infected by retrovirus produced by Phoenix packaging cells. Transfectants that expressed the most GFP were chosen for expression cloning experiments. LEC11B-Eco cells retained the LEC11B glycosylation phenotype as determined by their lectin resistance pattern and expression of cell surface Le X.

**cDNA library construction**

Total RNA was isolated from Pro–5 LEC18 cells using TRlzel Reagent (Life Technologies, Carlsbad, CA) and poly(A)+ RNA was prepared using an Oligo-dt column (New England Biolabs). First strand cDNA was synthesized from 5 μg poly(A)+ RNA using a NotI primer-adapter and SuperScriptII reverse transcriptase. EcoRI/BstXI adapters (Invitrogen) were ligated into the EcoRI/NorI sites of pREX-ires-GFP (a gift of Dr. Jon Chatterton, MIT). Double-stranded cDNAs were size-fractionated (>500 bp) using the SuperScript Plasmid System for cDNA Synthesis (Life Technologies) and digested with NotI before ligation into pREX-ires-GFP. The pREX-ires-GFP cDNA library (~10 ng) was transformed into DH 10B ElectroMax competent E. coli (Life Technologies) by electrotransformation of 1 ml bacteria in a 0.1-cm cuvette at 2.5 kV, 100 ohms, and 25 μF in a Bio-Rad gene pulser. Independent clones (9.4 × 105) were obtained. From plasmid DNA of 20 clones, the average insert size was 2.3 kb, and 2 contained empty vector. The library was checked using a Gene Checker Kit (Invitrogen) for the presence of large (~6 kb) and full-length transcripts. Five primer sets amplified part of the GAPDH gene, the 5’ and 3’ ends of the actin gene, and the 5’ (6 kb) and 3’ (2 kb) ends of the clathrin gene. All five PCR products were present at the expected size. Plasmids from the library were divided into nine subpools.

**Production of retroviral supernatants**

Retroviral supernatants were prepared by transfecting Phoenix cells with 10 μg pREX-GFP plasmid DNA (control) or each subpool of the library. Phoenix cells plated at 10⁶ cells per well in 5 ml Dulbecco’s modified Eagle medium 10% FBS in six-well dishes were ~80% confluent after 24 h incubation at 37°C in a 5% CO2 incubator. Before transfection, the medium was replaced with 5 ml Dulbecco’s
modified Eagle medium 10% FBS containing 24 μM chloroquine, and incubated for 1 h at 37°C in a 5% CO₂ incubator. Plasmid DNA (10 μg) was added to 1 ml HBS (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose, 21 mM HEPES, pH 7.03) in a 75 mm polypropylene tube and mixed gently. Subsequently, 125 μl 1 M CaCl₂ was added to each tube containing the DNA/HBS mixture, immediately mixed by inverting the tube several times followed by 5–10 min incubation at room temperature. The DNA/HBS/CaCl₂ mixture was added drop-wise with gentle swirling to the chloroquine-containing medium in the 6-well dish. The plates were incubated for 6–8 h at 37°C in a 5% CO₂ incubator. After 24 h, the medium was replaced in each well with 4 ml Dulbecco’s modified Eagle medium with10% FBS. After 24 h at 32°C in a 5% CO₂ incubator (48 h after transfection), the retrovirus supernatant was filtered through a 0.22-μm filter and used immediately or frozen rapidly on dry ice and stored at −80°C.

Flow cytometry analysis and fluorescence-activated cell sorting

For flow cytometry, 2–4 × 10⁵ cells were pelleted and washed once with 3 ml cold phosphate buffered saline (PBS)/2% BSA. Cells were resuspended in 100 μl PBS/2 % BSA, containing 10 μg/ml anti-SSEA-1 monoclonal antibody. After 30 min on ice, cells were washed with 3 ml cold PBS/2% BSA. Phycoerythrin or fluorescein isothiocyanate–conjugated secondary antibody (1 μg) was added in 100 ml PBS/2% BSA for 30 min on ice and removed by a 3-ml wash with PBS/2% BSA. Cells resuspended in PBS were subjected to flow cytometry using a Becton-Dickinson Immunocyto-metry System (San Jose, CA).

For cell sorting, cells transduced with the retrovirus cDNA library were resuspended in PBS at ~5 × 10⁶ cells per ml. Cells were sorted on a Becton-Dickinson Immunocyto-metry System at a rate of ~10⁵ cells per min, and green cells were collected in a medium 10% FBS containing penicillin and streptomycin.

