Methods and Protocols

Part 3

Chapter 33

Remodeling of Glycans Using Glycosyltransferase Genes

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Running head: Glycan Remodeling by CIRES

Summary

Remodeling of glycans on the cell surface is an essential technique to analyze cellular function of lectin-glycan ligand interaction. Here we describe the methods to identify the responsible enzyme (glycosyltransferase) regulating the expression of the glycan of interest and to modulate the glycan expression by overexpressing the glycosyltransferase gene. For the identification of the responsible enzyme, we introduce a new method, CIRES (correlation index-based responsible-enzyme gene screening), that consists of statistical comparison of glycan expression profile obtained by flow cytometry and gene expression profile obtained by DNA microarray.

Key words: Cellular glycan function, Lectin ligand, Biosynthesis pathway, Flow cytometry, DNA microarray, Statistical correlation, Transfection, Retrovirus infection

1. Introduction

Lectins usually function by interacting with their glycan ligands. Therefore, modulation of glycan ligand expression on the cells is useful for the functional analysis of lectins. However, sometimes it is difficult to efficiently regulate the targeted glycan expression by single gene overexpression because several different enzymes share common molecules as substrates and targets in the multi-step and highly branched glycan biosynthesis pathway. In this chapter, we introduce the method to find responsible (e.g., rate-limiting) enzyme in the biosynthesis pathway for effective glycan expression modulation before describing transfection of glycosyltransferase gene to mammalian cells for glycan remodeling.

To find the responsible gene for the biosynthesis of a certain glycan, we established new methodology called CIRES (correlation index-based responsible-enzyme gene screening) *(1)*. CIRES is novel quantitative phenotype-genotype correlation analysis, utilizing cell surface phenotypes (glycan expression) and gene expression profiles obtained from multiple cells (**Fig. 1**). Relative glycan expression profile among multiple cell lines is obtained using flow cytometry by staining the cells with glycan-specific probes/antibodies and calculating relative mean fluorescence intensity. The gene expression profile is concomitantly obtained by microarray *(2)*. Then, these glycan and gene expression profiles are assessed by calculating Pearson's correlation coefficient, and candidate genes are listed *(1, 3, 4)*. For the cross-comparison of multiple samples by microarray, each sample is compared with universal reference RNA (Clontech) to create a relative gene expression profile. Pearson's correlation coefficient is ranging from -1 (negative correlation) to 1 (positive correlation) and 0 means no correlation; thus, CIRES can be used to identify both positively and negatively regulating enzymes. Overexpression of positively correlated gene or knockdown of negatively correlated gene is expected to induce the expression of interested glycan (**Fig. 2**). The microarray data can be repeatedly used making it unnecessary to carry out microarray experiment as long as same set of cells is used for comparison. We have used 6 human B cell lines, Daudi, KMS-12BM, KMS-12PE, Namalwa, Raji, and Ramos for cross-comparison and deposited in gene expression database as GEO Series GSE 4407 *(1, 3)*, thus publically available for subsequent informatics analyses. This gene expression profiling was performed using a glycan-focused cDNA microarray (RIKEN human glycogene microarray, version 1) and a Gene Expression Omnibus (GEO) Platform (GPL#3465) *(2, 5)*.

Transfection of a glycosyltransferase gene can be done using standard transfection methods. Here we describe two different methods; a conventional method using liposome for transfection of adherent cells and a retrovirus method for both adherent and suspension cells (*see* **Note 1**). In general, a stronger expression level can be achieved by the use of plasmid vector-based expression and a more efficient gene transfer is achieved by retrovirus.

