Envelope Glycoproteins of Rauscher Murine Leukemia Virus: Isolation and Chemical Characterization†

Hans Marquardt,* Raymond V. Gilden, and Stephen Oroszlan

ABSTRACT: The envelope glycoproteins (designated gp70 and gp45) of the Rauscher strain of murine leukemia virus were solubilized by osmotic shock and freeze-thawing in chaotropic solutions. The viral glycoproteins were then purified by phosphocellulose chromatography and gel permeation chromatography on Bio-Gel A-1.5m. Yields by this procedure were 6.2% for gp70 and 1.3% for gp45 on a protein input basis. The apparent molecular weights were respectively 67 500 and 47 500 with a polypeptide chain molecular weight of approximately 45 000 for both glycoproteins. Amino acid analysis showed a high degree of similarity for both components, with some differences subject to further evaluation. The total carbohydrate content was approximately 32% for gp70 and 6–9% for gp45. In keeping with the amino acid compositional similarity suggesting relationships, alanine was found to be the amino-terminal amino acid of both glycoproteins, and cross-reactivity was demonstrated by immunologic tests. The data suggest that the chief difference between gp70 and gp45 lies in the carbohydrate content.

The major envelope glycoprotein (gp70)[1] of several murine leukemia viruses (MuLV)[2] has been recently isolated and partially characterized by immunologic methods.

Several procedures have been applied to solubilize gp70: sequential freezing and thawing in buffers of low ionic strength and sonication in buffers of high salt concentrations to disrupt RLV (Strand and August, 1973), osmotic shock to release the glycoprotein from Friend MuLV (Moenning et al., 1973) and diiodosalicylate to preferentially solubilize gp70 from AKR MuLV (Ihle et al., 1976). Although differences in molecular weights of gp70's isolated by the above methods have been noted, no attempts were made to chemically characterize the glycoproteins.

The presence of a minor glycoprotein component in MuLV with an apparent molecular weight of 45 000 (gp45) has also been reported (Ihle et al., 1973; Moennig et al., 1974). The origin and the characteristics of this glycoprotein have thus far not been described. The present studies utilized chaotropic agents to completely solubilize both RLV glycoproteins. The application of chaotropes provides a versatile methodology which can be applied to different RNA tumor viruses. Data on the chemical nature of these glycoproteins from RLV are presented and their immunological relationship is demonstrated.

Materials and Methods

Rauscher Virus. RLV was grown in monolayer cultures of chronically infected mouse (BALB/c) bone marrow, JLS-V9 cells (Wright et al., 1967). The virus-shedding cell line was originally obtained from Electronucleonics, Bethesda, Md.

Virus Purification. Tissue culture fluids were clarified by filtration through a membrane filter (Millipore Corp., Bedford, Mass., 1.2-μm pore size). The virus was concentrated by continuous flow centrifugation with isopycnic banding in a Tris-buffered (0.01 M, pH 7.4) sucrose gradient (20 to 50% w/w) at 4 °C. Fluid volumes of 50 to 100 L were collected at flow rates of 13 L/h on the K-6 continuous flow rotor of the Model K Mark II ultracentrifuge (Electronucleonics). The concentrated virus was thereafter diluted with TNE buffer (0.01 M Tris-HCl–0.1 M NaCl–0.001 M EDTA, pH 7.4) and rebanded a second time using a Ti-15 zonal rotor (Beckman) operating for 2 h at maximum speed (Olpin et al., 1974).

Extraction of Soluble Viral Glycoproteins. Solubilization by Osmotic Shock. Sucrose gradient purified RLV was diluted with TNE buffer from 36 to 15% sucrose concentrations. The viral residue was pelleted in a SW 25.1 rotor (Beckman) at 25 000 rpm for 2 h at 4 °C and the supernatant was then dialyzed against TNE buffer. The amount of solubilized protein was determined and the volume reduced by pressure membrane filtration (Amicon Corp., Lexington, Mass., type UM-2). The product was designated OS-E.

