# A Comparison of Membrane Glycoconjugates from Mouse Cells Transformed by Murine and Primate RNA Sarcoma Viruses†

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ABSTRACT: Kirsten murine sarcoma virus (Ki-MSV) transformed Balb/3T3 mouse cells (K-Balb) were found to have altered membrane glycoconjugates compared to normal Balb/3T3 cells. There were reduced amounts of mono- and disialogangliosides, $G_{M1}$ and $G_{D1a}$, and activity of the specific galactosyltransferase required for synthesis of these gangliosides was reduced to between 0 and 18.5% of normal in the several K-Balb clones examined. When fucose-labeled glycopeptides derived from the surfaces of Balb/3T3 and K-Balb cells were compared by gel filtration chromatography, the glycopeptides from the transformed cells were enriched in earlier eluting components. These differences were also observed when the glycopeptides were derived from the entire cell and were diminished when the surface or cellular glycopeptides from Balb/3T3 and K-Balb were digested with neuraminidase prior to chromatographic analysis. Changes in these membrane sialoglycolipids and sialoglycopeptides were not influenced by Rauscher leukemia virus infection. In marked contrast, these changes in membrane glycoconjugates were not observed in Wooley monkey sarcoma virus (WSV) transformed Balb/3T3 cells (W-Balb). Although W-Balb cells like K-Balb were transformed by tissue culture criteria, their ganglioside composition, galactosyltransferase activity, and glycopeptide patterns were similar to normal Balb/3T3. These findings have potential implications concerning the role of these complex carbohydrates in the phenotypic alterations of transformed cells.

For the last several years, interest has been focused on the role of membrane glycoconjugates in regulating cell growth and behavior and, correspondingly, on the alterations in complex carbohydrates observed following transformation of cultured cells (for a recent review see Brady and Fishman, 1974a). Glycosphingolipid changes have been well documented in numerous lines of cultured cells from various species transformed by different oncogenic agents (see Brady and Fishman, 1974b). In general, transformed cells exhibit a loss of components with more complex oligosaccharide chains due to an absence of specific glycolipid glycosyltransferase activities. Thus, BHK21[1] cells transformed by polyoma virus have less ganglioside $G_{M3}$ (Hakomori and Murakami, 1968) and less CMP-NAN:lactosylceramide sialyltransferase activity (Den et al., 1971). Other transformed hamster cells (Den et al., 1974) and mouse cells transformed by DNA tumor viruses (Brady and Mora, 1970; Cumar et al., 1970) are deficient in gangliosides more complex than $G_{M3}$ and in the UDP-GalNAc:$G_{M3}$ *N*-acetylgalactosaminyltransferase activity required for their synthesis. More recently, we demonstrated that Balb/3T3 cells transformed by Ki-MSV, chemical carcinogens or X-irradiation are deficient in gangliosides more complex than $G_{M2}$ and in UDP-Gal:$G_{M2}$ galactosyltransferase activity (Fishman et al., 1974; Coleman et al., 1975).

Glycoprotein changes have also been observed in a variety of transformed cells (Buck et al., 1970, 1971; Glick et al., 1973, 1974; and van Beek et al., 1973). The changes are characterized by an increase in the proportion of fucose-labeled glycopeptides of higher apparent molecular weight following transformation. Removal of sialic acid from the glycopeptides diminishes the differences between glycopeptides derived from normal and transformed cells (Warren et al., 1972; van Beek et al., 1973), and the asialoglycopeptides from transformed cells serves as an acceptor for a specific sialyltransferase activity which is elevated in the transformed cells (Warren et al., 1972).

In order to examine the possible effects that different host cell and tumor viruses interactions may have on the expression of these membrane phenomena, we investigated the membrane glycosphingolipids and glycopeptides of the highly contact-inhibited untransformed mouse embryo clonal cell line, Balb/3T3, and derivative lines transformed by the Kirsten murine sarcoma virus (Ki-MSV) and the Wooley monkey sarcoma virus (WSV). Thus, we were able to directly compare glycosphingolipids and glycopeptides in the same clonal cell line before and after transformation by two different RNA tumor viruses.

## Materials and Methods

*Cell Culture and Cell Lines.* Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.) at 37°C in a 10% $CO_2$ atmosphere. Unless otherwise specified, cells were cultivated in 60-mm or 100-mm plastic petri dishes (Falcon Plastics). A contact-inhibited clonal mouse embryo cell line, Balb/3T3 (Aaronson and Todaro, 1968), and subclone of this line, nonproductively transformed by

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[1] Abbreviations used are: BHK21, baby hamster kidney cells clone 21; Ki-MSV, Kirsten murine sarcoma virus; WSV, Wooley monkey sarcoma virus; R-MuLV, Rauscher murine leukemia virus; NAN, *N*-acetylneuraminic acid; $G_{M3}$, *N*-acetylneuraminylgalactosylglucosylceramide; $G_{M2}$, *N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]galactosylglucosylceramide; $G_{M1}$, galactosyl-*N*-acetylgalactosaminyl[*N*-acetylneuraminyl]galactosylglucosylceramide; $G_{D1a}$, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]galactosylglucosylceramide; EDTA, ethylenediaminetetraacetate.

the Kirsten strain of murine sarcoma virus, K-Balb-234 (Aaronson and Weaver, 1971), have been reported. Additional KiMSV nonproducer transformants K-Balb-521, K-Balb-B10, and K-Balb-E9 were derived following separate infections by KiMSV of single Balb/3T3 cells in microtiter wells and isolation by the microtiter procedure (Stephenson et al., 1972). Previous studies have demonstrated that a transforming virus isolated from a naturally occurring fibrosarcoma of a wooly monkey, wooly sarcoma virus (Theilen et al., 1971; Kawakami et al., 1971), transforms cells in the absence of infectious virus production (Aaronson, 1973; Scolnick and Parks, 1973). In the present studies, WSV-transformed Balb/3T3 cells were obtained by infection with a stock of WSV containing Rauscher murine leukemia virus as its helper virus. A transformed clonal line, producing both WSV and R-MuLV, was isolated by the microtiter procedure and was designated W-Balb(R). Balb/3T3 and K-Balb-521 clones, superinfected with R-MuLV, were designated Balb/3T3(R) and K-Balb(R), respectively.

