Enzymatic Basis for a Lectin-resistant Phenotype: Increase in a Fucosyltransferase in Mouse Melanoma Cells

JUKKA FINNE, MAX M. BURGER, and J.-P. PRIEELS
Department of Biochemistry, Biocenter, University of Basel, Switzerland and Laboratoire de Chimie Générale I, Faculté des Sciences, Université Libre de Bruxelles, Belgium

ABSTRACT In the search for the biochemical basis of the control of glycosylation of cell surface carbohydrates, revertant clones were isolated from previously characterized wheat germ agglutinin-resistant clones of B16 mouse melanoma cells by selection for resistance to Lotus tetragonolobus lectin or to ricin. Comparison of the wheat germ agglutinin-resistant clones with the parent and revertant clones indicated that this phenotype was correlated with an increased sensitivity to the Lotus lectin, a 60- to 70-fold increase in α1 → 3 fucosyltransferase activity and a decreased sialic acid content of the N-glycosidic chains of glycoproteins. The results suggest a novel type of control mechanism for lectin resistance, an increase in a glycosyltransferase activity. The presence of α1 → 3 bound fucose on N-acetylglucosamine residues would interfere with the addition of sialic acid by α2 → 3 linkages to galactose residues in the carbohydrate units, and this change could explain the resistance to wheat germ agglutinin and the increased sensitivity to the Lotus lectin. A change in a regulatory gene for the fucosyltransferase as a possible primary cause for the changed phenotype is discussed.

The availability of lectin-resistant cell lines with altered carbohydrate moieties of cell surface glycoproteins and glycolipids has provided a powerful tool for the study of the biosynthesis and function of the complex carbohydrates in animal cells (1–4). Some of the variant cell lines appear to have a block at some level of the biosynthetic pathway of protein-bound oligosaccharides, but the enzymatic basis for the changed phenotype is usually not known. For the study of the biological role of glycoproteins and glycolipids, it would be important to know how the expression of cell surface carbohydrates is regulated.

Many lectin-resistant variant cell lines differ from the parent lines with respect to more than one property (5–7). Because the occurrence of more than one genetic or epigenetic change even in single-step isolates cannot be ruled out, it is not known whether all the changes observed could be ascribed to one primary change. Other evidence should therefore be obtained by the isolation of independent variants or revertants (7, 8). The isolation of independent variants has so far been shown only for the lectin-resistant cells with a block in a specific N-acetylgalactosaminyltransferase (9, 10), whereas the isolation of revertants has usually not been successful (1, 7). We report here the isolation of variants and revertants that could be obtained repeatedly as well as their enzymic defect.

Previous work from this laboratory has described a wheat germ agglutinin (WGA)-resistant mouse melanoma cell line with many altered properties, including changes in cell adhesion and metastasis (11). Analysis of the protein-bound carbohydrates revealed a structural change involving increased fucose-content and decreased sialic acid-content (12). To reveal a possible correlation between these findings, new independent mutant and revertant clones were isolated, and analyses were carried out to reveal a biochemical basis for the changed phenotype. The results indicate that the changed properties could be explained by an increase in a specific fucosyltransferase activity. This suggests a new type of control mechanism in the regulation of complex carbohydrate biosynthesis in lectin-resistant cells.

MATERIALS AND METHODS

Cells

F1, a line of B16 melanoma cells, was obtained from Dr. I. J. Fidler (13). The Wa3 line and the Wa4 and Wa5 clones were isolated by selection for WGA resistance in 3, 4, and 5 subsequent steps, respectively, by Dr. T.-W. Tao (11). A line selected for ricin resistance from the Wa5 clone was also obtained from Dr. Tao and was found, in preliminary surface-labeling experiments, to be composed of a mixture of cells with different phenotypes. Several clones were isolated from the mixture, and two of them, Wa5Re and Wa5Rx, were chosen for further studies. For the selection for resistance to the lectin from Lotus tetragonolobus, Wa4 cells grown on plastic dishes were exposed to 800 μg/ml Lotus lectin for 7
d with one change of medium after 3 d. The medium was replaced by lectin-free medium, and from the surviving cells two clones, Wa4Lb and Wa4Ld, were isolated for further studies. The cloned parent line Fa was isolated by recloning from the uncloned Fl line and was similar in growth properties, morphology, and lectin sensitivity to the original Fl line. Clone FaWd was obtained in one step from the Fa clone by selection for WGA resistance (125 µg/ml), and clone FaRB by selection for ricin resistance (2 µg/ml) as described above.