Isolation and characterization of cDNAs from LEC11B revertants

Integrated retroviral cDNA inserts were isolated by PCR performed on genomic DNA using primers located within pREX-IRE-GFP vector. PS406 (5′ CCACGCCCC-CAAGTAGACG 3′) was from the upstream sequence of the 5′ multiple cloning site and PS407 (5′ CCAACT-TAATCGCCTTGCAGCA 3′) was from the IRES sequence located downstream of the 3′ multiple cloning site. Extend long template PCR system and ELONGASE polymerase were used. Premix 1 (20 μl) contained 0.2 mM of dNTPs, 10 pmol primers, and up to 500 ng genomic DNA template. Premix 2 (30 μl) consisted of 1 μl dimethyl sulfoxide, 2 μl ELONGASE enzyme mix, and buffer B (final concentration: 60 mM Tris-SO₄ [pH 9.1], 18 mM (NH₄)₂SO₄, with 2 mM MgSO₄). Samples were treated at 94°C for 30 s, 30 cycles of 94°C for 30 s, annealed at 60–65°C for 30 s, and elongated at 68°C for 1 min per kb of target. PCR products were separated on a 1.0% agarose gel, and those longer than 500 bp were recovered using a Qiagen gel extraction kit. cDNA inserts were grouped by size and were subsequently digested by Rsal. The Rsal digestion patterns were then analyzed on a 1.2% agarose gel. The inserts of the same size and the same Rsal digestion pattern were grouped together, and representatives from each group were sequenced.

The cDNA inserts recovered from the genomic DNA of RicK, GFP*, SSEA-1 colonies by PCR using PS406 and PS407 were ligated into the pCR3.1 mammalian expression vector, and clones in the correct orientation were identified by restriction digestion and by sequencing. For expression in the pREX-IRE-GFP retroviral vector, cDNAs in pCR3.1 were released by BamHI and NotI or EcoRI and cloned into the BamHI and NotI or EcoRI sites of the pREX-IRE-GFP vector. Restriction analysis was used to identify cDNAs in the correct orientation, and these were transfected into LEC11B-Eco cells or into Phoenix cells to produce retrovirus supernatant for infection of LEC11B-Eco cells. LEC11B-Eco cells expressing a candidate revertant cDNA were tested for ricin resistance, SSEA-1 antigen expression, and egFUT6β gene expression.

Cloning and sequencing of CHO Hdac5

To clone the CHO Hdac5 coding region, reverse transcrip-tase PCR was performed on Pro μF, Gat μ2 parent CHO and LEC11B total RNA (5 μg). An oligo-dT primer was used for first strand cDNA synthesis. cDNA products (2 μl) were added to an Extend Long Template PCR reaction. PCR amplifications were performed using primers designed according to the 5′ and 3′ UTR regions of mouse Hdac5 cDNA sequence. The forward primer was W1 (5′ CCCCCCT-CCCCGTCCCCAGCCCCAACGTCAGC 3′) and the reverse primer was W5 (5′ ATGAGGCCAAGGAT-GGGGCCCAGGGTG 3′). The annealing temperature of the PCR program was 63°C, and the elongation steps were for 4 min. The 3.3-kb band was gel purified and subeloned into pCR3.1 using the TA Cloning Kit (Invitrogen) and sequenced using six primer pairs designed to span the coding region according to the mouse and human HDAC5 coding sequences. Sequencing was performed by the Sequencing Facility at Albert Einstein College of Medicine.

Transfection of plasmid DNA into CHO cells

CHO cells were seeded at 1.6 × 10⁶ per 100 mm tissue culture dish in 10 ml of α medium 10% FBS the day before transfection. For transfection, 5–10 μg DNA prepared with LipofectAMINE in Opti-MEM I reduced serum medium according to the manufacturer’s instructions was added to cells that had been rinsed once with Opti-MEM I medium. After 6 h at 37°C in a CO₂ incubator, the DNA solution was replaced with fresh α medium 10% FBS. Stable transfectants were selected for resistance to G418 (1 mg/ml active weight) or hygromycin (700 μg/ml) added the next day. At day 4, the medium was replaced with fresh selection medium and colonies picked at 8–10 days.

Northern blot analysis

Total RNA preparation and northern blot analysis were performed as described previously (Chen et al., 2001). When more than one probe was used sequentially, blots were stripped by boiling in 0.1% SDS.
Lectin resistance test
To determine lectin resistance, 2000 CHO cells in α medium 10% FBS (100 μl) were added to each well of a 96-well plate. Lectins (100 μl) in α medium 10% FBS were added at increasing concentrations. After ~4 days at 37°C in the 5% CO2 incubator, when control wells were confluent, the medium was removed and the cells were fixed and stained with methylene blue in 50% methanol. The D10 value was the concentration of lectin that killed 90% of the cells.

Western blot
For western blot analysis, 50 μg of whole cell lysate were separated on a 7.5–10% SDS–PAGE under reducing conditions and transferred to polyvinylidene fluoride transfer membrane (PolyScreen, NEN Life Science Products). After blocking in 5% nonfat dry milk, first antibody was incubated with the membrane for 1.5 h at room temperature followed by washing with Tris-buffered saline and 0.05% NP-40 five times for 5 min. Secondary antibody conjugated to horseradish peroxidase as incubated with membrane at room temperature for 1.5 h. After washing, bound antibody was visualized by incubating the membrane for 1 min with western blot chemiluminescence reagent (Renaissance, NEN Life Science).

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Abbreviations
BSA, bovine serum albumin; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GFP, green fluorescent protein; Hdac5, histone deacetylase 5; SSEA-1, stage-specific embryonic antigen-1; MFI, mean fluorescence index; Ovccov1, ovarian cancer overexpressed 1; PBS, phosphate buffered saline; PCR, polymerase chain reaction; UTR, untranslated region.

References