*Doubling Time and Saturation Density.* Cells were innoculated onto 20-cm$^2$ petri dishes at a density of $5 \times 10^3$ cells/cm$^2$, and the medium was changed every 3 days. Cells from duplicate plates were counted with a hemocytometer at each time point. The doubling time was determined during growth in exponential phase. The saturation density was taken as the value where three successive cell counts at 2-day intervals showed no increase in cell number.

*Colony-Forming Assays.* Cell colony formation was measured on confluent monolayers of an adult human fibroblast strain, 501T, as previously described (Aaronson and Todaro, 1968). Cells were plated at serial tenfold dilutions and colonies counted with the aid of a dissecting microscope following fixation with formalin and staining with 1% hematoxylin. Colony formation in soft agar was determined according to methods reported previously (MacPherson and Montagnier, 1964).

*Glycolipid Analysis.* Glycolipids were extracted from subconfluent cell cultures and partitioned into neutral and acidic glycolipid fractions as previously described (Mora et al., 1969). Gangliosides were separated by thin-layer chromatography on silica gel-coated glass plates (E. Merck, Germany) with the developing solvent of chloroform, methanol, and 0.25% aqueous $CaCl_2$ (60:35:8, v/v), detected with resorcinol spray and quantitated by densitometry with a Zeiss chromatogram scanner (Brady and Mora, 1970).

*Enzyme Assays.* Cells were harvested, washed, lysed by freeze–thawing in 0.25 *M* sucrose containing 0.1% 2-mercaptoethanol, and assayed for the various glycosyltransferase activities as previously described (Fishman et al., 1974; Coleman et al., 1975). Incubation conditions for UDP-GalNAc:$G_{M3}$ *N*-acetylgalactosaminyltransferase were: 25 nmol of $G_{M3}$, 5 nmol of UDP[$^{14}$C]GalNAc (14 mCi/mmol), 1.25 $\mu$mol of sodium cacodylate buffer (pH 7.0), 0.5 $\mu$mol of $MnCl_2$, 100 $\mu$g of Nonidet P-40, and 40 $\mu$g of cell protein (whole lysate) in a total volume of 25 $\mu$l; incubations were for 2 hr at 37°C. For UDP-Gal:$G_{M2}$ galactosyltransferase, conditions were: 10 nmol of $G_{M2}$, 50 nmol of UDP[$^{14}$C]Gal (4 Ci/mol), 1.25 $\mu$mol of sodium cacodylate buffer (pH 5.3), 1.0 $\mu$mol of $MnCl_2$, 200 $\mu$g of Triton CF-54, 100 $\mu$g of Tween-80, and 250 $\mu$g of cell protein (600*g* pellet) in a total volume of 50 $\mu$l; incubations were for 3 hr at 37°C. For CMP-NAN:$G_{M1}$ sialyltransferase conditions were: 10 nmol of $G_{M1}$, 20 nmol of CMP[$^{14}$C]NAN (7 mCi/mmol), 1.25 $\mu$mol of sodium cacodylate buffer (pH 6.3), 100 $\mu$g of Triton CF-54, 50 $\mu$g of Tween-80, and 100 $\mu$g of cell protein (whole lysate) in a total volume of 25 $\mu$l; incubations were for 2 hr at 37°C. Formation of radioactive reaction products was determined by high-voltage electrophoresis for the sialyltransferase assay and by Sephadex column chromatography for the other two glycosyltransferases (Coleman et al., 1975). All enzyme activities were assayed under optimum conditions and linearity in terms of time of incubation and protein concentrations. Values expressed as nanomoles of [$^{14}$C]glycolipid product per milligram of protein per hour were corrected for endogenous activity as measured in the absence of added glycolipid acceptor.

*Labeling of Membrane Glycoproteins.* Cells were cultured for 72 hr during their exponential phase of growth in medium containing either L-[$^{14}$C]fucose (53 mCi/mmol; 1 $\mu$Ci/ml) or L-[$^3$H]fucose (84 mCi/mmol; 1.5 $\mu$Ci/ml); the fucose concentration was the same (18 $\mu M$) and the medium was not changed. The cells were routinely cultured with labeled medium in 100-mm plastic petri dishes and cells from five dishes were pooled for each analysis; in one experiment the cells were cultured in roller bottles. Following removal of labeled medium, the cell monolayers were washed three times with Tris-buffered saline (0.15 *M* NaCl–0.02 *M* Tris-HCl (pH 7.5)) and surface components removed by incubation with 0.1% trypsin dissolved in the same buffer for 30 min at 37°C (van Beek et al., 1973). Based on trypan blue exclusion, the transformed cells were 100% viable and the Balb/3T3 cells 90% viable following trypsinization. An equivalent amount of soybean trypsin inhibitor was then added and the cells were collected by centrifugation at 800*g* for 15 min at 4°C. The supernatants (trypsinates) and cells were then frozen at −20°C until digested with Pronase.

*Preparation of Glycopeptides.* The trypsinates were lyophilized, dissolved in 2–3 ml of distilled water, and dialyzed against the same at 4°C for 24 hr. In some experiments, the trypsinates were desalted on a 1 × 60 cm Bio-Gel P-2 (200–400 mesh) column eluted with distilled water. The desalted trypsinates were lyophilized and dissolved in 1 ml of 0.1 *M* Tris-HCl (pH 7.8) containing 2.5 m*M* $CaCl_2$. The labeled cell pellets were homogenized in 2 ml of the same buffer. Pronase was added to each sample at an initial concentration of 0.1% followed by a few drops of toluene. The samples were incubated at 37°C with fresh Pronase being added at 2 and 4 days. After 6 days the samples were centrifuged at 40000*g* for 30 min, and the supernatants were frozen at −20°C until analyzed.

