Rous Sarcoma Virus-transformed Baby Hamster Kidney Cells Express Higher Levels of Asparagine-linked Tri- and Tetraantennary Glycopeptides Containing [GlcNAc-β(1,6)Man-α(1,6)Man] and Poly-N-acetyllactosamine Sequences Than Baby Hamster Kidney Cells*

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The alterations in complex-type N-linked oligosaccharides that can occur when an animal cell line is transformed by two dissimilar viruses were examined by comparing the N-linked oligosaccharides of baby hamster kidney (BHK) cells, metabolically radiolabeled with [2-3H]mannose, to the same class of oligosaccharides from BHK cells separately transformed by Rous sarcoma virus (RS-BHK), an RNA retrovirus, and polyoma virus (PY-BHK), a DNA papovavirus. Based on experiments that utilized serial lectin affinity chromatography, glycosidase digestions, and methylolation analyses, both RS-BHK and PY-BHK cells demonstrated a significant increase in the relative amounts of tri- and tetraantennary complex-type N-linked oligosaccharides containing the branching sequence, [GlcNAc-β(1,6)Man-α(1,6)Man], compared to the nontransformed BHK cells. In addition, almost all of the poly-N-acetyllactosamine sequence, [GlcNAc-β(1,3)Gal-β(1,4)], was expressed on the tri- and tetraantennary N-linked oligosaccharides from BHK and RS-BHK cells that contain the sequence, [GlcNAc-β(1,6)Man-β(1,6)Man]. The increase in the relative amounts of this latter sequence in the transformed cells, therefore, most likely results in an increase in the amount of poly-N-acetyllactosamine sequence on the N-linked glycopeptides of these cells. The analysis of the degree of sialylation of the complex-type N-linked oligosaccharides from BHK and RS-BHK cells by ion exchange chromatography revealed no apparent differences, and in both of these cell types approximately 3% of the glycopeptide fraction radiolabeled with mannose was recovered in a highly negatively charged fraction that was identified by keratanase digestion to be keratan sulfate.

Transformation of cultured cells by any of several means can quantitatively alter the structures of oligosaccharides expressed on cell surface glycoconjugates (1, 2). Several studies have focused on the changes in [3H]Fuc-labeled glycopeptides released by trypsinizing fibroblasts and their transformants. Regardless of the means of transformation, whether by mutagenesis (3), viral transformation (4), or transfection with DNA from unrelated tumor cells (5), the transformed cells demonstrated a relative increase in the amounts of radiolabeled, trypsin-released glycopeptides that eluted with a lower V, upon Sephadex G-50 chromatography (3, 4). Yamashita et al. (6) recently analyzed in detail the asparagine-linked oligosaccharides of BHK cells and their polyoma virus transformants using hydrazinolysis to release the oligosaccharides from asparagine, followed by methylation analysis and gel-permeation chromatography. They concluded that the polyoma-transformed cells expressed higher levels of the Asn-linked complex-type oligosaccharides that contain an N-acetylgalactosamine residue linked β(1,6) to mannose. This increase was observed as an increase in both the tetraantennary and the triantennary oligosaccharides with this linkage (Scheme 1). Subsequently, these investigators (7) provided evidence that this quantitative change was due at least in part to the increased specific activity of a particular glycosyltransferase, UDP-N-acetylgalactosamine:α-mannoside β(1,6)-N-acetylgalactosaminyltransferase (8), which catalyses the synthesis of the GlcNAc-β(1,6)-Man linkage.

