Structural Differences in Envelope Glycoproteins Associated with Rat Leukaemia Virus Produced by Novikoff Hepatocellular Carcinoma and Spontaneously Transformed Wistar Rat Embryo Cells

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SUMMARY

Immunochemical and immunocytochemical techniques have been used to characterize viral glycoproteins and endogenous rat leukaemia viruses (RaLV) produced both by Novikoff hepatocellular carcinoma cells and spontaneously transformed Wistar rat embryo cells (WRC). Results from immunocytochemical analysis demonstrated that RaLV produced by Novikoff and WRC cells could be distinguished by their unique patterns of reactivity with xenoantisera raised against virus particles or viral glycoproteins. This differential labelling was unexpected since all the antisera tested had been shown by immunoprecipitation and immunodepletion analysis to be reactive with viral glycoproteins expressed on the cell surface. Since no significant differences in cell surface-associated viral glycoproteins and those shed from the cell surface were detected by pulse iodination analysis, it was concluded that the apparent discrepancy between immunoferritin labelling and immunoprecipitation analysis resulted from differences in antigen accessibility on intact virions caused by structural differences in the viral glycoproteins expressed on Novikoff and WRC cells. This conclusion was supported by results from ferritin–lectin labelling, affinity chromatography and neuraminidase digestion studies which demonstrated differences in the saccharide moieties on both virion and cell surface-associated viral glycoproteins. Further evidence of structural differences was provided by limited digestion with trypsin and V8 protease of the \( M_r 64,000 \) (Nov gp64) and \( M_r 68,000 \) (WRC gp68) viral glycoproteins immunoprecipitated from Novikoff and WRC cells, respectively, with either monospecific anti-Rauscher murine leukaemia virus anti-gp70 serum or monospecific antiserum against Nov gp64 (anti-gp64). Results from digestion studies showed that all the major cleavage fragments from WRC gp68 were of higher molecular weight than their Nov gp64-derived counterparts. Evidence that Nov gp64 and WRC gp68 both share structural homology with other murine viral gp70s was suggested by results from immunoprecipitation analysis with anti-gp70 and anti-gp64 sera under reducing and non-reducing conditions which demonstrated the presence of an interchain disulphide bond in both glycoproteins and showed that at least some of these molecules exist on the cell surface as disulphide-linked heterodimers of \( M_r 78,000 \) and \( 82,000 \).

INTRODUCTION

In the mouse, endogenous C-type retroviruses have been shown to be a polymorphic group of viruses that exhibit considerable diversity in host range, interference pattern and antigenicity (Pincus, 1980). Mouse leukaemia viruses can be subdivided on the basis of host range into three major groups: ecotropic viruses which preferentially replicate in mouse cells; xenotropic viruses which preferentially replicate in cells from species other than mice; dualtropic murine leukaemia viruses which can replicate in either mice or heterologous species (Pincus, 1980). Ecotropic and xenotropic mouse leukaemia viruses can also be distinguished from each other by...
structural and antigenic differences in their major envelope glycoproteins (Famulari & Jalalian, 1979; O'Donnell et al., 1980). Dualtropic viruses appear to arise as a result of genetic recombination between endogenous ecotropic and non-endogenous xenotropic leukaemia viruses (Hartley et al., 1977; Lung et al., 1980), a recombination which gives rise to viruses displaying enhanced leukaemogenicity (Cloyd et al., 1980; Rowe et al., 1980) and expressing novel envelope glycoprotein with unique structural and antigenic properties (Elder et al., 1977; Niman & Elder, 1980; Walff et al., 1982; O'Donnell & Nowinski, 1980; Schultz et al., 1983).

In contrast to those of the mouse, relatively little is known about the antigenic polymorphism of endogenous C-type retroviruses (rat leukaemia viruses, RaLV) in the rat. Indeed, in previous studies, no type-specific antigenic differences were detected on viral structural proteins as measured by immunofluorescence or neutralization kinetics (Bergs et al., 1972; Pearson et al., 1972). In the present report, we examined using immunochemical and immunocytochemical techniques (i) the structure and the surface properties of RaLV produced by Novikoff hepatocellular carcinoma cells and spontaneously transformed Wistar rat embryo cells (WRC) and (ii) the immunochromatographic properties of cell surface antigens related to the major envelope glycoproteins of these RaLV. Our results indicate that these viruses show unique patterns of reactivity with a battery of heteroantisera whose reactivity with rat viral envelope glycoproteins has been confirmed by immunoprecipitation and immunodepletion analysis. Novikoff and WRC RaLV can also be distinguished by differences in their reactivity with ferritin-conjugated plant lectins, differences that, in some cases, appear to be related to the degree of sialylation of the envelope glycoproteins. Results from immunochemical analysis further demonstrate that RaLV-associated cell surface antigens on Novikoff and WRC cells are sialoglycoproteins that, although closely related antigenically, show distinct differences in size and structure.

METHODS

Tumour cells. Novikoff (1957) hepatocellular carcinoma cells were maintained in ascitic form by intraperitoneal transplantation into 6- to 9-week-old female Sprague-Dawley rats (Harlan Sprague/Dawley, Madison, Wis., U.S.A.) at weekly intervals. WRC cells were obtained from Dr S. Kit, Baylor College of Medicine, Houston, Tx., U.S.A. These cells were grown in Dulbecco's modified Eagle's minimal essential medium, supplemented with 10% heat-inactivated foetal bovine serum (Reheis), and 50 μg gentamicin (Shering Corp.)/ml. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and were subcultured every 7 to 10 days using 0.25% trypsin (Gibco). WRC cells, spontaneously transformed after approximately 100 passages in vitro, were shown by electron microscopic examination to be producing type C virus particles (Chan et al., 1977). C6XL lymphoma cells producing ecotropic murine leukaemia virus (MuLV) were maintained in C57BL/6 mice as previously described (Allison et al., 1982).

V-16 and SD-1 cell cultures, maintained in Dulbecco's modified Eagle's minimal essential medium supplemented as described above, were subcultured at weekly intervals. V-16 cells, which produce large quantities of Rauscher MuLV, were obtained from Dr R. B. Arlinghaus (University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston). The SD-1 embryo culture, a cell line which produces endogenous ecotropic RaLV, was obtained from Dr S. Rasheed (Department of Pathology, University of Southern California Medical School, Los Angeles, Ca., U.S.A.). AKR 225J cells producing MuLV were obtained from Dr E. Richie, Science Park–Research Division. Normal hepatocytes were isolated by collagenase perfusion as previously described (Hixson et al., 1983). 252 hepatocellular carcinoma cells (252), obtained from Dr F. Becker at M.D. Anderson Hospital and Tumor Institute, were maintained in 6- to 9-week-old ACI rats by intraperitoneal transplantation.