2. Materials

2.1. Flow Cytometry

1. Phosphate-buffered saline (PBS): 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 136.8 mM NaCl.
2. FACS buffer: [1% bovine serum albumin, 0.1% NaN_3, PBS] or [1% bovine serum albumin, 0.1% NaN_3, 1 mM $CaCl_2$, Tris-buffered saline] (*see* **Note 2**). Tris-buffered saline: 10 mM Tris-HCl (pH 7.6), 154 mM NaCl.
3. Probes: Any kinds of probes that specifically detect glycan structure can be utilized; e.g., lectin-Fc chimera probe, antibody or toxin. If they are not fluorescently labeled, fluorescence-labeled (e.g., FITC, R-phycoerythrin-conjugated) secondary probe/antibody is needed for detection by flow cytometry.
4. 12 × 75 mm FACS tube (BD Biosciences), and 40 μm Cell Strainer (BD Biosciences) or nylon mesh: e.g., Polystyrene Round-Bottom Tube with Cell-Strainer Cap (BD Biosciences).

2.2. Transfection of Glycosyltransferase Gene – Lipofectamine Method

1. Plasmid DNA.
2. Transfection reagent: e.g., Lipofectamine (Life Technologies) (*see* **Note 3**).
3. Opti-MEM (Life Technologies).
4. Target cells and appropriate growth medium containing serum.

2.3. Transfection of Glycosyltransferase Gene – Retrovirus Method

1. Plasmid DNA: MSCV (mouse stem cell virus) vector.
2. Virus packaging cells: Plat-A for human cells, Plat-E for rodent cells *(6)*.
3. Cell culture medium for virus packaging cells (DMEM/FBS): DMEM containing 4.5 g/L glucose, 10% fetal bovine serum, 2 mM L-glutamine.
4. BD CalPhos Mammalian Transfection Kit (BD Biosciences): 2M Calcium Solution and 2× HEPES-Buffered Saline (HBS) are included.
5. 0.45 μm filter: Acrodisc 0.45 μm HT Tuffryn membrane (NIPPON Genetics).
6. Polybrene solution: 10 mg/mL polybrene in H_2O.
7. Target cells and appropriate growth medium containing serum.

3. Methods

3.1. Identification of Responsible Enzyme

1. Harvest cells, suspend them in FACS buffer and incubate for 15−20 min at room temperature (*see* **Note 4**).
2. Aliquot the cells in 1.5 mL tube as 2×10^5 cells/sample.
3. Spin down the cells and remove the supernatant.
4. Resuspend the cells in 100 μL of probe/antibody-containing FACS buffer for the staining (*see* **Note 5**).

5. Incubate for 30 min−1 h on ice.
6. Wash with 1 mL of PBS or FACS buffer (*see* **Note 6**).
7. If the primary probe/antibody is not labeled with fluorescence, repeat step 4−6 with fluorescence-labeled secondary probe/antibody (incubate for 30 min on ice) (*see* **Note 7**).
8. Resuspend the cells in 500 µL of FACS buffer and transfer to 12 × 75 mm FACS tube through 40 µm Cell Strainer or nylon mesh.
9. Obtain fluorescent intensity data by flow cytometry (*see* **Note 8**).
10. Calculate the relative staining signal as the ratio of sample mean fluorescent intensity (MFI) divided by the control MFI.
11. Calculate Pearson's correlation coefficient between glycan expression profiles (obtained by flow cytometry) and gene expression profiles (obtained by microarray, *see* **Note 9**) using the correlation coefficient test.

3.2. Transfection of Transferase Gene

3.2.1. Lipofectamine Method (for Adherent Cells)

1. Subculture target cells in 60 mm dish the day before transfection (*see* **Note 10**).
2. Dilute 4 µg of plasmid DNA (glycosyltransferase cDNA-containing plasmid) into 250 µL of Opti-MEM (*see* **Note 11**).
3. Dilute 10−20 µL of Lipofectamine reagent into 250 µL of Opti-MEM.
4. Combine diluted plasmid DNA and Lipofectamine reagent, and incubate for 15 min at room temperature.
5. Replace culture medium with 2 mL of Opti-MEM.
6. Add combined DNA-Lipofectamine complex (500 µL) to the cells. Gently move dishes back and forth to distribute transfection solution evenly (*see* **Note 12**).
7. Incubate the cells at 37°C for 1−5 h.
8. Add 2 mL of pre-warmed growth medium (appropriate growth medium used for the target cell culture) dropwise.
9. Incubate at 37°C overnight.
10. Replace the culture medium with fresh growth medium and culture at 37°C.
11. Harvest the cells 48 h after transfection for analysis (*see* **Note 13**).