Extraction with Chaotropic Agents. The viral pellet was subsequently resuspended in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl at a protein concentration of 1 mg/mL and quick frozen in dry ice–acetone and thawed at room temperature. Freezing and thawing were repeated six times. The viral residue was pelleted in a SW41 rotor (Beckman) and the amount of solubilized protein determined. The volume was reduced by pressure membrane filtration. The product was designated NaCl-E.

The remaining viral pellet was resuspended at a protein concentration of 1 mg/mL in 3.5 M KBr–2 mM dithiothreitol–1 mM EDTA and then reextracted as before. The supernatant obtained after 2 h centrifugation at 105 000g was then dialyzed against TNE buffer, and the volume was reduced by pressure membrane filtration. The product was designated KBr-E.

Purification of Viral Glycoproteins. Chromatography on Phosphocellulose. Solubilized gp70 and gp45 were isolated by chromatography of 20–40-mg samples of OS-E, NaCl-E, or

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[1] The nomenclature of oncornavirus proteins is that recommended by August et al. (1974).

[2] Abbreviations used are: MuLV, murine leukemia virus; RLV, Rauscher murine leukemia virus; TNE, Tris–NaCl–EDTA buffer; Bes, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

KBr-E on 1.5 × 6 cm columns of phosphocellulose (Whatman P-11) under conditions similar to those described previously (Strand and August, 1973). Elution was achieved by use of a linear gradient pumped from a two-chamber constant level device containing 100 mL of starting buffer (10 mM Bes-1 mM EDTA, pH 6.5) in the first chamber and 100 mL of limit buffer (10 mM Bes-1 mM EDTA containing 0.6 M KCl, pH 6.5). The column was operated at a flow rate of 35 mL/h at room temperature; the effluent was monitored continuously at 280 nm in a Beckman Model 25 spectrophotometer, and absorbance was recorded on a Beckman 10-in. linear log potentiometric recorder at a chart speed of 2 in./h. The column effluent was collected in 4-mL fractions. Pools of fractions comprising the gp70 and gp45 peaks (about 80 mL) were dialyzed against TNE buffer and concentrated by pressure membrane filtration.

Chromatography on Bio-Gel A-1.5m. Following ion-exchange chromatography, the glycoprotein fractions were further purified by gel permeation chromatography on a 1.9 × 136 cm column (385 mL bed volume) of Bio-Gel A-1.5m (200–400 mesh; Bio-Rad Laboratories). The column was equilibrated with TNE buffer containing 0.02% sodium azide. Samples of protein (1–6 mg) in TNE buffer (2 mL) were applied to the column. To ensure a constant flow rate, the column effluent was regulated at 10 mL/h with a peristaltic pump. The column effluent was monitored at 280 nm and 4-mL fractions were collected. Fractions representing the major portion of a given protein peak (excluding both front and tail) were pooled and concentrated as described above.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Protein samples were dialyzed against 0.05 M ammonium bicarbonate, lyophilized, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (1969). Gels were stained for carbohydrate using the periodic acid-Schiff procedure as described by Glossmann and Neville (1971).

Molecular weights for gp70 and gp45 were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously published (Segrest et al., 1971). The following proteins were used to construct standard plots of log molecular weight vs. mobility in the different acrylamide gel concentration studies: phosphorylase *a*, mol wt 94 000; bovine serum albumin, mol wt 68 000; catalase, mol wt 60 000; ovalbumin, mol wt 43 000; glyceraldehyde-phosphate dehydrogenase, mol wt 36 000; carbonic anhydrase, mol wt 29 000; chymotrypsinogen, mol wt 25 700; and myoglobin, mol wt 17 200.

Disc Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out essentially as described by Reisfeld et al. (1962).