Radioactive labelling of cells. Novikoff cells (5 x 10⁶) collected from ascites by centrifugation at 125 g, and WRC cells (2 x 10⁵ to 5 x 10⁵) collected from subconfluent monolayers suspended in PBS (140 mM-NaCl, 4 mM-KCl, 2 mM-K₂HPO₄, pH 7.4), containing 0.2% EDTA, were washed twice in PBS containing 1 μM-KI and resuspended in 0.5 ml of the latter buffer. Cells were then surface-labelled with 125I (Amersham, carrier-free) by a previously described modification (Hixson et al., 1983) of the method of Keski-Oja et al. (1977) and extracted in 0.5% Nonidet P40 (NP40) (Particle Data Inc.) as previously described (Hixson et al., 1983). When extracts were to be analysed under non-reducing conditions, cells were extracted in lysis buffer containing 10 mM-iodoacetamide.

Cell extracts were clarified at 10000g for 20 min and stored at -20 °C.

For pulse iodination studies, 125I-labelled cells were resuspended in L-15 medium (Gibco) supplemented with 1.5 mg D-glucose/ml, 15 μg gentamicin/ml, and 25 mM-HEPES, and incubated at 37 °C for 4 h. Cells were then removed by centrifugation at 70 g for 5 min, and the supernatant culture fluid, after being made 0.5% in NP40, was clarified at 10000 g for 10 min and stored at -20 °C.
Immunoprecipitation. Immunoprecipitation was accomplished by incubating aliquots of detergent lysates from radioiodinated cells or virus for 16 h at 4 °C with 5 to 10 μl of antiserum. Immune complexes were collected by adsorption to heat-killed, formalin-fixed Staphylococcus aureus Cowan I (SACI) (10% suspension) (New England Enzyme Center, Boston, Mass, U.S.A.) for 2 h at 4 °C. The SACI were then washed in a buffer containing 10 mM-Tris–HCl, 150 mM-NaCl, 0.5% NP40, 1 mg ovalbumin/ml, pH 7-4. Immune complexes were subsequently eluted either by suspending the washed SACI for 1 h in 9-5 M-urea containing 2% NP40, 2% ampholytes (1:2:2 mixture of pH 3 to 10, 5 to 7, and 3 to 5 LKB Ampholines), and 5% 2-mercaptoethanol (sample buffer for two-dimensional gel analysis), or by heating at 100 °C for 5 min in a buffer containing 63 mM-Tris–HCl, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, pH 6-8 (sample buffer for one-dimensional gel analysis).

Immunodepletion. To compare the reactivity of two different antisera that show reactivity with components of similar apparent molecular weights, aliquots of 125I-labelled Novikoff or WRC extracts were sequentially immunoprecipitated with the first antiserum until SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis indicated that all reactive components had been removed. The immunodepleted extracts were then immunoprecipitated with the second antiserum and the immunoprecipitates analysed by SDS–PAGE. If the second antiserum showed no reactivity with the depleted extract this was interpreted as evidence that the first antiserum was recognizing all components reactive with the second. If, on the other hand, the second antiserum still precipitated components from the depleted extract, this was taken as evidence that the two antisera were recognizing different components with similar molecular weights.

Immune sera. Rabbit anti-Novikoff RaLV serum was obtained from rabbits that had been immunized with virus collected from ascites fluid by centrifugation. Ascitic fluid was clarified at 500 g for 15 min followed by centrifugation at 10000 g for 20 min. A pellet containing Novikoff virus was obtained by centrifugation for 1 h at 100000 g. This pellet was resuspended in 0-5 ml of bacteriostatic saline, emulsified in complete Freund’s adjuvant, and injected intramuscularly at 2-week intervals. Serum was collected 10 days after each injection. The immunoglobulin-containing fraction from the second bleeding was precipitated from immune serum by 30% ammonium sulphate and re-dissolved in PBS and stored at -20 °C.

Antiserum reactive with the envelope glycoprotein of Nov RaLV was produced by immunizing a rabbit with component(s) immunoprecipitated by goat anti-Rauscher MuLV gp70 (anti-gp70) serum from an NP40 extract of Novikoff cells. Extract from 2.5 x 10^6 cells was incubated for 16 h with 20 μl of anti-gp70 serum. Immune complexes were then collected by adsorption to 400 μl of SACI (10% suspension). The SACI were washed extensively in lysis buffer to remove non-specifically bound material, followed by several washes in PBS to remove NP40. The SACI were subsequently suspended in 1 ml of sterile PBS and injected intramuscularly. Booster injections were given at 2-week intervals, and serum was collected 10 days after each injection. This antiserum was designated anti-gp64.

Goat anti-Rauscher MuLV gp70 serum and goat antiserum against RaLV from Sprague-Dawley, Wistar/Furth and Osborne/Mendel strain rats were obtained through the Viral Oncology Program Office of Resources and Logistics, National Cancer Institute, Bethesda, Md., U.S.A. For the purposes of this study, these antisera were designated as anti-gp70, anti-RaLV S/D, anti-RaLV W/F and anti-RaLV O/M, respectively.

Protease digestions. For trypsin digestion of detergent-solubilized components, samples (30 to 50 μl) of NP40 extracts from radioiodinated cells were incubated at 37 °C for 2, 5 or 10 min with 2 or 5 μg of TPCK-trypsin (Worthington). Digestion was stopped by the addition of 100 KIU of aprotinin and antiserum (5 μl) was added. After incubation at 4 °C for 18 h, immune complexes were collected by adsorption to SACI as described above for immunoprecipitation. For trypsin digestion of antibody-bound envelope glycoproteins, immune complexes collected on 100 μl of 10% SACI were incubated at 37 °C for 2, 5 or 10 min with 5 μg trypsin. The reaction was terminated by adding an appropriate volume of fivefold concentrated SDS sample buffer and boiling for 5 min.

Digestion with S. aureus V8 protease was performed essentially as described by Cleveland et al. (1977). Briefly, components immunoprecipitated from radioiodinated extracts with anti-gp70 serum were submitted to SDS–PAGE on 7.5% gels as described below. After a brief wash in methanol:acetic acid:water (46:8:46), gels were wrapped in polyethylene and placed on sheets of Kodak XR-I X-ray film. After a 2 to 4 h exposure at -70 °C the X-ray film was developed and the resulting autoradiogram used to localize areas in the gel corresponding to viral glycoproteins. These areas were subsequently cut from the gel, inserted into sample wells of a 12.5% slab gel and digested with 0.025 or 0.25 μg of V8 protease as described by Cleveland et al. (1977).