The cells were kept in culture as described by Fidler (13). All cell lines were regularly tested for mycoplasma and were found negative. The relative DNA content and homogeneity of the cell lines was determined after staining with ethidium bromide by flow cytometry (14).

**Determination of Lectin Sensitivity**

For the determination of lectin sensitivity, 1 x 10⁴ cells were plated on 35-mm dishes and grown for 24 h in normal medium. The lectins were added to the medium from sterile stock solutions at the appropriate concentrations. After 48 h the numbers of adherent cells were counted from duplicate samples using a Coulter counter. Control samples of each line were grown similarly, but without lectin. In preliminary experiments the concentrations of each lectin were determined as those which give no or only slight decrease (0-35%) in the number of cells with the lectin-resistant phenotypes and a clear decrease (67-100%) in the cell number of the lectin-sensitive cell lines. Subsequently, these concentrations of lectin (50 µg/ml WGA, 1.0 µg/ml ricin, 100 µg/ml Lotus lectin) were used to test each cell line for lectin resistance.

**Glycopeptide Analysis**

Cells (4 x 10⁶) were plated on 35-mm dishes and, after 24 h, metabolically labeled by growing them in the presence of 4 µCi/ml of [N-¹⁴C]glucosamine for 48 h. The cells were delipidated, and glycopeptides were prepared by digestion with pronase as previously described (15). Glycosaminoglycans and nucleic acids were removed from the pronase digests by precipitation with cetylpyridinium chloride in the presence of 0.4 M NaCl (16) (this salt concentration prevents the precipitation of the most acidic glycopeptides but does not affect glycosaminoglycans). To all melanoma cell lines do not contain hyaluronic acid (12). The samples were lyophilized, dissolved in 0.1 ml of 50 mM pyridine-acetate buffer (pH 5.0), and purified by centrifugation (17) through columns (bed volume, 1 ml) of Bio-Gel P-2 (200-400 mesh, Bio-Rad Laboratories, Richmond, Calif.) prepared in the same buffer. The glycopeptides were treated with 0.05 M NaOH in 1.0 M NaBH₄ at 45°C for 16 h, and the O-glycosidicoligosaccharides were separated from the N-glycosidic glycopeptides by gel filtration on Sephadex G-50 in 0.1 M pyridine-acetate buffer (pH 5.0) (15). The N-glycosidic glycopeptide fraction was further fractionated by anion-exchange chromatography on DEAE-Sephadex A-25 eluted with a linear gradient of pyridine-acetate buffer (18). Treatment of the glycopeptides with *Vibrio cholerae* neuraminidase was performed as described previously (12).

**Gel Electrophoresis**

Labeling of cell surface components and gel electrophoresis were performed as described previously (12). Total cellular samples were also analyzed after electrophoresis by staining with radioactive WGA and autoradiography (19). Control gels treated in the presence of 0.1 M N-acetylgalactosamine did not produce labeled bands.

**Glycosyltransferase Assays**

The frozen, packed cells were homogenized and solubilized, on ice, by addition of 2 vol of 0.1 M sodium cacodylate buffer (pH 6) containing 25% glycerol, 0.15 M NaCl and 2% Triton CF-54 (wt/vol). After vortexing for 2 min, the cells were centrifuged at 4°C for 15 min at 9000 g. The supernatant was used for glycosyltransferase assays and protein determination (Lowry).

The incubation mixture for fucosyltransferase assay contained in a final volume of 100 µl: GDP-[³H]fucose 1 nmol (20,000 cpm), sodium morbillino ethane sulfonate (pH 5.5), 5 µM; MnCl₂, 0.5 µmol; NaCl, 10 µmol; and lactose, 16 µmol. Final concentration of glycerol and Triton CF-54 were brought to 25 and 2%, respectively. 20 µl of extract was added, and the mixture was incubated at 37°C for 15 min. For each cell extract, a control for endogenous or pyrophosphatase activities was included by omitting lactose in the assay. Control determinations were also done by mixing Triton extracts from cells with the high and low activity to reveal possible inhibitory factors. The reaction was stopped by addition of 1 ml of ice-cold water. The reaction mixture was then transferred to a column made of a Pasteur pipette containing 1 ml of settled Dowex 1-X8 resin (200 x 400 mesh) in the Cl⁻ form (20). The tube was rinsed with another 1 ml of water, which was also transferred to the Dowex column. The eluate was directly collected in a scintillation vial and counted after addition of scintillation fluid.