*Neuraminidase Digestion.* Aliquots of the labeled supernatants were heated for 5 min at 100°C to inactivate Pronase. The samples were then incubated overnight at 37°C with 0.03 unit of neuraminidase[2] in 0.1 *M* sodium acetate buffer (pH 5.0). The samples were then heated at 100°C for 5 min to inactivate the neuraminidase.

*Gel Filtration Chromatography.* Aliquots of $^{14}$C- and $^3$H-labeled material were admixed and the samples (0.4 ml) in 0.25 *M* sucrose were layered on 1 × 100 cm columns (1.5 × 90 cm in some experiments) of Bio-Gel P-10 (200–400 mesh). The columns were equilibrated and eluted with 0.1 *M* Tris-acetate buffer (pH 9.0) containing 0.1% sodium dodecyl sulfate, 0.01% EDTA= AND ]/[% mercaptoetha-

[2] Commercial neuraminidase (*Cl. Perfringens* Type VI from Sigma) was further purified 50-fold on an affinity column by the method of Cuatrecasas and Illiano (1971). The column was kindly provided by Dr. Gilbert Ashwell, NIAMDDS. The purified enzyme had a specific activity of 29 IEU per mg of protein with fetuin as a substrate.

Table I: Comparison of Tissue Culture Properties of KiMSV and WSV Transformed Balb/3T3 Cells.[a]

| Cell line | Doubling Time[b] (hr) | Saturation Density[c] (cells/cm$^2$ × $10^{-5}$) | Colony-Forming Ability[d] Monolayers (%) | In agar (%) |
|---|---|---|---|---|
| Balb 3T3 | 26 | 0.5 | 0.001 | 0.001 |
| K-Balb 234 | 22 | 2.5 | 15 | 5.0 |
| W-Balb (R) | 24 | 2.7 | 12 | 3.5 |

[a] The tissue culture properties of Balb/3T3 and its transformed subclones, K-Balb-234 and W-Balb (R), were determined as described under Materials and Methods. [b] $5 \times 10^3$ cells/cm$^2$ were inoculated onto 20-cm$^2$ petri dishes under conditions where medium containing 10% calf serum was changed every 3 days. Cell counts were performed in duplicate every 2 days and the doubling times were determined during growth in the exponential phase. [c] The saturation density was taken as that value where three successive cell counts at 2-day intervals showed no increase in cell number. [d] Average of values obtained from two or more experiments where cells were inoculated at serial tenfold dilutions either onto confluent monolayers of adult human fibroblasts or in suspension in soft agar. Colonies were counted at 12–15 days and are expressed as the percent of the cells inoculated.

Table II: Distribution of Gangliosides in Normal, RNA Leukemia Virus Infected and RNA Sarcoma Virus Transformed Balb/3T3 Mouse Cells.[a]

| Cell Line | % Distribution of Sialic Acid: Ganglioside $G_{M3}$[b] | $G_{M2}$ | $G_{M1}$ | $G_{D1a}$ | Total (nmoles of Sialic Acid/mg of Protein) |
|---|---|---|---|---|---|
| Balb/3T3 | 12.8 | 30.5 | 9.4 | 47.3 | 3.74 |
| Balb/3T3 (R) | 11.3 | 42.1 | 10.9 | 35.7 | 4.96 |
| K-Balb (R) | 12.4 | 78.5 | 6.6 | 2.9 | 2.42 |
| W-Balb (R) | 8.0 | 27.8 | 20.3 | 43.9 | 3.55 |

[a] Glycolipids were extracted from each cell line when in the log phase of growth, the ganglioside fraction was separated by thin-layer chromatography, and the individual gangliosides were detected by resorcinol reagent and quantitated by direct densitometry as described under Materials and Methods. [b] Values for $G_{M3}$ include amounts recovered in the non-ganglioside (lower phase) fraction.

nol at a rate of 3 ml/hr. Fractions were made acidic with 50 μl of glacial acetic acid and dissolved in Aquasol. Radioactivity was measured with a Beckman Model LS 250 liquid scintillation spectrometer set for simultaneous $^3$H and $^{14}$C counting. The uncorrected counts were analyzed by a computer program which corrected the counts for background, quenching, cross channel spill, and counting efficiency. The results as dpm of $^3$H and $^{14}$C were plotted as the percent of total radioactivity of each isotope recovered from the columns.

*Materials.* Radioisotopes and Aquasol were obtained from New England Nuclear. Unlabeled UDP-galactose, L-fucose, trypsin (Type III), soybean trypsin inhibitor (Type I-S), neuraminidase (Type VI), and Pronase (protease, Type VI) were from Sigma. Bio-Gel P-2 and P-10 and Sephadex G-25 superfine were purchased from Bio-Rad Laboratories and Pharmacia, respectively. The sources of glycolipids and detergents used in the enzyme assays have been described (Fishman et al., 1972, 1974).

## Results

*Comparison of Tissue Culture Properties of Balb/3T3 with KiMSV and WSV Transformants of Balb/3T3.* Previous reports have demonstrated that cell culture properties including the ability to form colonies on a monolayer of contact-inhibited cells (Aaronson and Todaro, 1968) or in soft agar (MacPherson and Montagnier, 1964) correlate well with the in vivo malignancy of tissue culture lines. In the present studies, the biologic properties of parental Balb/3T3 were compared with those of its KiMSV and WSV transformed subclones. As shown in Table I, the doubling times of each were similar (22–26 hr); however, both sarcoma virus transformants reached much higher saturation densities than the parental Balb/3T3 clone. While Balb/3T3 failed to form colonies on confluent monolayers or in soft agar, the sarcoma virus transformed lines were able to form colonies with similar high efficiencies.