Polyacrylamide gel electrophoresis. Viral proteins immunoprecipitated by anti-gp70 or anti-gp64 serum were analysed as previously described (Hixson et al., 1983) by either one-dimensional SDS–PAGE according to the procedure of Laemmli (1970) on 7.5 and 10% slab gels or two-dimensional electrophoresis as described by O’Farrell (1975). Both one- and two-dimensional gels were stained overnight in 0.2% Coomassie Brilliant Blue R-250, destained for 7 or 8 h and rehydrated as previously described (Glenney et al., 1979). Gels were then dried under vacuum and visualized by autoradiography on Kodak XR-1 X-ray film at -70 °C on DuPont Cronex Lightning Plus Intensifying screens. Relative mobilities were determined from molecular weight standards [β-galactosidase (M, 116000), bovine serum albumin (M, 68000), ovalbumin (M, 43000) and carbonic anhydrase (M, 29000)] run concurrently with radiolabelled samples.
Affinity chromatography. Lectin affinity chromatography was performed on Sepharose-bound *Ricinus communis* agglutinin I (RCAI). RCAI was prepared by a previously described procedure (Hixson et al., 1979). The specific activity of the purified RCAI, as determined by the haemagglutination assay of Smith et al. (1973), was 40000 haemagglutinating units/mg. RCAI was coupled to Sepharose 4B using the method of Cuatrecasas (1970). Coupling was performed in the presence of 0-2 m-lactose.

Isolation of tryptic peptides by lectin affinity chromatography was accomplished by suspending 20 to 50 μl of Sepharose-bound RCAI for 1 h at 4 °C in 50 μl of PBS containing 0.5% NP40 and 2 × 10^5 to 5 × 10^5 cts/min of radioiodinated cell extract (10^5 to 2.5 × 10^6 cell equivalents) that had been previously digested with trypsin. After extensive washing in lysis buffer, bound components were released by suspending the Sepharose-bound RCAI for 30 min at 4 °C in 100 μl of lysis buffer containing 0-2 m-lactose. Viral glycopeptides were subsequently isolated by immunoprecipitation with anti-gp64 or anti-gp70 antisemur.

Neuraminidase digestion. Neuraminidase digestion of NP40-solubilized components from radioiodinated Novikoff cells was accomplished by incubating detergent extracts from 3 × 10^6 cells for 1 h at 37 °C in 200 μl of lysis buffer containing *Vibrio cholerae* neuraminidase (Behring Diagnostics) at a concentration of 10 or 25 U/ml. Aprotinin was added (20 KIU/ml) prior to enzyme digestions to minimize protease activity during incubation. Digestion was terminated by the addition of 25 μl of 0-2 m-EDTA and 100 μl of a 20% suspension of SACI to the reaction mixture.

Neuraminidase digestion of Novikoff cells with *V. cholerae* neuraminidase was performed essentially as described by Neri et al. (1976). Briefly, cells were washed three times in PBS pH 7-0, and incubated for 10 min at 37 °C prior to enzyme digestion. Cells were then digested at 37 °C in *V. cholerae* neuraminidase or heat-activated enzyme (1 h at 65 °C) at a concentration of 12 U/ml. After incubation for 1 h with shaking, the cell suspension was cooled to 4 °C and cells were washed three times in PBS pH 7-4. Viability following enzyme digestion was greater than 80% as measured by exclusion of trypan blue.

Immunoferritin and ferritin–lectin labelling. Conjugates intended for ferritin–lectin (Fer–lectin) labelling studies were isolated, purified, and conjugated to ferritin by previously described methods (Hixson et al., 1979). Labelling of cells by ferritin linked to concanavalin A, wheat germ agglutinin and *R. communis* agglutinin II (Fer–ConA, Fer–WGA and Fer–RCAI, respectively) was accomplished as previously described (Hixson et al., 1979). Labelling with Fer–RCAI was performed by layering 1 × 10^6 cells suspended in 50 μl PBS onto 50 μl Fer–RCAI solution containing 5% (w/v) sucrose. Cells were allowed to settle under gravity into the Fer–RCAI solution for 15 min. The upper layer of PBS was then removed and the cells allowed to incubate for an additional 5 min. Following several washes in PBS, labelled cells were fixed in 1% acrolein and 3% glutaraldehyde, followed by 2% osmium tetroxide, and processed by standard methods for electron microscopy. Silver sections, stained with uranyl acetate and lead citrate, were examined in an RCA EMU 3E or Philips 201C electron microscope.

For immunoferritin labelling, cells (2 × 10^6) were pre-fixed for 5 min in 0-5% acrolein, washed once in 0-1 m-glycine in PBS, then twice in PBS, and incubated for 45 min at 23 °C in 100 μl of appropriately diluted antiserum. Cells were then washed twice in PBS, once in PBS containing 0-5 mg apoferritin/ml and either 1% normal rabbit serum or 1% normal goat serum. After a final wash in PBS, cells were incubated for 45 min in 50 μl of ferritin-conjugated rabbit anti-goat immunoglobulin (Cappel Laboratories, Cochraneville, Pa., U.S.A.) or ferritin-conjugated goat anti-rabbit immunoglobulin (Cappel). Cells were subsequently washed in PBS, fixed in 3% glutaraldehyde, and processed for electron microscopy as previously described (Hixson et al., 1979).

Dot hybridization analysis. Dot blots were performed as described by Thomas (1980) on nitrocellulose membranes. High molecular weight DNA, purified as described by Nairn et al. (1982), was sheared to uniform size by four passes through a 25-gauge needle and adjusted to have an absorbance at 260 nm of 5-0. Ten μl samples of successive dilutions of 1:5 of each DNA sample were applied to the nitrocellulose membrane. After air-drying and baking for 2 h at 80 °C, the membrane was processed as described by Wahl et al. (1979) and probed with ^32P-labelled nick-translated plasmid p1700, containing the 3' half of the *env* region of a molecular clone of ecotropic MuLV (Chattopadhyay et al., 1982).