To begin an investigation of the mechanisms by which viral transformation alters oligosaccharide structure, we have characterized the Asn-linked complex-type oligosaccharides of Rous sarcoma virus-transformed BHK cells (RS-BHK) and their parental BHK cell line and, as a point of comparison, we have also studied this class of oligosaccharides in BHK cells transformed by polyoma virus (PY-BHK), whose Asn-linked oligosaccharides have been previously characterized (6). Rous sarcoma virus is an RNA retrovirus whose transforming ability can be localized to the src gene. By contrast, polyoma virus is a DNA papovavirus which contains two transforming genes. Comparing the changes in Asn-linked oligosaccharides observed in BHK cells transformed separately by each virus should, therefore, allow us to determine if the two viruses cause similar alterations in Asn-linked oligosaccharide structures. Earlier studies suggested that the [2-3H]Fuc-labeled, trypsinizable glycopeptides from both RS-BHK and PY-BHK cells eluted with a lower V, than the corresponding glycopeptides from BHK cells; however, the RS-BHK glycopeptides have not been characterized in detail (3).

For this analysis we have utilized the protocol of radiolabeling cells metabolically with [2-3H]Man, fractionating their

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1 The abbreviations used are: BHK, baby hamster kidney cells; LCA, Lens culinaris agglutinin; L-PHA, phytohemagglutinin-L;DSA, Datura stramonium agglutinin; BSA, bovine serum albumin; GlcNAc, N-acetylgalactosamine; Gal, galactose; Man, mannose; Fuc, fucose; PBS, phosphate-buffered saline; RS-BHK, Rous sarcoma virus; PY-BHK, polyoma virus; HPLC, high performance liquid chromatography.
glycopeptides by serial lectin-affinity (9) and QAE-Sephadex chromatography, and also subjecting to methylation analysis the metabolically radiolabeled O-methylhexitols present in the glycopeptide fractions. The results of this study demonstrate that both transformed cell types, RS-BHK and PY-BHK, expressed more relative amounts of Asn-linked oligosaccharides containing the sequence GlcNAc-\(\beta(1,6)\)Man-\(\alpha(1,6)\)Man than did the parental BHK cells. These studies thus confirm the earlier results for PY-BHK cells (6) and demonstrate that the increase in the Asn-linked antenna with the sequence [GlcNAc-\(\beta(1,6)\)-Man] is not viral specific. RS-BHK cells, however, contained the highest relative levels of glycopeptides with this sequence. The Asn-linked glycopeptides in all fractions after serial lectin affinity chromatography contained similar degrees of sialylation. Based on chromatography of the metabolically radiolabeled Asn-linked glycopeptides of BHK and RS-BHK cells on immunobilized Datura stramonium lectin (10), the increase in the relative amounts of the glycopeptides that contain the sequence, [GlcNAc-\(\beta(1,6)\)Man-\(\alpha(1,6)\)Man] also results in an increase in the transformed cells of the repeating sequence [GlcNAc-\(\beta(1,3)\)-Gal\(\beta(1,4)\)], which often terminates this antenna.

MATERIALS AND METHODS AND RESULTS

The Asn-linked oligosaccharides of both BHK and RS-BHK cells types appeared to be qualitatively similar by several criteria. Methylation analysis of radiolabeled glycopeptide fractions separated by serial lectin chromatography demonstrated similar patterns of mannose substitution. QAE-chromatography of three glycopeptide fractions from both cell types before and after mild acid treatment demonstrated a remarkably similar pattern of sialylation. In addition, similar amounts of a fraction of the glycopeptides from both cells that bound to no lectin was found to contain keratan sulfate, based on keratanase sensitivity. Keratan sulfate has been demonstrated to be linked to biantennary Asn-linked oligosaccharides in bovine cornea (20) and in the III glycoprotein of human erythrocytes (19), which contains both Asn-linked poly-N-acetyllactosamine and keratan (21). The results of two earlier studies that utilized metabolic labeling of the glycopeptides from RS-BHK and BHK cells (32) and a murine tumor cell line (22) are both consistent with the presence of both poly-N-acetyllactosamine and keratan sulfate on the Asn-linked oligosaccharides of these cells.