*Glycosphingolipid Composition of Balb/3T3 and RNA Virus-transformed Subclones.* Thin-layer chromatograms of neutral glycosphingolipids extracted from the various cell lines indicated no substantial differences in agreement with previous results (Fishman et al., 1974). Thin-layer chromatograms of gangliosides extracted from the various cell lines showed considerable differences. Both Balb/3T3 and Balb/3T3(R) had a complete complement of gangliosides whereas the K-Balb clones and K-Balb(R) cells were deficient in gangliosides corresponding to $G_{M1}$ and $G_{D1a}$ and had increased amounts of gangliosides corresponding to $G_{M2}$. However, W-Balb(R) cells contained a ganglioside pattern very similar to that of normal Balb/3T3. The distribution of gangliosides in the various cell lines is given in Table II. Although the total ganglioside content varied considerably, the distribution of gangliosides in Balb/3T3, Balb/3T3(R), and W-Balb(R) was similar but very distinct from that observed with K-Balb (Fishman et al., 1974) and K-Balb(R). In the latter two cell lines the proportion of $G_{M1}$ and $G_{D1a}$ was decreased and that of $G_{M2}$ increased.

Although the individual gangliosides were not identified further, gangliosides from Balb/3T3 cells had been identified previously by gas–liquid chromatographic analysis (Dijong et al., 1971). When gangliosides extracted from W-Balb(R) cells were treated with purified bacterial neuraminidase,[2] there was a complete disappearance of gangliosides comigrating with authentic $G_{M3}$ and $G_{D1a}$ standards and an increase in the ganglioside corresponding to $G_{M1}$. Under our incubation conditions, terminal sialic acids are cleaved by neuraminidase while the inner sialic acid on $G_{M1}$ and $G_{M2}$ is resistant. We had previously demonstrated that gangliosides from Balb/3T3 cells corresponding to $G_{M3}$ and $G_{D1a}$ are neuraminidase-sensitive whereas gangliosides from K-Balb cells corresponding to $G_{M2}$ are resistant (Fishman et al., 1974).

*Glycosyltransferase Activities in Normal and RNA Virus-Transformed Mouse Cells.* The activities of three glycosyltransferases involved in the sequential synthesis of $G_{D1a}$ from $G_{M3}$ were determined in normal and virus-transformed Balb/3T3 cell lines (Table III). The results are in agreement with the observed ganglioside patterns. KiMSV-transformed cells are deficient in galactosyltransferase activity and have normal levels of *N*-acetylgalactosaminyl- and sialyl-transferase activities. In contrast, WSV-transformed cells have enzyme activities similar to normal Balb/3T3. This same decrease in UDP-galactose:$G_{M2}$ galactosyltransferase was observed in each of the KiMSV-transformed Balb/3T3 clones examined. The activity in the

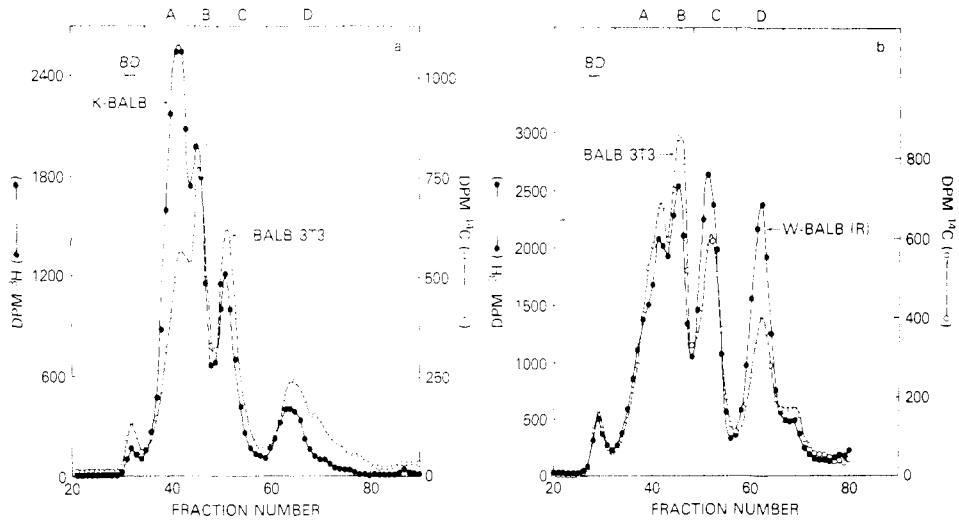


FIGURE 1: Elution profiles of fucose-labeled surface glycopeptides from normal and RNA virus-transformed Balb/3T3 cells cochromatographed on Bio-Gel P-10 columns. Trypsinates were isolated from log phase cells grown 72 hr without a change of medium in the presence of either 1 $\mu$Ci of [$^{14}$C]fucose (Balb/3T3 cells) or 1.5 $\mu$Ci of [$^{3}$H]fucose (K-Balb and W-Balb(R) cells) per ml of medium; the trypsinates were desalted and digested with Pronase as described under Materials and Methods. Labeled glycopeptides were admixed and cochromatographed on a 1.5 × 90 cm column of Bio-Gel P-10 (200–400 mesh) eluted with 0.1 *M* Tris-acetate buffer (pH 9.0) containing 0.1% sodium dodecyl sulfate, 0.1% mercaptoethanol, and 0.01% EDTA. Fractions of 1 ml were collected and analyzed for $^{3}$H dpm (●) and $^{14}$C dpm (○). Scale for each isotope is proportional to the total radioactivity of that isotope recovered from the column. BD, blue dextran 2000 containing fractions; A, B, C, and D, regions where major peaks of glycopeptides were eluted. (a) Profiles of surface glycopeptides from Balb/3T3 and K-Balb cells; (b) profiles of surface glycopeptides from Balb/3T3 and W-Balb(R) cells.

Table III: Glycosyltransferase Activities in Normal and RNA Virus-Transformed Balb/3T3 Cells.[a]

| | nmol per mg of Protein per hr[b] | | |
|---|---|---|---|
| | Cell Line | | |
| Glycosyltransferase | Balb/3T3 | K-Balb (R) | W-Balb (R) |
| *N*-Acetylgalactosaminyltransferase | 2.58 | 3.25 | 3.12 |
| Galactosyltransferase | 0.54 | 0.10 | 0.46 |
| Sialyltransferase | 0.34 | 0.47 | 0.29 |

[a] Enzyme assays were performed on the various cell extracts as described under methods; for galactosyltransferase assays, the 600*g* resuspended pellet was used as the enzyme source; for the other two assays the whole cell lysate was used. The cells were subconfluent and growing. [b] Values represent synthesis of product from exogenous glycolipid acceptor.

various K-Balb clones ranged from less than 2 to 18.5% of that of control Balb/3T3 cells.