RESULTS

Reactivity of anti-gp64 and anti-gp70

Cell surface antigens related to the viral envelope glycoproteins were isolated from extracts of ^125I-labelled cells by immunoprecipitation with anti-gp64 or anti-gp70 serum and analysed by SDS–PAGE. As shown in Fig. 1, both antisera immunoprecipitated a single major component of 64000 Mr from detergent lysates of ^125I-labelled Novikoff cells. To determine whether both antisera were reacting with the same 64000 Mr component, immunoprecipitations were carried out on extracts that had been immunodepleted by sequential immunoprecipitation with either anti-gp70 or anti-gp64 serum. In Fig. 1 it can be seen that immunodepletion of extracts with one...
Fig. 1. Reactivity of anti-gp64 and anti-gp70 sera. $^{125}$I-labelled surface components were isolated by immunoprecipitation and analysed by SDS–PAGE. (a) Lanes 1 and 2, and lanes 4 and 5, components isolated from Novikoff cell extracts by the first or third sequential immunoprecipitations with anti-gp70 or anti-gp64, respectively; lanes 3 and 6, anti-gp64 or anti-gp70 immunoprecipitates from anti-gp70 or anti-gp64 depleted extracts. (b) Lanes 1 and 2, and lanes 4 and 5, components isolated from WRC extracts by the first or third sequential immunoprecipitations with anti-gp70 or anti-gp64, respectively; lanes 3 and 6, anti-gp64 or anti-gp70 immunoprecipitates from anti-gp70 or anti-gp64 depleted extracts. (c) Components immunoprecipitated from extracts of SD-1 cells with anti-gp70 (lane 1) or anti-gp64 (lane 2). (d) Components immunoprecipitated with anti-gp70 (lane 1) or anti-gp64 (lane 2) from extracts of V-16 cells.

antiserum could remove all components reactive with the other, thus demonstrating that anti-gp64 and anti-gp70 sera are indeed reactive with the same protein. When immunoprecipitation analysis was performed on extracts of $^{125}$I-labelled WRC cells, a protein of 68 000 $M_r$ was the only component precipitated by anti-gp64 and anti-gp70 sera (Fig. 1). Results from immunodepletion analysis again confirmed that both antisera were reacting with the same 68 000 $M_r$ component (Fig. 1). These virus-associated cell-surface components from Novikoff and WRC cells will henceforth be designated as Nov gp64 and WRC gp68, respectively.

When tested by immunoprecipitation for reactivity with SD-1 cells, both anti-gp70 and anti-gp64 showed strong reactivity with a single component of 68 000 $M_r$ (Fig. 1). Neither antiserum showed any reactivity with detergent lysates of $^{125}$I-labelled normal rat hepatocytes, cultured rat embryo cells (passage 10) or 252, a non-producing transplantable hepatocellular carcinoma line (data not shown). In addition, anti-gp64 showed no reactivity with MuLV envelope glycoproteins which were readily immunoprecipitated by anti-gp70 from detergent extracts of $^{125}$I-labelled V-16 cells (Fig. 1) and C6XL lymphoma cells (data not shown).

Characterization of antisera by radioimmunoprecipitation analysis

The reactivity of antisera raised against various strains of RaLV was characterized by radioimmunoprecipitation analysis of $^{125}$I-labelled cell extracts. SDS–PAGE profiles of components immunoprecipitated by these antisera from Novikoff and WRC extracts are shown in Fig. 2. It can be seen that three major groups of components with $M_r$ 64 000 or 68 000 (gp64 or gp68), 80 000 (p80) and 116 000 (p116) were immunoprecipitated by these antisera from each of the three different $^{125}$I-labelled cell extracts. Immunodepletion analysis was performed on each of the $^{125}$I-labelled cell extracts to determine the antigenic relationships of these various components to the 64 000 and 68 000 $M_r$ components immunoprecipitated by anti-gp70 and anti-gp64 sera. Results from immunodepletion analysis of Novikoff (Table 1) and WRC (Table 2) cell extracts indicated that each of the antisera tested was reacting with the same $M_r$ 64 000 or 68 000 component immunoprecipitated by anti-gp70 and anti-gp64 sera. The results also indicated that, with the exception of anti-RaLV O/M serum, each of the anti-RaLV sera was recognizing the same $M_r$ 80 000 and 116 000 components. These higher molecular weight components, however, were apparently unrelated antigenically to gp64 or gp68 since they were not removed by sequential immunoprecipitation with either anti-gp64 or anti-gp70 serum but
Fig. 2. Reactivity of anti-RaLV sera. (a) Components immunoprecipitated from Novikoff extracts with anti-Novikoff RaLV (lane 1), anti-gp70 (lane 2), anti-RaLV O/M (lane 3) or anti-RaLV W/F (lane 4) serum. (b) Components immunoprecipitated from WRC extracts with anti-gp70 (lane 1), anti-RaLV W/F (lane 2), anti-Novikoff RaLV (lane 3) or anti-RaLV O/M (lane 4) serum.

Table 1. Immunodepletion analysis of Novikoff cell extracts*

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<tr>
<td>gp64 p80 p116</td>
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<tr>
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* See Table 1 footnote.

Table 2. Immunodepletion analysis of WRC cell extracts*

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* See Table 1 footnote.

were immunoprecipitated from detergent lysates of $^{125}$I-labelled hepatocytes and non-producer hepatocellular carcinoma cells by the various anti-RaLV sera (data not shown). When tested for reactivity with NP40 extracts of $^{125}$I-labelled V-16 cells, the three anti-RaLV sera showed little or no reactivity with components which comigrated with gp70 immunoprecipitated by anti-gp70 serum (data not shown).

Immunoferritin labelling studies

As shown in Fig. 3 and Table 3, WRC cells were labelled by all five of the antisera tested. Novikoff-associated virus particles, however, were only labelled by homologous serum raised
RaLV glycoprotein differences

Fig. 3. Immunoferritin labelling of RaLV. Panels (a) to (e) and (f) to (j) show Novikoff and WRC virus particles, respectively, labelled by the indirect immunoferritin labelling technique with anti-Novikoff RaLV (a, b, f), anti-RaLV W/F (c, g), anti-gp70 (d, h) or anti-gp64 (e, i).

Table 3. Immunoferritin labelling of RaLV and MuLV

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Anti-Novikoff RaLV</th>
<th>Anti-RaLV O/M</th>
<th>Anti-RaLV W/F</th>
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<td>&lt; 10</td>
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<td>ND</td>
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* Approximate percentage of labelled virions, based on observations made on at least 30 virus particles.
† ND, Not determined.

against either Novikoff virus particles or Nov gp64 isolated from cell extracts. When immunoferritin labelling was repeated on unfixed Novikoff virus particles, identical results were obtained even when viruses were treated with undiluted antiserum. Results in Table 3 also demonstrate that RaLV produced by SD-1 cells are labelled by both anti-gp64 and anti-gp70 sera while MuLV produced by AKR 225J mouse lymphoma cells and V-16 cells are only labelled by anti-gp70 and show no reactivity with anti-gp64 serum.