3.2.2. Retrovirus Method (for Both Suspension and Adherent Cells)

1. Subculture packaging cells (e.g., Plat-A for human cells, Plat-E for rodent cells) in 10 cm dish the day before transfection (*see* **Note 14**).
2. 0.5 h prior to transfection, replace culture medium with 10 mL of pre-warmed fresh medium (DMEM/FBS).

3. Dilute 30 µg of plasmid DNA (MSCV vector plasmid containing glycosyltransferase cDNA) to 876 µL with sterile water and add 124 µL of 2 M Calcium Solution (*see* **Note 15**).
4. Add DNA-calcium solution dropwise to 1 mL of 2× HBS with constant bubbling (*see* **Note 16**).
5. Incubate the transfection solution (DNA-calcium mixture in HBS) at room temperature for 20 min to form calcium crystal conjugated with DNA.
6. Gently vortex the transfection solution.
7. Add the transfection solution dropwise to the packaging cells. Gently move dishes back and forth to distribute transfection solution evenly (*see* **Note 12**).
8. Incubate at 37°C.
9. Remove medium 6 h after transfection and add 4 mL of fresh medium (DMEM/FBS).
10. Incubate at 37°C for 24 h.
11. Collect and filter (Acrodisc 0.45 µm filter) culture supernatant which contains retrovirus. Aliquot the virus and store at –80°C until infection (*see* **Note 17**).
12. Add 4 mL of fresh medium (DMEM/FBS) to the cells and incubate for another 24 h (*see* **Note 18**).
13. Collect and filter culture supernatant as in **step 11**. Directly proceed to infection step (**step 14**) or freeze collected virus at –80°C.
14. If the virus is frozen, thaw it in 37°C water bath and immediately place it on ice once it is thawed. Add polybrene (6 µg/mL) to the retrovirus-containing culture supernatant and incubate for 10 min on ice (*see* **Note 19**).
15. If target cells are suspension, prepare the target cells during this incubation time (*see* **next step**, **15a**). If target cells are adherent, go to **step 15b**.

    15a. In the case of suspension cells, harvest the cells, count the number of cells and aliquot into a 1.5 mL tube per infection sample (e.g., 4×10^5 cells/sample for infection in 24-well plate; *see* **Note 20**). Spin down the cells and remove the supernatant. Then, resuspend the cells in 500 µL of retrovirus-polybrene mixture (from **step 14**), transfer to 24-well culture plate, and spin-infect the cells with virus (1,120 × *g*, 90 min, 32°C) (*see* **Note 21**). After centrifugation, add 500 µL of fresh growth medium (appropriate growth medium used for the target cell culture) to the cells (no pipetting) (*see* **Note 22**).

    15b. In the case of adherent cells, add virus-polybrene mixture directly to the culture.
16. Incubate at 32°C overnight.
17. Add fresh growth medium (scale up to larger well or dish, if needed) and incubate at 37°C.
18. Harvest the cells >48 h after infection for analysis (*see* **Note 13**).