*Profiles of Labeled Glycopeptides from the Surface of Normal and RNA Virus-Transformed Mouse Cells.* When fucose-labeled glycopeptides derived from the surface of exponentially growing Balb/3T3 and K-Balb cells were cochromatographed on Bio-Gel P-10, distinct elution profiles were observed (Figure 1a). Glycopeptides from K-Balb cells were highly enriched in earlier eluting material (peak A) whereas Balb/3T3 had slightly more later eluting glycopeptides (peaks C and D). However, the glycopeptides isolated from the surface of growing W-Balb(R) were not enriched in peak A glycopeptides (Figure 1b) and were similar to those of Balb/3T3 except for an increase in later eluting material (peaks C and D). These same results were observed in three separate cell labeling experiments including one in which the isotopes were reversed.

Because the WSV-transformed clone contained R-MuLv, and produced viruses, we isolated surface glucopeptides from K-Balb cells infected with R-MuLV. These glycopeptides cochromatographed identically with glycopeptides from K-Balb (data not shown). When K-Balb(R) and W-Balb(R) derived glycopeptides were cochromatographed on Bio-Gel P-10, the elution profiles were distinct (Figure 2). Glycopeptides from K-Balb(R) were enriched in earlier eluting labeled components compared to W-Balb(R).

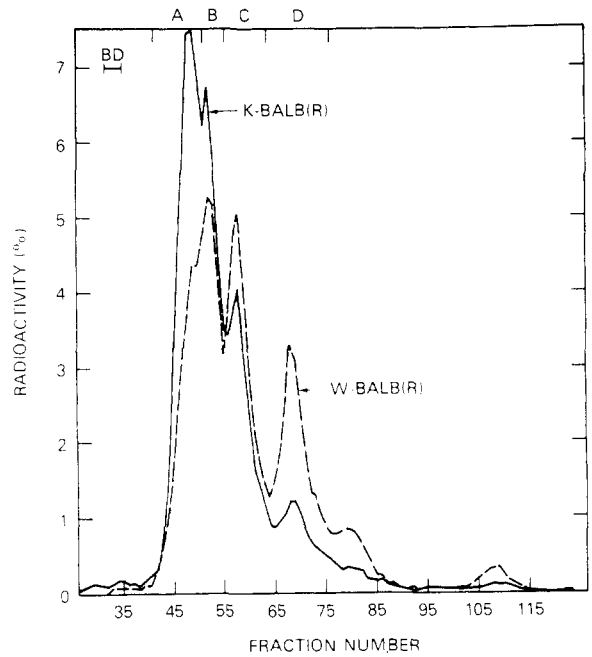


FIGURE 2: Elution profiles of fucose-labeled surface glycopeptides from K-Balb(R) and W-Balb(R) cells. Details are the same as in Figure 1 except log phase K-Balb(R) cells (—) were labeled with [$^{3}$H]fucose and log phase W-Balb(R) cells (- -) with [$^{14}$C]fucose and the Pronase-digested trypsinates were cochromatographed on a 1 × 100 cm column of Bio-Gel P-10. Fractions of 0.5 ml were collected and analyzed. Radioactivity in each fraction is expressed as the percent of the total radioactivity eluted from the column. BD, blue dextran; A, B, C, and D, regions where major glycopeptide peaks were eluted from the column.

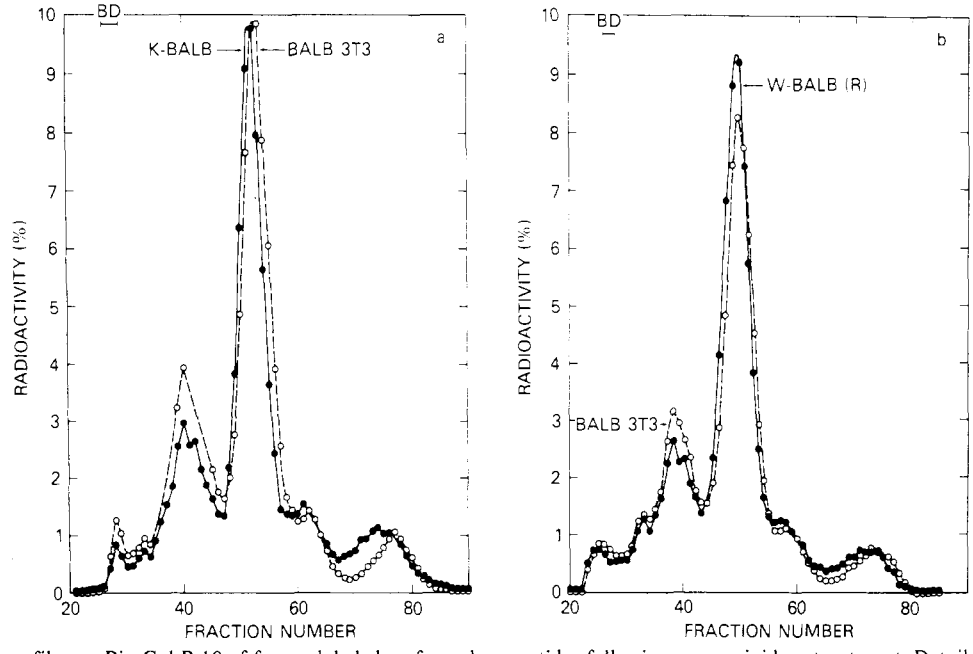


FIGURE 3: Elution profiles on Bio-Gel P-10 of fucose-labeled surface glycopeptides following neuraminidase treatment. Details are the same as in Figure 1 except the Pronase-digested trypsinates from the log phase cells were treated with purified neuraminidase as described under Materials and Methods. Radioactivity in each fraction is expressed as the percent of the total radioactivity recovered from the column. BD, blue dextran containing fractions. (a) Profiles of neuraminidase-treated surface glycopeptides from [$^3$H]fucose-labeled K-Balb (●) and [$^{14}$C]fucose-labeled (○) Balb/3T3 cells. (b) Profiles of neuraminidase-treated surface glycopeptides from [$^3$H]fucose-labeled W-Balb(R) (●) and [$^{14}$C]fucose-labeled Balb/3T3 cells (○).