Ferritin–lectin labelling

Results shown in Fig. 4 and Table 4 show that Novikoff-associated virus particles were poorly labelled by ConA, RCA₁ or RCA₁₇ but were consistently labelled (50%) by WGA. Virus particles associated with WRC cells were poorly labelled by ConA and RCA₁. However, unlike Novikoff virus particles, particles associated with WRC cells were strongly labelled by RCA₁₇, but only poorly by WGA (Fig. 4, Table 4). Since we have shown in previous studies that terminal sialic acid residues can block the binding of RCA₁ to galactose on membrane-associated, but not detergent-solubilized, cell-surface glycoproteins (Glenney et al., 1979), it seemed likely that sialic acid would also be involved in determining the binding of Fer–RCA₁ and Fer–RCA₁₇ to Novikoff RaLV. This possibility was confirmed by results shown in Table 5 in which it can be seen that digestion with 10 units of neuraminidase decreased labelling by WGA, a lectin which binds to both N-acetyl-d-glucosamine and sialic acid residues (Bhavanandan & Katlic, 1979), but greatly increased labelling by both RCA₁ and RCA₁₇. With the exception of Fer–RCA₁₇ on Novikoff cells, surface labelling of Novikoff and WRC cells was apparent with all the Fer-
Fig. 4. Ferritin–lectin labelling of cell-associated RaLV. (a to e) Novikoff virus labelled with Fer-ConA (a), Fer-WGA (b, c), Fer-RCAII (d) or Fer-RCAI (e). (f to i) WRC virus labelled with Fer-ConA (f), Fer-WGA (g), Fer-RCAII (h) or Fer-RCAI (i).

<table>
<thead>
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<th>WRC</th>
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* Labelling was classified as follows: ++ if >75% of the particles examined were either unlabelled or sparsely labelled; + if 50 to 80% of the particles examined were uniformly labelled; ++ if >80% of the particles examined were uniformly labelled.

lectins. This was not unexpected since the plasma membranes of many cells contain glycoproteins capable of binding Fer–lectins (Hixson et al., 1979).

**Pulse iodination analysis**

To characterize further the surface antigens expressed by RaLV associated with Novikoff and WRC cells, we initially attempted to analyse, using immunocytochemical techniques, detergent extracts of virus purified from ascites or tissue culture fluids. However, attempts at purification were hampered by the low virus production and the correspondingly high cell densities that were
RaLV glycoprotein differences

Fig. 5. Pulse iodination analysis. (a, d) Two-dimensional gel analysis of anti-gp70 reactive components released from $5 \times 10^6$ $^{125}$I-labelled WRC (a) or $2 \times 10^7$ Novikoff (d) cells during a 4 h post-labelling incubation. (b, c) Two-dimensional gel analysis of components immunoprecipitated from NP40 extracts ($5 \times 10^6$ WRC and $2 \times 10^6$ Novikoff cell equivalents) of $^{125}$I-labelled WRC (b) or Novikoff (c) cells.

Fig. 6. Resolution of components reactive with anti-gp70 or anti-gp64 by SDS-PAGE under non-reducing and reducing conditions. $^{125}$I-labelled cells were extracted in the presence of iodoacetamide. Components reactive with anti-gp70 or anti-gp64 were then isolated by immunoprecipitation and analysed by SDS-PAGE in the presence (lanes 5 to 8) or absence (lanes 1 to 4) of 2-mercaptoethanol. Lanes 1 and 5, anti-gp64 immunoprecipitates and lanes 3 and 7, anti-gp70 immunoprecipitates from WRC extracts; lanes 2 and 6, anti-gp64 immunoprecipitates and lanes 4 and 8, anti-gp70 immunoprecipitates from Novikoff extracts.

Table 5. Labelling of Novikoff virus by ferritin-conjugated lectins*

<table>
<thead>
<tr>
<th>Ferritin–lectin conjugate</th>
<th>WGA</th>
<th>RCA$_{II}$</th>
<th>RCA$_{I}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control digest</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Neuraminidase digest</td>
<td>−15</td>
<td>−38</td>
<td>−20</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>42</td>
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<td></td>
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<td></td>
<td>22</td>
<td>36</td>
<td>42</td>
</tr>
</tbody>
</table>

* The extent of labelling on virus particles associated with either neuraminidase or heat-inactivated enzyme was determined from examination of coded specimens without prior knowledge of enzyme treatment or type of Ferritin–lectin. At least 100 particles were classified subjectively as either unlabelled (−), sparsely labelled (±) or uniformly labelled (+).

needed to generate workable virus concentrations. At high concentrations, both ascites and tissue culture supernatants became contaminated with cell debris, resulting in virus preparations which contained significant quantities of membrane fragments. This persistent membrane contamination precluded the isolation by immunoprecipitation of envelope glycoproteins that were unequivocally of viral origin. A further problem in isolating envelope glycoproteins from purified virus preparations was the rapid loss of intact viral glycoproteins during purification, a phenomenon previously observed by Witte & Weissman (1976).

Since these problems prevented the direct analysis of purified virus, the pulse iodination-chase technique of Witte & Weissman (1976) was used as an alternative method for enriching the amount of virus-associated glycoproteins in material immunoprecipitated by anti-gp70 serum. In this procedure cells were radioiodinated and subsequently incubated for 4 h at 37 °C in
complete medium without foetal calf serum. During this incubation, approximately 30 to 50% of the cell surface-associated radioactivity was released. Two-dimensional electrophoretic analysis (Fig. 5) of material immunoprecipitated by anti-gp70 serum showed that a major component with an apparent 64000 Mr was released from Novikoff cells, while a similar component with a slightly higher molecular weight of approximately 68000 was shed by WRC cells. It can also be seen by comparison of Fig. 5(a) and (d) with (b) and (c) that these released components not only exhibited the same apparent molecular weights as Nov gp64 and WRC gp68 immunoprecipitated from detergent extracts of 125I-labelled cells, but also showed an identical isoelectric focusing pattern and appeared as a family of proteins with pIs ranging from approximately 4.0 to 5.5.

**Electrophoretic analysis of gp64 and gp68 under non-reducing conditions**

Results shown in Fig. 6 demonstrate that in the absence of reducing agent a variable proportion of Nov gp64 and WRC gp68 migrated as larger molecules of 78000 and 82000 Mr, respectively. These higher molecular weight forms were extremely labile and could only be detected under non-reducing conditions using fresh extracts that had never been frozen.

**Partial proteolytic digestion of Nov gp64 and WRC gp68**

The structural and antigenic relatedness of Nov gp64 and WRC gp68 was determined by comparing the electrophoretic patterns of peptide fragments immunoprecipitated with anti-gp64 and anti-gp70 sera from NP40 extracts that had been partially digested with trypsin. As seen in Fig. 7, treatment with 5 μg trypsin converted Nov gp64 into two major peptides of 45000 Mr and 26000 Mr, both of which were reactive with anti-gp70 and anti-gp64 sera. These peptides will be designated as Nov gp45 and Nov gp26 respectively. Fig. 7 also shows that digestion of WRC gp68 with trypsin produced two major immunoreactive peptide fragments, both of which were significantly larger than the corresponding peptides from gp64. These fragments of Mr 48000 and 30000 will be designated as WRC gp48 and WRC gp30 respectively.