Neuraminidase Treatment. Purified gp70 (1 mg) in 0.05 M sodium acetate buffer, pH 5.5, containing 5 mM calcium chloride and 0.15 M sodium chloride (0.5 ml) was incubated with 10 units of *Vibrio cholerae* neuraminidase (Behring Diagnostics) for 6 h at 37 °C. The enzymatic hydrolysate was then dialyzed against 0.1 M ammonium bicarbonate and lyophilized.

Determination of N-Terminal Amino Acids. The procedure described by Weiner et al. (1972) was used without major modifications for the determination of N-terminal amino acids. Purified proteins or mixtures of proteins (20–60 μg) were dissolved in 100 μl of 0.1 M sodium bicarbonate containing 2% sodium dodecyl sulfate and dansylated for 30 min at 37 °C. The reaction mixture was then dialyzed against 0.1 M ammonium bicarbonate, lyophilized, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fluorescence of the gels was observed under a portable ultraviolet lamp, and the identified protein bands were excised, hydrolyzed, and prepared for chromatography. Two-dimensional thin-layer chromatography was performed on double-layered Cheng-Chin polyamide sheets (Gallard-Schlesinger, Carle Place, N.Y.) using a solvent system of heptane-1-butanol-formic acid (10:10:1, v/v/v) as the first solvent and 0.15 M aqueous ammonium hydroxide as the second solvent.

Quantitative Determinations. Amino Acid Analyses. Samples were hydrolyzed at 110 °C for 24 h in constant boiling 6 N HCl under nitrogen in sealed tubes. The amino acid contents of the hydrolysates were determined on a single column automatic amino acid analyzer (Model 121H, Beckman Instruments, Inc.) equipped with an automatic sample injector. Peak areas were determined electronically with an Autolabs System AA computing integrator (Oroszlan et al., 1975). Tryptophan was determined after alkaline hydrolysis (Hugli and Moore, 1972). Half-cystine was determined as cysteic acid and methionine as the sulfone after performic acid oxidation (Hirs, 1956). Norleucine was used as internal standard.

Hexosamine Analyses. The hexosamines, glucosamine and galactosamine, were determined on the amino acid analyzer after hydrolysis with 4 N HCl at 100 °C for 6 h.

Neutral Monosaccharides and Neuraminic Acid. The carbohydrate composition of purified gp70 was determined by gas–liquid chromatography of the trimethylsilyl methyl glycosides (Laine et al., 1972) using arabitol as internal standard. Glycoprotein preparations (0.1–0.2 mg) were methanolyzed with 0.5 ml of 1.5 N anhydrous methanolic hydrochloric acid at 80 °C for 24 h under nitrogen (Chambers and Clamp, 1971). Methods for re-N-acetylation conditions and the preparation of trimethylsilyl derivatives from methyl glycosides were adopted from Etchinson and Holland (1975). The trimethylsilylated methyl glycosides were dissolved in 15–50 μL of hexane and 1–3 μL was injected into the gas chromatograph. A Packard-Becker 420 gas chromatograph with flame ionization detectors and a linear temperature programmer was used. Separations were performed on an ⅛ in. × 10 ft coiled stainless steel column packed with 3% OV-17 on 80–100 mesh Chromosorb WHP (Supelco, Inc., Bellefonte, Pa.). The gas chromatograph injector temperature was 240 °C, the detector temperature was 270 °C, and the column temperature was programmed from 125 to 250 °C at 5 °C per min from the time of injection. Nitrogen at a flow rate of 30 mL per min was used as carrier gas.

Protein Determination. Total protein was determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard. A nitrogen determination (Kjeldahl) of the standard bovine serum albumin was performed, after being thoroughly dialyzed against phosphate buffered saline, and centrifuged for 2 h at 105 000*g* at 4 °C. The total protein content of the purified glycoproteins was also determined by quantitative amino acid analysis. The two methods gave similar results.