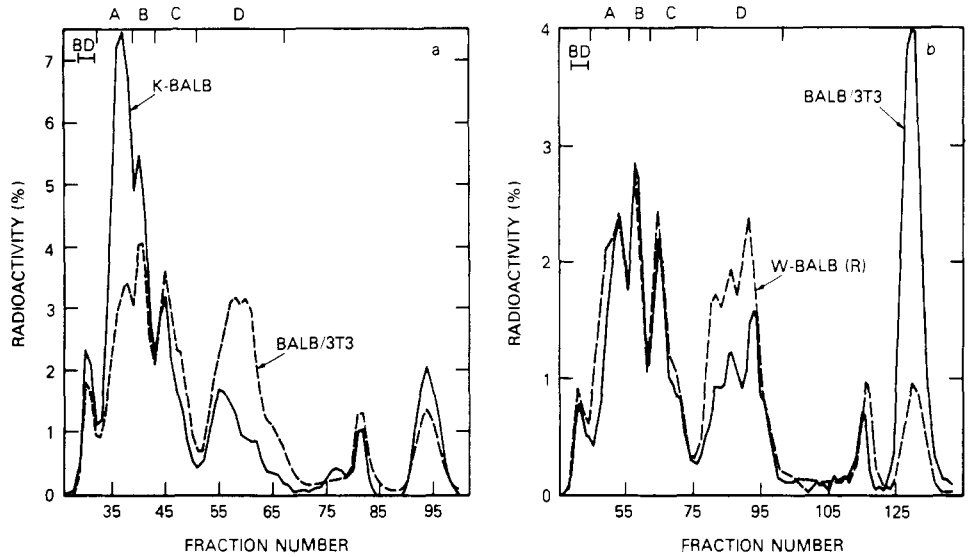


FIGURE 4: Elution profiles of fucose-labeled cellular glycopeptides from normal and RNA virus-transformed Balb/3T3 cells cochromatographed on Bio-Gel P-10 columns. Log phase cells were grown for 72 hr without a change of medium in the presence of [$^{14}$C]fucose (- -) or [$^3$H]fucose (—). After digestion with trypsin, the cells were digested with Pronase as described under Materials and Methods. The cellular glycopeptides were admixed and cochromatographed on 1 × 100 cm columns of Bio-Gel P-10 (200–400 mesh) as described in Figure 2. The radioactivity in each fraction is expressed as the percent of the total radioactivity eluted from the column. BD, blue dextran A, B, C, and D, regions containing major glycopeptide peaks. (a) Profiles of cellular glycopeptides from Balb/3T3 (- -) and K-Balb (—) cells. (b) Profiles of cellular glycopeptides from Balb/3T3 (—) and W-Balb(R) (- -) cells.

*Effect of Neuraminidase on Surface Glycopeptides.* When the surface glycopeptides were treated with neuraminidase and chromatographed on Bio-Gel P-10, the radioactive profiles were simplified and differences between Balb/3T3 and K-Balb were minimized (Figure 3a). The glycopeptide profile of W-Balb(R) was also similar to those of Balb/3T3 and K-Balb (Figure 3b) and the major glycopeptide peak from all three cell lines now chromatographed with a glycopeptide peak observed in the samples prior to neuraminidase digestion (peak C in Figure 1).

*Profiles of Labeled Glycopeptides Derived from Trypsinized Normal and RNA Virus-Transformed Mouse Cells.* Cellular glycopeptides were obtained by Pronase digestion of the trypsinized cells. When cochromatographed on Bio-Gel P-10, glycopeptides from K-Balb cells were enriched in earlier eluting components compared to Balb/3T3 cells (Figure 4a); a similar enrichment was not observed with cellular glycopeptides derived from W-Balb(R) (Figure 4b). There were differences in later eluting material (region D in Figure 4a and b); W-Balb cells were enriched and K-

Table IV: Distribution of Radioactivity in Glycopeptides Derived from Normal and RNA Virus-Transformed Balb/3T3 Cells.[a]

| Cell Line | L-Fucose Isotope | Source of Glycopeptides[b] | % Distribution of dpm in Glycopeptide Region[c] A | B | C | D |
|---|---|---|---|---|---|---|
| Balb/3T3 | $^{14}C$ | Surface | 29.4 | 24.6 | 28.1 | 17.9 |
| K-Balb | $^{3}H$ | Surface | 46.8 | 22.3 | 19.8 | 11.1 |
| K-Balb (R) | $^{3}H$ | Surface | 42.8 | 24.0 | 22.4 | 10.8 |
| W-Balb (R) | $^{3}H$ | Surface | 26.7 | 20.1 | 27.3 | 25.9 |
| Balb/3T3 | $^{14}C$ | Cell | 21.2 | 17.6 | 24.4 | 36.8 |
| K-Balb | $^{3}H$ | Cell | 43.9 | 22.1 | 18.9 | 15.1 |
| W-Balb (R) | $^{14}C$ | Cell | 24.4 | 15.2 | 20.6 | 39.8 |

[a] The elution profiles of fucose-labeled glycopeptides chromatographed on Bio-Gel P-10 columns were divided into regions A through D which represent the major populations of glycopeptides (see Figure 1 for example). The distribution of radioactivity in each of these regions is expressed as the percent of the total dpm eluted in regions A through D. [b] Surface glycopeptides were obtained by Pronase digestion of desalted trypsinates and cell glycopeptides by Pronase digestion of the trypsinized cells as described under Materials and Methods. [c] The radioactivity in dpm recovered in regions A through D represented 89–97% (for surface glycopeptides) and 75–81% (for cellular glycopeptides) of the total radioactivity in dpm eluted from the columns.

Balb cells diminished in these labeled glycopeptides compared to Balb/3T3 cells.