Further information about the antigenic and structural properties of Nov gp64 and Nov gp68 was obtained by examining the degree of protection against proteolysis provided by anti-gp64 or anti-gp70 antibodies. As seen in Fig. 7, Nov gp45 was the major peptide protected by anti-gp70 under conditions (5 μg trypsin for 10 min) that almost completely converted Nov gp64 into peptides of Mr < 10000. Conversely, the major peptides protected by anti-gp64 antibodies were Nov gp26 and a new peptide of 40000 Mr (Nov gp40) that was not evident when digestions were done without protecting antibodies (Fig. 7).

Protection of WRC gp68 by anti-gp64 antibodies was similar to that seen with Nov gp64 and involved primarily the smaller fragment WRC gp30 and a new fragment of 40000 Mr (WRC gp40). As shown in Fig. 7, WRC gp30 was protected in the presence of anti-gp64 antibodies even after a 10 min digestion with 5 μg trypsin, an incubation period normally sufficient to allow almost complete conversion of this peptide to lower molecular weight fragments (Fig. 7). Anti-gp70 antibodies, however, offered considerable protection to WRC gp48, but little or no protection to WRC gp30 (Fig. 7).

**Affinity chromatography on Sepharose-RCA1**

Information regarding the nature of the carbohydrate associated with Nov gp64 and WRC gp68 was obtained by radioimmunoprecipitation analysis of 125I-labelled extracts that had been partially digested with trypsin and fractionated by lectin affinity chromatography on Sepharose-bound RCA1. It can be seen in Fig. 8 that Nov gp64 and WRC gp68 and their major tryp tic peptides were bound to Sepharose-RCA1. This demonstrated that Nov gp64 and WRC gp68, as well as their major tryp tic fragments, are glycoproteins that contain galactosyl residues capable of binding to Sepharose-RCA1 on both of their major tryp tic fragments.

**Analysis of tryptic peptides of Nov gp64 and WRC gp68 on two-dimensional gels**

The ability of anti-gp64 serum to protect both Nov gp26 and WRC gp30 against digestion by trypsin made it possible to produce sufficient quantities of these peptides for two-dimensional
Fig. 7. Tryptic peptides of Nov gp64 and WRC gp68 reactive with anti-gp70 and anti-gp64. (a) Tryptic peptides isolated by immunoprecipitation with anti-gp70 or anti-gp64 from extracts digested with trypsin as described in Methods. Lanes 1 to 8 show SDS–PAGE profiles of anti-gp70 (lanes 1 to 4) or anti-gp64 (lanes 5 to 8) immunoprecipitates from Novikoff extracts digested for 0 min (lanes 1 and 5), 2 min (lanes 2 and 6), 5 min (lanes 3 and 7) or 10 min (lanes 4 and 8) with 5 μg trypsin/ml. (b) Lanes 1 to 8 show SDS–PAGE profiles of anti-gp70 (lanes 1 to 4) or anti-gp64 (lanes 5 to 8) immunoprecipitates from WRC extracts digested for 0 min (lanes 1 and 5), 2 min (lanes 2 and 6), 5 min (lanes 3 and 7) or 10 min (lanes 4 and 8) with 5 μg trypsin/ml. (c) Tryptic peptides resulting from digestion of Nov gp64 complexed with anti-gp70 (lanes 1 to 3) or anti-gp64 (lanes 4 to 7) antibodies. Digestion was for 0 min (lanes 1 and 4), 2 min (lane 5), 5 min (lanes 2 and 6) or 10 min (lanes 3 and 7). (d) Tryptic peptides resulting from digestion of WRC gp68 complexed with anti-gp70 (lanes 1 to 4) or anti-gp64 (lanes 5 to 8) antibodies. Digestion was for 0 min (lanes 1 and 5), 2 min (lanes 2 and 6), 5 min (lanes 3 and 7) or 10 min (lanes 4 and 8).

gel analysis. Comparison of Fig. 9(a) and (b) shows that Nov gp26 and WRC gp30 exhibited an ever greater range of charge heterogeneity than their undigested parent polypeptides, Nov gp64 and WRC gp68.

Effect of neuraminidase digestion on gp64 and gp68

Digestion of Nov gp64 with 10 units of neuraminidase shifted its isoelectric point to a more alkaline pH relative to the undigested control and actually increased the charge heterogeneity, separating Nov gp64 into a family of 12 distinct components (Fig. 10a). This increased heterogeneity most likely results from incomplete removal of sialic acid residues by neuraminidase digestion, an interpretation consistent with the further shift in pI following digestion with a higher concentration of neuraminidase (data not shown). Neuraminidase digestion of WRC gp68 resulted in an even more dramatic change in pI, with some forms appearing at or near the basic end of the isoelectric focusing gel (Fig. 10b).
Fig. 8. Interaction of Nov gp64 and WRC gp68 and their tryptic peptides with Sepharose-bound RCA. NP40 extracts from 125I-labelled cells were digested with trypsin and subjected to affinity chromatography on Sepharose-RCA. Bound components eluted in 0.2 M-lactose and immunoprecipitated with anti-gp64 or anti-gp70 serum were then analysed by SDS–PAGE. Lanes 1 and 3, anti-gp70 immunoprecipitates from bound fractions of WRC (lane 1) and Novikoff (lane 3) extracts. Lanes 2 and 4, anti-gp64 immunoprecipitates from bound fractions of WRC (lane 2) and Novikoff (lane 4) extracts.

Fig. 9. Two-dimensional gel analysis of tryptic peptides of Nov gp64 and WRC gp68. Tryptic peptides produced in the presence of anti-gp64 antibodies were analysed by SDS–PAGE. (a) Peptides from Nov gp64; (b) peptides from WRC gp68.

Peptide maps produced by digestion with V8 protease

Nov gp64 and WRC gp68, isolated by immunoprecipitation with anti-gp70 serum and purified by SDS–PAGE, were digested with V8 protease according to the method of Cleveland et al. (1977). It was found that digestion of Nov gp64 with 0.025 μg V8 protease produced three major peptide fragments of Mr 45000, 31000 and 23000 (Fig. 11). Similar digestion of WRC gp68 produced three major peptides, but each of these had a slightly higher mobility, exhibiting Mr’s of 47000, 34000 and 25000 (Fig. 11). Digestion with a tenfold higher concentration of protease converted WRC gp68 into primarily small peptides (Mr < 10000), but resulted in a large increase in the 23000 Mr fragment from gp64, suggesting that this fragment lacked a V8 protease-susceptible site. These results were thus consistent with previous findings from trypsin digestion studies and suggest there are significant structural differences between Nov gp64 and WRC gp68.