Although the Asn-linked oligosaccharides of BHK and RS-BHK are similar in several respects, RS-BHK cells consistently demonstrated a 2-fold increase in the relative amounts of glycopeptides with the [Gal-\(\beta(1,4)\)GlcNAc-\(\beta(1,6)\)Man] sequence, those which bind to immobilized L-PHA. This increase (Table I) has been expressed in terms of per cent of total radioactivity in glycopeptide. The increase calculated in this manner is most likely an underestimate, however, since the high-mannose glycopeptides (ConA fraction III + IV) have an average of 7-8 mannose residues/molecule, while the bi-, tri-, and tetraantennary glycopeptides have an average of 3-4 mannose plus fucose residues. Therefore, the contribution of the non-high-mannose glycopeptides to the total glycopeptide radioactivity is somewhat underestimated by expressing changes of the amounts of glycopeptides that bind to L-PHA simply as an increase in the amount of radioactive glycopeptide that binds to L-PHA relative to the total radioactivity in glycopeptide.

Chromatography of the fractionated, radiolabeled BHK and RS-BHK glycopeptides on immobilized D. stramonium agglutinin revealed that about 40-50% of the glycopeptides that bind to L-PHA from either cell expressed the repeating N-acetyllactosamine sequence, determined by their sensitivity to endo-\(\beta\) galactosidase. Little of this repeating sequence was found on antenna that did not bind to L-PHA, which suggests that this repeating sequence is expressed preferentially on those glycopeptide antenna that bind to L-PHA, those which contain the sequence [Gal-\(\beta(1,4)\)GlcNAc-\(\beta(1,6)\)Man]. In support of this observation, recent experiments (23) suggest that the N-acetylglucosaminyltransferase that is responsible, with galactosyltransferase, for the synthesis of the repeating poly-N-acetyllactosamine sequence, transfers GlcNAc preferentially to the N-linked oligosaccharide antenna that contain GlcNAc-\(\beta(1,6)\)Man to Man.

An increase in antenna with the [Gal-\(\beta(1,4)\)GlcNAc-\(\beta(1,6)\)Man] sequence has been reported for PY-BHK cells by Yamashita et al. (6), who compared the Asn-linked oligosaccharides of BHK and PY-BHK cells by methylation analysis of the total N-linked acidic oligosaccharides and their subsequent fractionation by gel permeation chromatography. The present study utilized metabolically labeled glycopeptides and lectin fractionation to confirm that compared to BHK cells, PY-BHK cells express quantitatively more of the antenna that bind to L-PHA. Since RS-BHK cells show a similar quantitative increase of these antenna, these results demonstrate that transformation of BHK cells by either an RNA or DNA tumor virus causes a similar phenotypic change in the ASN-linked oligosaccharides of these cells. The results of a study by Santer et al. (5) suggest that the fucose-labeled glycopeptides of NIH 3T3 cells show an increased binding to immobilized L-PHA when these cells were transfected with naked DNA from two unrelated tumor cell lines, suggesting that transformation by transfection increases the amounts of glycopeptides with the binding determinant that is recognized by L-PHA. Transformation of fibroblasts by two dissimilar viruses, by transfection, and most likely by mutagenesis (5) appears to cause similar Asn-linked structural alterations.

The same molecular mechanisms may, therefore, cause these specific, quantitative changes.

Homogenates of PY-BHK cells have been shown to express about twice as much UDP-N-acetylgalactosamine:\(\alpha\)-mannoside \(\beta(1,6)\)-N-acetyllactosaminyltransferase activity as do BHK cells, while the specific activities of the other five N-acetyllactosaminyltransferases were similar (7). The higher specific activity of the \(\alpha\)-mannoside \(\beta(1,6)\)-N-acetyllactosaminyltransferase in the transformed cells is thus consistent with the hypothesis that the increases in the antenna expressing [GlcNAc-\(\beta(1,6)\)Man-\(\alpha(1,6)\)Man] can be explained at least in part by an increase in the specific \(\alpha\)-mannoside \(\beta(1,6)\)-N-acetyllactosaminyltransferase activity. It has not as yet been demonstrated, however, that this increase is sufficient to explain the phenotypic increase in this specific oligosaccharide structure. If this hypothesis is correct, then the mechanism by which the transformation of fibroblasts by any of several means causes a specific increase in only one of the N-acetyllactosaminyltransferase activities must be investigated in detail.