The cellular glycopeptide profiles were more heterogeneous than the surface glycopeptides. There was more material eluting in the void volume of the column (blue dextran containing fractions) which probably represented incompletely digested glycopeptides, and there was more later eluting material (beyond region D) which probably consisted of free fucose or low molecular weight conjugates of fucose. The amounts of these high and low molecular weight components varied from experiment to experiment. The cellular glycopeptides were also altered by neuraminidase digestion in a manner similar to the surface glycopeptides.

*Incorporation of Radioactive Fucose into Surface and Cellular Glycopeptides from the Various Mouse Cell Lines.* The ratio of surface to cellular glycopeptides was relatively constant for the various cell lines and approximately 14–18% of the recovered glycopeptides are derived from the cell surface. Buck et al. (1970) reported that 15–22% of the total incorporated radioactivity was recovered in the trypsinates of fucose-labeled hamster cells. Our data suggest that there is no preferential release of radioactive glycopeptides from the surfaces of the various cell lines. There also appears to be no selective loss of labeled components due to incomplete Pronase digestion. When the Pronase digests were centrifuged at 40000*g*, 92–100% of the radioactivity from the desalted trypsinates and 87–99% of the cellular radioactivity were recovered in the supernatant fractions.

*Distribution of Fucose-Labeled Glycopeptides Derived from Normal and RNA Virus-Transformed Balb/3T3 Cells.* We chose to compare the elution profiles of glycopeptides derived from the various cells by plotting the dpm of each isotope in each fraction as the percent of the total dpm of that isotope eluted from the column. Other ways of displaying the data would be to adjust the scales to correspond to the maximum peak for each isotope (Buck et al., 1970), or to maximize differences between particular peaks (Buck et al., 1974). In order to quantitate changes in the distribution of fucose-labeled glycopeptides derived from the various cell lines, we calculated the distribution of radioactivity in the four regions, A through D, of the elution profiles which contained the major populations of labeled glycopeptides (see Figures 1 and 4 for examples).

There was a significant increase in the proportion of glycopeptides derived from Ki-MSV transformed Balb/3T3 cells eluted in region A compared to normal or WSV-transformed Balb/3T3 cells (Table IV). This was observed both for surface and cellular glycopeptides. There was little variation in the proportion of region B glycopeptides derived from the various cell lines. K-Balb cells contained proportionally less labeled glycopeptides eluting in regions C and D compared to Balb/3T3 and W-Balb(R) cells. Finally, W-Balb(R) cells were enriched in region D glycopeptides compared to Balb/3T3. These four regions accounted for 89–97% of the total surface and for 75–81% of the total cellular radioactive material eluted from the columns. Although this analysis only applies to the distribution of radioactive fucose among the various classes of glycopeptides, the long labeling period (72 hr) should have compensated for any variations in the turnover of different glycopeptides from the same or different cell lines.

## Discussion

Balb/3T3 cells transformed by the Kirsten murine sarcoma virus demonstrate changes in membrane glycoconjugates that have been observed in numerous other transformed cells. These include: (1) a simplification of sialic acid containing glycosphingolipid components due to reduced activity of a specific galactosyltransferase required for the synthesis of gangliosides $G_{M1}$ and $G_{D1a}$ (Fishman et al., 1974; Coleman et al., 1975); (2) an increase in fucose-labeled glycopeptides of apparently higher molecular weight (Buck et al., 1970, 1971; van Beek et al., 1973; Glick et al., 1973, 1974). In the present report, these alterations were shown to occur in several independently isolated Ki-MSV transformed clones (in the case of ganglioside changes, in four independently isolated clones utilizing two different isolation procedures) and were not dependent on or correlated with the presence or absence of leukemia "helper" virus replication.

Neuraminidase digestion simplifies the glycopeptide profiles of both normal and transformed Balb/3T3 cells in agreement with the work of others (Warren et al., 1972; van Beek et al., 1973) and these results suggest that differences between Balb/3T3 and K-Balb glycopeptides are due in part to an increase in sialic acid content. Warren has demonstrated that virus-transformed hamster and chick cells have elevated levels of a specific sialyltransferase activity (Warren et al., 1972) but recently has reported that there are additional differences between the glycopeptides from normal and transformed cells (Warren et al., 1974).

The difference observed in surface glycopeptides from Balb/3T3 and K-Balb could also be found in the glycopeptides derived from the rest of the cell. These results are consistent with previous studies on BHK cells (Keshgegian and Glick, 1973; Buck et al., 1974) and indicate that when changes in these glycoproteins occur in transformed cells, they are not restricted to the cell surface but also occur in the various subcellular organelles. Thus, glycopeptides obtained by Pronase digestion of whole cells can be used to explore differences in glycopeptide composition.

Balb/3T3 cells transformed by the wooley monkey sarcoma virus did not exhibit these distinct changes in glycosphingolipids or glycopeptides although the cells behave in

culture very much like Ki-MSV-transformed cells. They grow to a high saturation density, grow in agar and on confluent fibroblast monolayers. Thus, they were very similar to K-Balb in these properties. The loss of contact or density dependent inhibition of growth and anchorage dependence as measured by these assays are two of the characteristic features of transformed cells and have been shown to correlate well with tumor formation (Aaronson and Todaro, 1968); K-Balb is known to be highly tumorigenic (Stephenson and Aaronson, 1972). W-Balb(R) could not be meaningfully tested for tumorigenicity because such testing is complicated by the presence of Rauscher leukemia virus. In the presence of this helper virus, sarcoma virus is produced, and tumor formation could be due to the spread of virus and not to the growth of the transformed cells. Thus, the relationship of membrane glycoconjugates to the malignant potential of W-Balb cells cannot be stated.[3] However, it can be concluded that the loss of growth control and anchorage dependence in vitro by W-Balb cells was not associated with detectable alterations in these surface components.