Dot blot hybridization analysis

DNA samples from mouse and rat cells were probed for the presence of MuLV proviral env sequences by dot blot hybridization with the 32P-labelled 3’ half of the env region of a molecular clone of ecotropic MuLV. As shown in Fig. 12, MuLV env sequences were readily detected in MuLV producer (AKR 225J lymphoma cells) and non-producer (normal SENCAR epidermal) mouse cells. However, no sequences showing homology with the MuLV env gene probe were detected in either RaLV producer (SD-1, Novikoff and WRC cells) or non-producer (normal hepatocytes) rat cells. These results in combination with earlier studies of Benveniste & Todaro (1973) demonstrate that the env gene sequences coding for Nov gp64 and WRC gp68 are not of mouse origin.
Fig. 10. Two-dimensional gel analysis of neuraminidase-digested Nov gp64 and WRC gp68. Extracts of radioiodinated cells were digested with neuraminidase and immunoprecipitated with anti-gp70. Reactive components were then analysed by SDS–PAGE. (a, b) Anti-gp70 immunoprecipitates from undigested (a) or neuraminidase-digested (b) Novikoff extracts. Digestion in (b) was for 60 min. (c, d, e) Anti-gp70 immunoprecipitates from undigested (c) or neuraminidase-digested (d, e) WRC extracts. Digestion in (d) and (e) was for 10 and 20 min, respectively.

Fig. 11. Peptide maps produced by V8 protease digestion. Peptides produced by V8 digestion of electrophoretically purified WRC gp68 and Nov gp64 are shown in lanes 1 to 3 and lanes 4 to 6, respectively. Lanes 1 and 4 show undigested proteins, while lanes 2 and 5 and lanes 3 and 6 show peptides produced following digestion with 0.025 μg or 0.25 μg V8 protease, respectively.

Fig. 12. Dot blot hybridization of DNA samples from mouse and rat tissues. Lane 1, SENCAR mouse epidermal tissue; lane 2, SD-1 cells; lane 3, Novikoff cells; lane 4, WRC cells; lane 5, normal rat hepatocytes; lane 6, AKR 225J cells; lane 7, p1700 DNA (15 pg/ml).

**DISCUSSION**

The results presented here demonstrate that endogenous RaLV produced by Novikoff and WRC cells can be distinguished by their unique patterns of reactivity with ferritin-conjugated lectins and with xenoantisera raised against either virus particles or isolated virus glycoproteins. This differential reactivity with xenoantisera was unexpected, since all the antisera used for immunoferritin labelling had been shown by immunoprecipitation or immunodepletion analysis to be reactive with viral glycoproteins expressed on the surface of Novikoff and WRC cells. It seems possible that the apparent inconsistency between immunoferritin labelling and immunoprecipitation analysis could result from antigenic differences between viral glycoproteins expressed on the cell surface and those present on virus particles associated with Novikoff
cells. These antigenic differences could arise as a result of some further modification of gp64 after it is shed from the cell surface. Alternatively, the viral envelope form of gp64 may be present on the cell surface but not accessible to radioiodination and consequently not detectable by immunoprecipitation. However, neither of these possibilities was supported by results from pulse iodination analysis which indicated that the majority of gp64 shed from the cell surface of Novikoff cells undergoes no major structural modifications that significantly alter its reactivity with anti-gp70 serum or that can be detected by two-dimensional gel electrophoretic analysis.

A more likely explanation for these inconsistencies is suggested by recent studies of Pinter et al. (1982). These investigators found that there were differences in iodination of gp70 on the surface of ecotropic AKR MuLVs and gp70 solubilized in non-ionic detergent (Pinter et al., 1982). These results were interpreted as evidence that there were regions of the gp70 molecule that were inaccessible in the intact virions but were exposed after solubilization in detergent. The discrepancy between immunoferritin labelling and immunoprecipitation analysis in the present studies could also be explained by a similar situation in which determinants accessible to antibodies on solubilized Nov gp64 molecules are buried in the interior of multimeric knob structures on the surface of intact virions. This interpretation raised the possibility that the differential reactivity of Novikoff and WRC RaLV with xenoantisera might reflect structurally related differences in accessibility. To explore this possibility further, we used a variety of immunological and biochemical techniques to characterize Nov gp64 and WRC gp68 immunoprecipitated from detergent extracts of 125I-labelled cells.

Aside from the differences in mobility, however, further PAGE analysis did not reveal any significant differences between Nov gp64 and WRC gp68. When analysed by two-dimensional PAGE, both glycoproteins demonstrated a similar range of pl's. Both glycoproteins also showed a similar increase in mobility when analysed by SDS–PAGE in the absence of reducing agent, suggesting that like MuLV gp70 (Naso et al., 1976; Pinter & Fleissner, 1977; Witte et al., 1977) at least some forms of Nov gp64 and WRC gp68 are present on the cell surface as disulphide-linked complexes with a protein of approximate $M_r$, 15000. In addition, we also observed that the electrophoretic mobility of both glycoproteins increased under non-reducing conditions, resulting in $M_r$ values of 62000 and 57000 for WRC gp68 and Nov gp64, respectively. Pinter & Fleissner (1977) have previously reported that murine gp70s also exhibit increased mobility under non-reducing conditions and have attributed this to a conformational change due to internal disulphide bonds. It seems likely, therefore, that both Nov gp64 and WRC gp68 also possess interchain disulphide bonds.

Direct evidence for structural differences between Nov gp64 and WRC gp68 was provided by results of limited protease digestion with trypsin or V8 protease. Digestion with V8 protease produced three major cleavage fragments from both WRC gp68 and Nov gp64, while trypsin digestion produced two major fragments from each of these glycoproteins. However, all of the V8 protease fragments, as well as both of the tryptic fragments from WRC gp68, were of higher molecular weight than their Nov gp64-derived counterparts. Digestion with higher concentrations of V8 protease demonstrated that all of the cleavage products of WRC gp68 contained additional cleavage sites and could be degraded to lower molecular weight peptides ($M_r < 10000$), while, in contrast, the cleavage product from Nov gp64 of $M_r$, 23000 appeared to be resistant to further proteolysis.

Although one-dimensional peptide maps revealed significant structural differences in Nov gp64 and WRC gp68, protease digestion in the presence of anti-gp70 or anti-gp64 antibodies suggested that there was a close similarity in the antigenic properties of these molecules. As one would expect, anti-gp64 and anti-gp70 antibodies altered digestion patterns in a manner dependent upon their reactivity with the various epitopes present on the Nov gp64 and WRC gp68 molecules. When Nov gp64 or WRC gp68 complexed with anti-gp70 antibodies was digested with trypsin, Nov gp45 and WRC gp48 were the major products. The nature of the protection offered by anti-gp64 antibodies, however, was quite different from that observed with anti-gp70 antibodies. When complexed with Nov gp64, anti-gp64 antibodies did not protect Nov gp45 but did protect Nov gp28 and a new Nov gp40 component from further proteolytic digestion. It appears, therefore, that anti-gp64 does not block the trypsin cleavage
site necessary for production of Nov gp26. The pattern of protection of WRC gp68 by anti-gp64 antibodies was similar to that seen with Nov gp64 in the presence of the antibody, i.e. protection was provided to WRC gp32 and a new WRC gp40 component, but not WRC gp48.