Preparation of Antisera. Guinea Pig Antiserum to RLV gp70 (GPS). The antiserum was prepared in guinea pigs by repeated injections of gp70. The antigen (25 μg/mL) was incorporated into complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Initially, animals were injected with 0.25 mL of the emulsion into each footpad; subsequent injections of 1.0 mL at six separate subcutaneous sites were made on days 10, 20, and 30. Animals were bled and antisera harvested 7 days after the last injection.

Goat Antiserum to RLV gp70 (GoS). Antiserum to RLV gp70 was prepared by immunizing a goat with RLV gp70

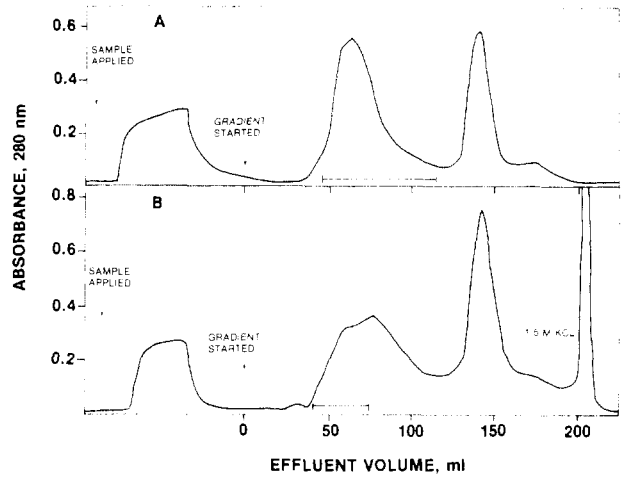

FIGURE 1: Ion exchange chromatography on phosphocellulose. (A) Elution pattern of 35 mg of viral protein solubilized with NaCl by freeze-thawing of RLV (NaCl-E). Elution was achieved with 0.010 M Bes–0.001 M EDTA (pH 6.5) using a linear salt gradient of KCl from 0 to 0.6 M. The total volume of the gradient was 200 ml. The gp70-containing fractions which eluted at 0.12 to 0.35 M KCl were pooled as shown by the bar (11.3 mg); p30 eluted at 0.43 M KCl. (B) Elution pattern of 38 mg of viral protein solubilized with KBr by freeze-thawing of preextracted RLV (KBr-E). Elution was achieved by employing conditions identical with those described above. Proteins not resolved by this procedure were eluted at the designated volume by increasing the KCl concentration to 1.5 M. The gp45 containing fraction eluted at 0.12 to 0.21 M KCl as indicated by the bar. Seventy percent of the applied gp70 eluted in the 40–120-ml fraction (7.1 mg of total protein) at 0.12 to 0.35 M KCl; p30 eluted at 0.43 M KCl.

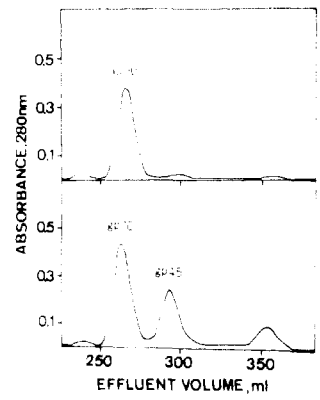

FIGURE 2: Gel permeation chromatography on Bio-Gel A-1.5m (385 ml column). Upper panel: Elution pattern of 4 mg of protein of the 40–120-ml portion of the phosphocellulose effluent (Figure 1A). Lower panel: Elution pattern of the 40–120-ml portion of the phosphocellulose effluent (Figure 1B). The elution was performed with 0.050 M Tris-HCl–0.10 M NaCl–0.001 M EDTA–0.02% NaN_3 (pH 7.4).

purified from sucrose gradient banded virus. A method described by Strand and August (1973) was used with the addition of an isoelectric focusing step (Oroszlan et al., in preparation).

Immunodiffusion Analyses. Ouchterlony analyses were done as previously described (Oroszlan et al., 1974). When virus particles or cells were tested, Triton X-100 (0.1% final concentration) was added to the virus or cell preparation which was then treated with a Branson Sonifier (Model S125, Heat Systems-Ultrasonics, Plainview, N.Y.) for a total of 1 min in an ice-water bath.