Incubation conditions for galactosyltransferase assay were identical to those described for the fucosyltransferase assay except for the substrates which were, respectively, N-acetylgalactosamine, 0.5 µmol and UDP-[¹³C]galactose, 10 nmol (50,000 cpm). Controls did not contain N-acetylgalactosamine. Reaction products were recovered as described for the fucosyltransferase assay.

Incubation conditions for sialyltransferase assays were identical to those described for the fucosyltransferase assay, except that CMP-[¹³C]sialic acid, 0.1 nmol (50,000 cpm) replaced the GDP-fucose. Incubation time was 30 min, and the products were recovered as described for the fucosyltransferase assay, except that the Dowex resin was in the phosphate form and that the reaction was stopped by addition of ice-cold sodium phosphate 5 mM pH 6.8 (21).

**Materials**

Ricin was purchased from Miles-Yeda, Ltd. (Rehovot, Israel) and *Lotus tetragonolobus* lectin from Sigma Chemical Co. (St. Louis, Mo.). Wheat germ agglutinin, pronase (B grade), and *Vibrio cholerae* neuraminidase were obtained from Calbiochem AG (Lucerne, Switzerland). GDP-[¹³C]fucose, UDP-[¹³C]galactose, and CMP-[¹³C]sialic acid were obtained from New England Nuclear (Boston, Mass.), and D-[U-¹⁴C]glucosamine (239 mCi/mmol) from The Radichemical Centre (Amersham, England).

**RESULTS**

Selection and Properties of Lectin-resistant Cells and Their Revertants

Previous multiple-step selections with WGA produced the lines Wa3, Wa4 and Wa5 from the parent Fl melanoma line (11). The step from Wa3 to Wa4 was accompanied by an abrupt change in WGA resistance, whereas little additional change was observed in the next selection step giving rise to the Wa5 clone (Table I). The structural change in the Wa4 glycoproteins has been shown to involve a decrease in sialic acid content and an increase in fucose content (12). As expected, the Wa4 cells showed an increased sensitivity to the fucose-binding lectin of *Lotus tetragonolobus*. This lectin was therefore used to attempt selection of revertants by the approach of collateral sensitivity (8). The clones obtained, Wa4Lb and Wa4Ld, were found to be reverted with respect to the WGA resistance and *Lotus* lectin sensitivity concurrently (Table I). Because the decrease in sialic acid content of Wa4 glycoproteins was accompanied by a corresponding increase in the proportion of terminal galactose residues (12), an attempt was made to find revertants with the galactose-binding lectin.

| Cell line | Selected from | Selective lectin | WGA | Lotus | Ricin | DNA |
|-----------|---------------|------------------|-----|-------|-------|-----|
| Fa        | -             | -                | -   | -     | -     | 1.0 |
| Wa3       | Wa2           | WGA              | -   | +     | -     | 1.0 |
| Wa4       | Wa3           | WGA              | +   | +     | -     | 1.4-1.6 |
| Wa4Lb     | Wa4           | Lotus lectin     | -   | +     | +     | 1.4-1.6 |
| Wa4Ld     | Wa4           | Lotus lectin     | +   | +     | -     | 1.4-1.6 |
| Wa5       | Wa4           | WGA              | +   | -     | -     | 1.4-1.6 |
| Wa5Rex    | Wa5           | Ricin            | +   | +     | +     | 1.4-1.6 |
| Wa5Rx     | Wa5           | WGA              | +   | +     | +     | 1.4-1.6 |
| Fa        | Wa            | WGA              | ++  | -     | -     | 1.0 |
| FaWd      | Fa            | WGA              | -   | +     | -     | 1.0 |
| FaRb      | Fa            | Ricin            | ++  | -     | -     | 1.0 |

The lectin sensitivities were determined as described in Materials and Method. The degree of lectin resistance is expressed as the percentage of surviving cells: (-) 0-33%; (+) 33-67%; (+++) 67-100%. The DNA contents are expressed as relative to that of the Fl line.
tein. The Wa5 clone, which was similar in properties to the Wa4 clone, was used as an independent source for the selection. The ricin-selected clones obtained, Wa5Re and Wa5Rx, were also found to revert with respect to their sensitivities to both lectins (Table I).