4. Notes

1. Here we introduce the method for gene overexpression; however, if the negatively correlated gene is selected to increase glycan expression, knockdown that gene by RNAi.
2. Serum is often used as blocking reagent; however, they include an array of glycoproteins. Therefore, it is recommended to use non-glycosylated bovine serum albumin instead in the case of glycan staining. Some lectins such as selectins require calcium cation for their binding to glycan ligand. If this is the case, FACS buffer should contain calcium cation. Higher concentration of calcium could alter FSC/SSC value in flow cytometry analysis. Thus, calcium concentration should be suppressed to minimal. We use 1 mM $CaCl_2$ in such a case. Since calcium could cause salt precipitation, use Tris-buffered saline instead of PBS. Filter FACS buffer using 0.22 μm filter such as Stericup (Millipore) to remove precipitate.
3. Other reagents can also be used, although here we describe the method using Lipofectamine. Please refer to the manufacture's protocol.
4. Choice of cell lines for CIRES is important. Choose cells showing different expression level of focused glycan. It is not necessary to use the cells whose glycan expression is planned to be modified after identification of the responsible enzyme. Use of suspension cells is recommended because the fluorescent peak of staining is usually sharper in suspension cells than adherent cells, resulting in more reliable profiling. If it is required to use adherent cells, harvest the cells with 1−5 mM EDTA/PBS or cell scraper instead of trypsin to prevent trimming of cell surface proteins carrying glycans, and then pass-through 40 μm Cell Strainer. Since cellular status affects the expression of glycans, use cells that are in log phase.
5. The probe/cells ratio is important. Before obtaining comparison data among various cell lines, perform titration using strongly positive cells.
6. If staining is performed in $CaCl_2$-containing FACS buffer, wash with FACS buffer.
7. Lectins often bind weakly to their ligands and therefore are easy to be washed out. It is recommended to pre-complex the primary and secondary probes if possible.
8. To cross-compare staining signals among various cell lines, adjust the mean fluorescent intensity (MFI) of the background (negative control sample) to around 10 for each cell line. Fluorescent signal could be affected by small difference in the set up of experiments. Therefore, it is important to normalize the signal.
9. For the comparison among multiple samples, we use universal reference RNA (Clontech) for comparison to each sample. Then, relative expression level against reference RNA is compared among various cell lines. For a sample size of six, a correlation coefficient of 0.81 indicates a statistical significance level of 5%.
10. Prepare the cells to reach 70−90% confluence on the day of transfection.
11. Filter plasmid DNA using 0.22 μm filter to prevent contamination of bacteria into the culture. To obtain control cells, prepare empty vector plasmid and use this to transfect cells. In the case of stable transfection, use linearized DNA for transfection (DNA amount can be reduced to 0.5−1 μg).
12. Do not rotate the dish as this will concentrate transfection precipitate in the center of the dish.

13. If stable clones should be established, start selection with selection reagent. If cells are sorted using cell sorter, culture the cells for 2 weeks before the sorting to make sure that the gene is stably integrated into the genome.
14. Cell density affects viral titer. Prepare the cells to reach 70% confluence at transfection.
15. Filter plasmid DNA using 0.22 μm filter to prevent contamination of bacteria into the culture. To obtain control virus, prepare empty MSCV vector plasmid and use this to transfect cells.
16. Keep blowing bubbles into 2× HBS with a 1 mL pipette and an autopipettor while adding DNA-calcium solution dropwise. Constant motion of bubbles keeps overgrowth of calcium phosphate crystal. Alternatively, slowly vortex 2× HBS while adding DNA-calcium solution.
17. Use of fresh virus (just after collection, not frozen) shows the highest titer, although the virus can be stored at –80°C for a long period. The virus-containing culture medium often colors yellow to orange.
18. The titer of virus collected at 48 h is usually higher than that at 24 h. Usually, both of them show enough titer for infection.
19. We use DOTAP Liposomal Transfection Reagent (10 μL/1 mL of virus, Roche) instead of polybrene for infection of mouse primary B cells stimulated with lipopolysaccharide.
20. Use cells covering ~95% of the area of the well, depending on the size of cells. During the centrifugation, the cells are pressed against the bottom of the wells and viruses are precipitated on them. To get stable transfectants, use 96- or 48-well plate. In the case of transient expression, use 24-well plate (if needed, prepare multiple wells per sample).
21. If high temperature centrifuge is not available, use normal centrifuge by setting temperature at 32˚C (during centrifugation, the temperature will rise).
22. No need to resuspend the cells by pipetting. After the centrifugation, viruses are attached to the cells.