The increased levels of antenna with [GlcNAc-\(\beta(1,6)\)Man-
α(1,6)Man] in RS-BHK and PY-BHK transformed cell lines documented in this study and for PY-BHK cells (6) could explain earlier observations (5) concerning the relative increase of Asn-linked oligosaccharide size upon transformation. The observed increase in size is undoubtedly accentuated by the increase of repeating poly-N-acetyllactosamine sequences, since these structures appear to be expressed preferentially on the [GlcNAc-β(1,6)Man] antenna. Studies by Glick et al. (24) demonstrated a correlation between the tumorigenicity of transformed hamster fibroblasts and the increased expression of trypsin-released, fucose-labeled glycopeptides with a lower V, than the majority of radiolabeled glycopeptides. It is not known as yet if such a correlation with tumorigenicity can be demonstrated for specific increases in either the [GlcNAc-β(1,6)Man-α(1,6)Man] antenna or the repeating poly-N-acetyllactosamine sequence. The expression of the repeating sequence has been documented on several cell types other than erythrocytes, including F9 teratocarcinoma cells (25) and mouse embryos (26), and in the case of the teratocarcinoma cells, this sequence appears to be directly involved in intercellular adhesion. Other studies have shown that this sequence is present on fibronectin from placenta and amniotic fluid (27), and its presence on placental fibronectin can significantly alter the interaction of fibronectin with gelatin (28).

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REFERENCES
SUPPLEMENTARY MATERIAL

Supplementary Table 1

<table>
<thead>
<tr>
<th>Lectin</th>
<th>RS-BHK</th>
<th>BHR</th>
<th>BHX</th>
</tr>
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<tr>
<td>Con A</td>
<td>20.0</td>
<td>4.3</td>
<td>29.9</td>
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<tr>
<td>III+IV</td>
<td>8.4</td>
<td>50.0</td>
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<tr>
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<tr>
<td>L-PHA</td>
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**Figure 1.** High Performance Liquid Chromatography of Methylated Derivatives from RS-BHK Glycopeptide Fractions. All the glycopeptides contained in fraction IIA were permethylated, hydrolyzed, and reduced with borohydride. The sample was eluted and chromatographed at 45°C on a reverse-phase octylsorbosphere column equilibrated with water and an organic phase of MeOH:AcOH (99:1). The column was developed with an increasing acetonitrile gradient as described in Experimental Procedures. Elution was monitored by absorbance at 260 nm, and the fraction was determined by reverse-phase high performance liquid chromatography (HPLC) of a representative glycopeptide fraction. The results are shown in Figure 1.
were summed. (RS-BHK aamigning the
ner.bolic.lly radiolabeled plysopeptides from
10776
methylation analyses. the asn-linked glycopeptides from
found in fractions
Man (25%). Peak IAI from both cells contained some glycopeptides with
glycopeptides. because of the presence of other substituents that blocked binding
in Table
contained GlcNAc linked
linked oliaosaccharides of these and
repeating polyN-acetyllactosamine structure, the glycopeptides were
to elute in the void volume.
followed by glycosidase digestions, it was important to confirm these
this repeating sequenoe on the am-linked glycopeptides from both
(T10). This treatment abolishes the binding of oligosaccharides
2 ml fractions were collected. The bound glycopeptides were then eluted with
chitobioae. Some enmples of glycopeptides were treated with
Tri- and tetraantennary Oligosaccharides most commonly contain
this fractionated [2-14C]-Man-labeled
and L-PHA and subsequent
Transformation Elevates [GlcNAc-8(1,6)Man-α(1,6)Man] Sequence
Transformation Elevates fGlcNAc-β(1,6)Man-(α,1,6)Man Sequence