The Wa4 clone and all the clones derived from it were found to have undergone a major change in their DNA content, as compared to the parent F1 line (Table I). Because such changes may produce difficulties in interpretation, the isolation of new WGA-resistant variants was attempted. Also, single-step mutants would be preferable to multistep mutants (8). To produce an independent parental line, the originally uncloned F1 line was cloned, and the clone Fa was used for new selections. The clone FaWd was obtained in a single step by selection for WGA resistance and was found to be similar in lectin sensitivities to the Wa4 and Wa5 clones (Table I). The relative DNA content was now, however, similar to that of the parental clone. For comparative purposes, a ricin-resistant clone, FaRb, was also isolated from the Fa clone by single-step selection and did not change its DNA content either (Table I).

All the cell lines except for FaWd kept their lectin sensitivities unchanged during prolonged culture (at least 2 mo). There was a gradual loss of the WGA resistance and, concomitantly, of the Lotus-lectin sensitivity of the FaWd clone, which became apparent after a few weeks of culture. Therefore, only batches of recently thawed cells taken from a frozen stock were used for the analyses.

As indicated by the two independent selections for WGA resistance, and by two independent selections for revertants, the Lotus-lectin sensitivity was correlated with the WGA-resistant phenotype in the cell studies (Table I). For ease of discussion, the cells with this property (Wa4, Wa5, FaWd) are referred to from this point as the WGA-R/Lo<sup>3</sup> cells.

**Glycosyltransferase Assays**

Chemical analyses of WGA-R/Lo<sup>3</sup> cells (Wa4 clone) (12) suggested either an increased fucosylation reaction (by 1 → 3 linkages to N-acetylgalcosamine residues) or a decreased sialylation reaction (by α2 → 3 linkages to galactose residues) as two possible enzymatic mechanisms responsible for the changed phenotype. Glycosyltransferase assays were therefore performed to investigate these possibilities. There was a 60- to 70-fold increase in the level of fucosyltransferase activity in the WGA-R/Lo<sup>3</sup> cells as compared to the parental or revertant cells (Table II). Using 2'-fucosyl lactose as an acceptor, the product was identified as lactofucotetraose, which indicated that the transferase was of the species transferring fucosyl residues by α1 → 3 linkages to glucose and N-acetylgalcosamine residues (22) (a detailed description of the properties and substrate specificity of this transferase will be published elsewhere).

In contrast to the fucosyltransferase, there was only slight variation in the levels of sialyl- and galactosyltransferases measured (Table II). The product of the sialyltransferase assay with lactose as acceptor was predominantly (92–99%) 3'-sialyl lactose in all cell lines. It is, however, possible that this activity corresponds to the transferase specific for galactosyl-(β1 → 3)-N-acetylgalactosamine (23) and does, therefore, not necessarily reflect the sialylation of the N-glycosidic chains of glycoproteins.

**Glycopeptide Analysis**

To find out whether the decrease in α2 → 3 sialyl linkages detected for the Wa4 clone (12) is correlated with the increased fucosyltransferase activity and the WGA-R/Lo<sup>3</sup> phenotype, a glycopeptide analysis was performed. As seen in Fig. 1, the total glycopeptide can be fractionated into two parts by gel filtration after mild alkaline borohydride treatment. The first peak corresponds to N-glycosidic glycopeptides and the second to O-glycosidic oligosaccharides (15). Gel filtration of samples from each of the cell lines indicated a decrease in the apparent molecular size of the N-glycosidic glycopeptides of the WGA-R/Lo<sup>3</sup> cells as compared to the parent and revertant cells. The molecular sizes of the O-glycosidic oligosaccharides were similar in all cell lines.