References

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Figure captions

Fig. 1. Concept of CIRES: example of comparison among 6 cell lines (a−f).

Relative gene expression profile obtained by DNA microarray and relative glycan expression profile obtained by flow cytometry are compared to find responsible enzyme for the glycan biosynthesis. In the web graphs, the edge of the polygon corresponds to the strongest expression. The polygons in the left web graphs represent the relative gene expression profiles of 8 different genes. The same set of cell lines is examined for cell surface glycan expression by flow cytometry (here, the relative mean fluorescence intensity is also expressed as web graph). Similarities and dissimilarities between the profiles are assessed using Pearson's correlation coefficient which has values ranging from –1 (inverse correlation) to 1 (perfect correlation). (Figure was modified from **Ref**. *1*)

Fig. 2. Remodeling of the cell surface glycan expression by α1,4-galactosyltransferase (*A4GALT*) overexpression.

(**A**) Cell surface expression of neutral glycosphingolipid Gb3 (CD77) was positively correlated with the gene expression of *A4GALT*. In order to have appropriate control, retrovirus vector used here expresses glycosyltransferase cDNA and EGFP via an internal ribosome entry site (IRES); thus, virus-infected cells were distinguished from non-infected cells by EGFP signal. Gb3 negative Namalwa cells were infected with retroviral vector encoding *A4GALT* and stained with anti-Gb3 monoclonal antibody. Positively correlated A4GALT expression efficiently remodeled cell surface Gb3 expression with single gene manipulation.

(**B**) Cell surface expression of ganglioside GM1 was negatively correlated with the gene expression of *A4GALT*. GM1-positive Namalwa cells were infected with retroviral vector encoding *A4GALT* and stained with cholera toxin B subunit (ChTxB) that binds to GM1. Although A4GALT is not directly involved in the GM1 biosynthesis, induction of A4GALT suppressed GM1. Further analysis revealed that A4GALT controls LacCer synthase to dominantly compete out GM3 synthase in the glycosphingolipid biosynthesis in human B cells (*see* **Ref**. *4*). Focusing on the negative correlation could result in the build up of hypothesis, which is otherwise impossible to reach. The strength of CIRES is to rationally evaluate biological systems such as biosynthetic pathway under the unbiased and robust statistical calculation, which sometimes goes beyond researcher's gut feelings.