These results indicate that there are differences in the reactivity of anti-gp64 and anti-gp70 antibodies and demonstrate the utility of digestion in the presence of antibody for defining differences in the reactivity of anti-gp64 and anti-gp70 antibodies with Nov gp64 and WRC gp68 molecules. In regard to this last point, it should be noted that the differences in the protection by anti-gp70 and anti-gp64 sera of the lower molecular weight tryptic fragments from Nov gp64 and WRC gp68 are consistent with the reactivity of these antibodies shown in Fig. 7, i.e. anti-gp64 showed a much stronger reaction than anti-gp70 with the lower molecular weight fragments. In contrast, the reactivity of these antisera with Nov gp45 and WRC gp48 appeared to be similar (Fig. 7). Analysis of the tryptic fragments formed by digestion in the presence of these antibodies revealed, however, that the site of reactivity within the larger peptide fragments were quite different for each of these antisera.

These differences in protection are also consistent with the method of immunization used to produce anti-gp64 antibodies. Our intention in using *Staphylococcus*-bound immune complexes of gp64 and anti-gp70 antibodies as an immunogen was to generate antibodies against rat-specific determinants on gp64 by blocking determinants common to mouse and rat with anti-gp70 antibodies. That this approach was successful is suggested not only by the differences in protection offered by anti-gp70 and anti-gp64 antibodies but also by the lack of reactivity of anti-gp64 with gp70 molecules on V-16 and C6XL lymphoma cells and virus produced by V-16 and AKR 225J lymphoma cells.

Immunoprecipitation analysis demonstrated that all the anti-RaLV sera were reactive with proteins of Mr 80000 and 116000. Results from immunodepletion analysis, however, indicated that these proteins were not antigenically related to Nov gp64 or WRC gp68. Whether these proteins are related to other structural proteins of RaLVs has not yet been determined. This seems unlikely, however, since components with similar mobilities were immunoprecipitated by the anti-RaLV sera from rat cells not producing virus (unpublished data). It also seems unlikely that the reactivity against these higher molecular weight components is involved in the labelling of virus particles since the data in Table 1 indicate that the higher molecular weight components recognized by anti-Novikoff RaLV, an antiserum which labelled Novikoff virus particles, are also recognized by anti-W/F RaLV, an antiserum which did not.

Our results demonstrated differences in the RCA-binding properties of detergent-solubilized and virus-associated viral glycoproteins from both Novikoff and WRC cells. If we assume that this apparent discrepancy does not result from differences in the degree of glycosylation of cell surface and viral envelope-associated glycoproteins, we can infer from these results and the known specificities of RCA (Baenzinger & Fiete, 1979) that Nov gp64 and WRC gp68 contain a saccharide chain with either a free terminal D-Gal or sialyl α2–6 D-Gal moiety that is accessible on detergent-solubilized gp64 or gp68 but not on intact virus particles. The dramatic increase in Fer-RCA labelling of Novikoff virus particles following neuraminidase digestion further suggests that some of the RCA binding sites expressed only on detergent-solubilized molecules may be exposed on intact virions by conformational changes in gp64 following removal of negatively charged sialic acid residues (Neri *et al.*, 1976; Glenney *et al.*, 1979). Alternatively, these neuraminidase-dependent sites may represent distinct saccharide chains which contain penultimate galactose residues that can serve as binding sites only after removal of a (α2–3)-linked sialic acid residue (Baenzinger & Fiete, 1979).

Results from lectin affinity chromatography and two-dimensional gel analysis of tryptic peptides indicated that there was a similar distribution of carbohydrate chains on Nov gp64 and WRC gp68. Both major tryptic fragments of Nov gp64 and WRC gp68 were glycosylated and comparable fragments from each molecule exhibited similar charge distributions on two-dimensional gels.

Results from Fer-lectin labelling studies, on the other hand, suggested differences in the saccharide moieties on these two glycoproteins. This conclusion was supported by the differential effect of neuraminidase digestion on two-dimensional gel patterns. As shown in Fig.
8, neuraminidase digestion separated Nov gp64 and WRC gp68 into families of 12 and 9 components, respectively and caused a greater shift in pI for WRC gp68 than for Nov gp64. It seems likely, based on results from our previous studies (Glenney & Walborg, 1980), that this increased heterogeneity results from incomplete removal of sialic acid residues. If one assumes, therefore, that the most acidic component has had the fewest sialic acid residues removed and that each progressively more basic component has had one more additional sialic acid residue removed than its nearest acidic neighbour, then one would estimate that limited neuraminidase digestion is accompanied by a loss of at least 12 and 9 sialic acid residues from Nov gp64 and WRC gp68. These numbers most likely represent an underestimate, since they are based on the assumption that the most basic component resulting from digestion has lost all of its sialic acid residues. That this is not the case is suggested by the increasing shift to more basic pIs with increasing neuraminidase concentration or increasing time of digestion.

Elucidation of the structural and antigenic features of the murine gp70 molecules has been an area of intense investigation in recent years. From these studies has emerged a detailed picture of the antigenic and structural features of the MuLV gp70 molecule. When the results of the present studies are compared with those of Krantz et al. (1977), Kemp et al. (1978), Niman & Elder (1980), and Pinter et al. (1982), it becomes apparent that both WRC gp68 and Nov gp64 show strong structural homology not only to each other but to other MuLV gp70s. It is also clear, however, that Nov gp64 and WRC gp68 are structurally distinct molecules that can be distinguished not only by differences in the size of their trypsin and V8 protease cleavage products, but also by the resistance of these products to further proteolysis. Our results further suggest that there may be qualitative or quantitative differences in the oligosaccharide chains on these viral glycoproteins. Additional studies are needed to determine if structural differences exist in cell-surface, env gene-related glycoproteins from other transformed rat cells. In this regard, we have recently obtained results which suggest that viral glycoproteins expressed by three hepatocellular carcinoma lines established in our laboratory from primary hepatic tumours induced by ethionine in ACI rats may differ structurally from those produced by Novikoff and WRC cells. Using immunoprecipitation analysis with anti-gp64 serum, we have found that two of these tumour lines express viral glycoproteins of Mr 71000 while a third expresses two different components of Mr 71000 and 66000 (unpublished data).

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