The N-glycosidic glycopeptides were further analyzed by ion-exchange chromatography. Most of the glycopeptides from the parent F1 line were bound to the DEAE-Sephadex column (Fig. 2A). After removal of the sialic acid residues by neuraminidase treatment and gel filtration, the glycopeptides no longer bound to the column (Fig. 2A), indicating that the binding was due to sialic acid.

Analysis of the N-glycosidic fractions from other cell lines revealed that a significant portion of the glycopeptides from WGA-R/Lo<sup>3</sup> cells were not bound to the column, and that those bound were enriched in the earlier-eluting components as

| Cell line | Fucosyl | Sialyl | Galactosyl | Size in gel filtration | Sialic acid (ion-exchange chromatography) |
|-----------|---------|--------|------------|----------------------|------------------------------------------|
| F1        | 1.45    | 3.15   | 8.8        | WT*                  | WT                                       |
| Wa3       | 0.77    | 2.18   | 8.7        | WT                   | WT                                       |
| Wa4       | 92.9    | 2.11   | 10.5       | Decreased            | Decreased                                |
| Wa4Lb     | 1.46    | 2.58   | 9.7        | WT                   | WT                                       |
| Wa4Ld     | 1.12    | 2.52   | 10.0       | WT                   | WT                                       |
| Wa5       | 99.0    | 1.87   | 12.3       | Decreased            | Decreased                                |
| Wa5Re     | 1.09    | 2.21   | 13.3       | WT                   | WT                                       |
| Wa5Rx     | 0.73    | 1.94   | 7.5        | WT                   | WT                                       |
| Fa        | 1.56    | 3.70   | 12.2       | WT                   | WT                                       |
| FaWd      | 94.8    | 3.54   | 11.1       | Decreased            | Decreased                                |
| FaRb      | 0.71    | 3.85   | 8.5        | WT                   | WT                                       |

* WT, wild-type.

![FIGURE 1](image)
compared to the glycopeptides from the parent or revertant cells (Fig. 2B–D). This indicated that the glycopeptides contained a decreased amount of sialic acid, and that this property was correlated with the increase in the fucosyltransferase and the \( \text{WGA}^{\text{Lot}^\text{b}} \) phenotype. The results of the gel filtrations and ion-exchange chromatography are summarized in Table II.

**Gel Electrophoresis of Cellular and Cell Surface Glycoproteins**

Fig. 3 shows the autoradiogram obtained for electrophoretically separated total cellular proteins stained with radio-iodinated WGA. As compared to the parent and revertant cell lines, there was a decreased binding of the lectin to some of the bands in the \( \text{WGA}^{\text{Lot}^\text{b}} \) cells. However, there was only a slight reduction in total amount of WGA bound, which is correlated with the finding that there was only an ~25% reduction in the total amount of protein-bound sialic acid, as determined for the Wa4 clone (12).

Cell surface labeling and gel electrophoresis revealed a similar pattern for all the cell lines except for a slightly increased mobility of some bands in the \( \text{WGA}^{\text{Lot}^\text{b}} \) cells (Fig. 4). This is in accordance with the reduced size of the carbohydrate chains in these cell lines (Fig. 1), and also with similar findings in other lectin-resistant cell lines with defects in protein glycosylation (2, 24, 25).

**DISCUSSION**

Although many different lectin-resistant cell lines have been isolated from a variety of sources, there are few examples where a clear correlation can be demonstrated between a biochemical change and a lectin-resistant phenotype. Independent evidence should be obtained to demonstrate that the pleiotropic changes often found could be due to one genetic change (7). Such evidence can be obtained by the repeated and reproducible selection of isolates of independent origin. Additional support would come from revertants if they could be shown to exhibit coordinate changes in different properties. By both criteria, a strong correlation is indicated in the cell lines studied for the following findings: increased fucosyltransferase activity, decreased sialylation of \( \text{N-glycosidic} \) chains of glycoproteins, decreased WGA binding to electrophoretically separated cellular glycoproteins, increased electrophoretic mobility of surface proteins, as well as WGA resistance and *Lotus*-lectin sensitivity.