Figure 1

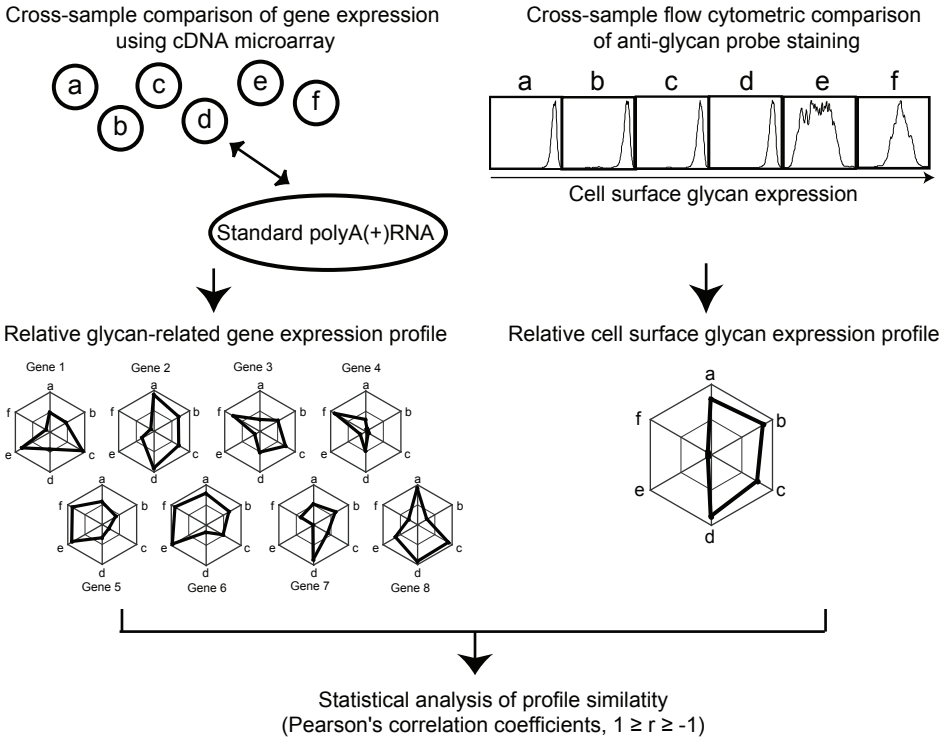
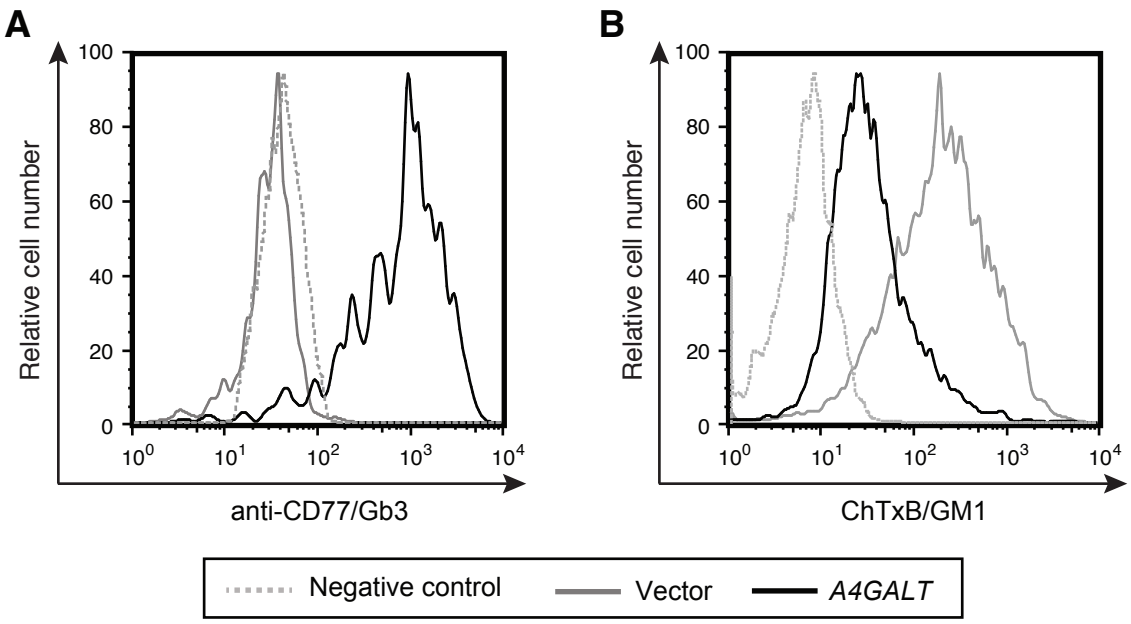

Cross-sample comparison of gene expression using cDNA microarray
a
b
c
d
e
f
Standard polyA(+)RNA
Cross-sample flow cytometric comparison of anti-glycan probe staining
a
b
c
d
e
f
Cell surface glycan expression
Relative glycan-related gene expression profile
Gene 1
Gene 2
Gene 3
Gene 4
Gene 5
Gene 6
Gene 7
Gene 8
Relative cell surface glycan expression profile
Statistical analysis of profile similatity
(Pearson's correlation coefficients, 1 ≥ r ≥ -1)

Figure 2


A
B
Relative cell number
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
anti-CD77/Gb3
ChTxB/GM1
Negative control
Vector
A4GALT