TABLE IV
QAE-SEPHADEX CHROMATOGRAPHY OF METABOLICALLY RADIOLABELED GLYCOPROTEINS

<table>
<thead>
<tr>
<th>Type Cell</th>
<th>Fraction 2</th>
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<th>70</th>
<th>140</th>
<th>200</th>
<th>250</th>
<th>300</th>
<th>500</th>
<th>750</th>
<th>1000</th>
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<td>0</td>
<td>0</td>
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<td>1</td>
<td>11</td>
<td>33</td>
<td>64</td>
<td>67</td>
<td>76</td>
<td>87</td>
<td>95</td>
<td>100</td>
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</tr>
<tr>
<td>BHK-21</td>
<td>2A1</td>
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<td>0</td>
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<tr>
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<td>0</td>
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Fractionated, metabolically radiolabeled glycoproteins were subjected to QAE-Sephadex chromatography as described in Figure 1. Those fractions designated "R" were hydrolyzed in 2.0 M acetic acid for 1 hr at 100°C prior to chromatography to release sialic acid.

TABLE V
QAE-SEPHADEX CHROMATOGRAPHY OF THE BULKY CHANGED GLYCOPROTEINS IN FRACTION RS-SEPHAROSE BEFORE AND AFTER REVERSAL TREATMENT

<table>
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<tr>
<th>Treatment</th>
<th>Fraction 2</th>
<th>20</th>
<th>70</th>
<th>140</th>
<th>200</th>
<th>250</th>
<th>500</th>
<th>750</th>
<th>1000</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>1A1</td>
<td>1</td>
<td>11</td>
<td>33</td>
<td>64</td>
<td>67</td>
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<td>25</td>
<td>4</td>
<td>1</td>
<td>13</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

Metabolically radiolabeled glycoproteins from fraction RS-SEPHAROSE that were eluted from QAE-Sephadex at 0.1 M NaCl concentration of greater than or equal to 1 mg ml⁻¹ with S. aureus, 7.5). Essentially identical results were obtained using either method.

Preparation of Radilabelled Glycopeptides—Radilabelled cells were pelleted by centrifugation, washed twice with a cold saline solution, and then kept at 0°C until they were resuspended in 2 ml Tri-alanine pH 8.0, and processed as described above. The glycopeptide fraction from the RNase digestion was then hydrolyzed in 2 M acetic acid for 1 hr at 100°C, and the hydrolysate was separated into 20 fractions by chromatography on QAE-Sephadex as described in Figure 2. Those fractions designated "R" were hydrolyzed in 2.0 M acetic acid for 1 hr at 100°C prior to chromatography to release sialic acid.

Preparation of Sialylated Glycopeptides—Radilabelled cells were pelleted by centrifugation, washed twice with a cold saline solution, and then kept at 0°C until they were resuspended in 2 ml Tri-alanine pH 8.0, and processed as described above. The glycopeptide fraction from the RNase digestion was then hydrolyzed in 2 M acetic acid for 1 hr at 100°C, and the hydrolysate was separated into 20 fractions by chromatography on QAE-Sephadex as described in Figure 2. Those fractions designated "R" were hydrolyzed in 2.0 M acetic acid for 1 hr at 100°C prior to chromatography to release sialic acid.

Methods and Materials—Protein was assayed by the method of Lowry et al. (28). Chromatography of glycopeptides on QAE-Sephadex was performed by the method described by Cummings et al. (18). The t-N-acetylglucosaminidase inhibitors, 2-naphthyl-N-acetylglucosamine, was a gift from Dr. C. Benitez, Department of Chemical, University of Alberta, and was synthesised by the method of Despeux et al. (20). This inhibitor was stored under nitrogen at 2°C and was stable in enzyme incubations at pH 7.0. The inhibitor was stored under nitrogen at 2°C and was stable in enzyme incubations at pH 7.0.

In the Tantreni process, the glycopeptide fraction was subjected to a series of modifications that eliminated or modified sialic acid and other carbohydrate moieties. The treated cell lysate was then subjected to further analyses, including enzymatic digestion, methylation analysis, and chemical modifications. The treated cell lysate was then subjected to further analyses, including enzymatic digestion, methylation analysis, and chemical modifications.