It is also possible that even though W-Balb cells contain a normal complement of gangliosides and glycopeptides, their organization in the membrane has been altered. It has been proposed that there is an increase in membrane fluidity in transformed cells (for a recent review see Nicolson, 1974). Thus, normal and transformed cells have similar numbers of concanavalin A binding sites but transformed cells are more readily agglutinated presumably due to increased mobility of the binding sites. Another possibility is that while normal membrane glycoconjugates are present in W-Balb cell, they are no longer functional. Although the function of these membrane components is unclear, a model has been proposed that gangliosides act as membrane receptors for effector molecules that invoke a cellular response through cyclic nucleotides (Fishman, 1974). This hypothesis is based on observations on the action of cholera toxin on cultured mouse cells (Hollenberg et al., 1974). Cholera toxin inhibits DNA synthesis in these cells by binding to ganglioside $G_{M1}$ on the cell membrane and activating membrane adenylyl cyclase. The response of the various mouse cell lines to cholera toxin depended on their $G_{M1}$ content. K-Balb cells in which $G_{M1}$ cannot be detected are very insensitive to cholera toxin.[4] W-Balb cells which contain $G_{M1}$ receptors may be defective in cyclic nucleotide metabolism as are some transformed cells (Pastan and Johnson, 1974) or in some other component required for a transmembrane information transmission system.

Our observations on W-Balb cells suggest that the loss of normal cell properties such as contact inhibition and anchorage dependence cannot be explained solely on the basis of changes in the chemical composition of these membrane glycoconjugates. Hypotheses involving a purely physical interaction between these membrane components and their associated membrane-bound glycosyltransferase activities would appear to be less tenable (Roseman, 1970; Chipowsky et al., 1973). It is more probable that these membrane glycoconjugates serve as specific receptor sites for external signals (as suggested above) or serve as internal switches for coordinating cell division and DNA synthesis during the cell cycle (Stoker, 1974). Because of the substantial body of evidence supporting some role for membrane glycoconjugates in controlling cell growth and behavior, these possibilities must be explored before such a role is dismissed. Primate sarcoma virus-transformed cells may be a valuable tool in such studies and prove useful in determining the function of membrane glycoconjugates.

[3] Glick et al. (1973) observed that chemically transformed hamster cells had surface glycopeptide profiles similar to hamster embryo secondary cells whereas tumor cells isolated from tumors formed from the transformed cells did contain increased amounts of peak A glycopeptides. They concluded that changes in surface glycopeptides were correlated with tumorigenesis and not in vitro transformation.

[4] M. D. Hollenberg, personal communication.

Acknowledgments

The excellent technical assistance of Mr. Roy M. Bradley is gratefully acknowledged. The authors thank Dr. Gilbert Ashwell, NIAMDD, for use of the neuraminidase affinity column.

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# ATPase of *Escherichia coli:* Purification, Dissociation, and Reconstitution of the Active Complex from the Isolated Subunits†

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ABSTRACT: A simple procedure for the purification of $Mg^{2+}$-stimulated ATPase of *Escherichia coli* by fractionation with poly(ethylene glycols) and gel filtration is described. The enzyme restores ATPase-linked reactions to membrane preparations lacking these activities. Five different polypeptides ($\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$) are observed in sodium dodecyl sulfate electrophoresis. Freezing in salt solutions splits the enzyme complex into subunits which do not possess any catalytic activity. The presence of different subunits is confirmed by electrophoretic and immunological methods. The active enzyme complex can be reconstituted by decreasing the ionic strength in the dissociated sample. Temperature, pH, protein concentration, and the presence of substrate are each important determinants of the rate and extent of reconstitution. The dissociated enzyme has been separated by ion-exchange chromatography into two major fragments. Fragment $I_A$ has a molecular weight of about 100000 and contains the $\alpha$, $\gamma$, and $\epsilon$ polypeptides. The minor fragment, $I_B$, has about the same molecular weight but contains, besides $\alpha$, $\gamma$, and $\epsilon$, the $\delta$ polypeptide. Fragment II, with a molecular weight of about 52000, appears to be identical with the $\beta$ polypeptide. ATPase activity can be reconstituted from fragments $I_A$ and II, whereas the capacity of the ATPase to drive energy-dependent processes in depleted membrane vesicles is only restored after incubation of these two fractions with fraction $I_B$, which contains the $\delta$ subunit.

The membrane-associated $Mg^{2+}$-stimulated adenosinetriphosphatases from mitochondria, chloroplasts, and bacteria appear to function in energy transduction reactions. Several extensive reviews have appeared (see, e.g., Senior, 1973; Beechey and Cattell, 1973; Baltscheffsky and Baltscheffsky, 1974; Racker, 1970; Abrams and Smith, 1974). The available information about these enzymes shows a remarkable similarity with respect to catalytic mechanism, molecular weight, and overall subunit composition. The enzyme complexes exist as multimeric proteins with molecular weights of 300000–400000 and contain five different polypeptides.

The ATPase (EC 3.6.1.3) of *Escherichia coli* has been purified by several workers. Two preparations yielded an enzyme which appeared to contain five polypeptide chains ($\alpha$–$\epsilon$) by sodium dodecyl sulfate electrophoresis, ranging in molecular weight from about 60000 to about 10000 (Bragg and Hou, 1972; Futai et al., 1974). These enzyme preparations were active as "coupling factor" in reconstituting energy-linked reactions on incubation with ATPase-depleted membranes. Several other purification procedures yielded an enzyme containing only four polypeptides, the $\delta$ subunit being the missing component. These preparations were not capable of restoring energy coupling (Evans, 1970; Kobayashi and Anraku, 1972, 1974; Hanson and Kennedy, 1973; Nelson et al., 1974). Direct evidence for the involvement of the $\delta$ subunit in the attachment of the ATPase to the membrane was obtained recently (Smith and Sternweis, 1975). These authors showed that the coupling factor capacity of the four-subunit enzyme could be restored by addition of a fraction containing mainly the $\delta$ and $\epsilon$ polypeptide chains.

The stoichiometry of the polypeptide chains in the ATPases from beef heart (Senior and Brooks, 1971) and rat liver (Catterall et al., 1973) was suggested to be $\alpha_3\beta_3\gamma\delta\epsilon$, based on staining intensities after dodecyl sulfate electro-

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