Among lectin-resistant cells so far characterized, the \( \text{WGA}^{\text{Lot}^\text{b}} \) cells are unique in having a dramatic increase in a glycosyltransferase activity. An increased sialyltransferase activity has previously been reported for a ricin-resistant mouse L cell line, but the increase was less marked and its contribution to the lectin-resistant phenotype was not entirely clear (24). Many lectin-resistant cell lines described, including a mouse lymphoma line with defective fucose metabolism (26), appear in contrast to have a block in the pathway of oligosaccharide biosynthesis (6, 7).

There are several possible explanations for the increase in fucosyltransferase activity. One is an increased activity due to a mutation in the structural gene of the fucosyltransferase.
Another possibility is that the gene of the fucosyltransferase is present in multiple copies in the variant cells. The latter mechanism has been shown for drug-resistant cell variants (27). However, with these mutants the resistance is gradually increased with successive selections, in contrast to the WGA\textsuperscript{R}\textsuperscript{Lot}\textsuperscript{s} cells, which are found after one selection step. The FaWd clone, however, resembles the drug-resistant mutants in that the resistance is gradually lost upon prolonged culture, a phenomenon ascribed to an extrachromosomal location of the altered gene (27). A third possible explanation for the increased fucosyltransferase could be a change in a putative regulatory (inhibitory) gene. The existence of such a gene for the fucosyltransferase is indicated by the differential expression of the fucosyl linkages in different tissues. In the rat, brain glycopeptides resemble those of the WGA\textsuperscript{R}\textsuperscript{Lot}\textsuperscript{s} cells in having a high proportion of the \(\alpha 1 \rightarrow 3\) linked fucose residues, whereas other tissues found in glycopeptides of some other tissues such as liver or kidney or plasma (28, 29). Furthermore, the similarity in the level of the changes of the transferase in the three variant lines (Wa4, Wa5, and FaWd), as well as the similarly low levels in the parent and revertant cells, also suggests that the effects may be due to a regulatory phenomenon.

The observed decrease in the sialic acid content of the N-glycosidic oligosaccharide chains provides an indication as to the substrate specificity of the sialyltransferase. Previous chemical analyses revealed that the sialic acid is bound mainly by \(\alpha 2 \rightarrow 3\) linkages to galactose residues in the parent F1 cells, and that the increase in fucosyl \(1 \rightarrow 3\) linkages to \(N\)-acetylglucosamine in the Wa4 cells was accompanied by a specific decrease of these sialyl residues (12). This would indicate that the presence of the fucose residues interferes with the addition of the sialic acid residues. In the Lotus-lectin selected revertants (Wa4Lb, Wa4Ld) the absence of fucose would thus lead to increased sialylation. The importance of the fucose residues in controlling sialylation is underlined in the case of the ricin-selected variants (Wa5Re, Wa5Rx). Although ricin does not bind to fucose, the activity of the fucosyltransferase was decreased to the normal level in these cells. It thus appears that low fucosyltransferase activity not only results in increased sialylation (Lotus-revertants) but that the low activity is a prerequisite for a complete sialylation (ricin-revertants).

The influence of the \(\alpha 1 \rightarrow 3\) fucosyl residues on the \(\alpha 2 \rightarrow 3\) sialyltransferase could be analogous to the observations made on the specificity of an \(\alpha 2 \rightarrow 6\) sialyltransferase, which has been shown to be unable to transfer sialyl residues to acceptors with this fucose substitution (21). It seems, however, that in the case of the \(\alpha 2 \rightarrow 3\) sialyltransferase the block is not complete, because structures containing both the sialyl and fucose substitution have been described (28, 30). Alternatively, the occurrence of the latter structures could indicate the presence of another \(\alpha 2 \rightarrow 3\) sialyltransferase for the fucosylated structures. The observations on the sialylation specificity are of particular interest, because it has not yet been possible to isolate the \(\alpha 2 \rightarrow 3\) sialyltransferase. The observations also indicate that it may be fruitful to use variant cells to study the control of glycosyltransferase reactions under the actual intracellular conditions, as a complementary approach to studies performed with purified transferases and substrates under in vitro conditions.

The glycosylation change observed for the WGA\textsuperscript{R}\textsuperscript{Lot}\textsuperscript{s} cells is in accordance with their WGA resistance. This lectin is known to have specificity for sialic acid and \(N\)-acetylglucosa-
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