Immunoelectrophoresis. The micromethod described by Scheidegger (1955) using glass slides coated with 1% agarose in sodium borate buffer, pH 8.6, $I = 0.1$ at protein concentrations of 500 μg/mL was used. The conditions for electrophoresis were 5 V/cm for 90 min at room temperature.

Results

Extraction of Soluble Viral Glycoproteins. Based on previous reports (Strand and August, 1973; Moennig et al., 1973) we combined osmotic shock and freeze-thawing procedures and introduced the use of chaotropic reagents of increasing potency to solubilize the viral envelope glycoproteins. The sequential solubilization procedure is described in Materials and Methods. Three extracts were obtained for further purification and analysis: OS-E, produced by dilution of purified RLV from 36 to 15% sucrose concentrations, contained 4.7% of the input viral protein in soluble form; NaCl-E, produced by treatment of the pellet obtained from the osmotic shock procedure with 0.15 M NaCl, pH 7.4, contained 17.3% of the total input protein in soluble form; KBr-E produced by treatment of the second pellet with 3.5 M KBr, contained an additional 25.3% of the input protein in soluble form. Several freeze-thawing cycles were required for complete solubilization of the glycoproteins. The yield of protein rapidly declined after the third cycle. Each of the three soluble extracts was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Each of the extracts had several common bands, one of which stained predominantly with the periodic acid–Schiff reagent. This component had an apparent molecular weight of 68 000 on 10% polyacrylamide gels and will be referred to here as gp70. Osmotic shock was ineffective in releasing the second glycoprotein (gp45) which was clearly seen after extraction with the chaotropic reagents. These procedures also released other virion components.

All extracts gave positive reactions in immunodiffusion with specific antisera to gp70. Significant amounts of gp70 or gp45 were not found in the remaining pellet after the sequential solubilization procedure.

Purification of Viral Glycoproteins. Ion-Exchange Chromatography on Phosphocellulose. The sodium chloride extract (NaCl-E) was dialyzed against 10 mM Bes containing 1 mM EDTA (pH 6.5), clarified by centrifugation and then chromatographed on phosphocellulose under conditions which retained gp70 but allowed removal of 45% of the input protein in an initial wash with the above dialysis buffer. gp70, gp45, and p30, and other proteins were eluted by use of a linear salt gradient. A representative chromatogram is illustrated in the upper panel of Figure 1. The first absorbance peak eluting shortly after application of the gradient contained mainly gp70. This peak "tailed" into the fractions eluted from the phosphocellulose by increasing the KCl concentration of the eluent to 0.6 M. The relatively broad absorbance peak coincided with gp70 immunoreactivity suggesting charge heterogeneity. Analysis of several fractions taken across the gp70 peak showed a constant amino acid composition suggesting, as shown below, that this charge heterogeneity resulted from the heterogeneity in the carbohydrate portion of the glycoprotein. The second major absorbance peak contained p30 as the predominant component. Fractions containing gp70, as indicated with a bar in Figure 1A, were pooled, dialyzed against TNE buffer, and concentrated by pressure filtration for further purification by gel permeation chromatography on Bio-Gel A-1.5m.

The phosphocellulose elution pattern of gp70 released by osmotic shock from banded RLV resembled that observed for gp70 in NaCl-E, although the gp70 peak appeared somewhat less heterogenous.

KBr-E was prepared for phosphocellulose chromatography and proteins were eluted by use of a linear salt gradient as

TABLE I: Recovery of gp70 and gp45 after Phosphocellulose and Gel Permeation Chromatography.

Preparation	gp70[a]	gp45[a]
OS-E	2.23	0.09
NaCl-E	1.93	0.29
KBr-E	2.03	0.91
Total	6.19	1.29

[a] In mg of protein (determined by Folin method) per 100 mg of total viral protein.

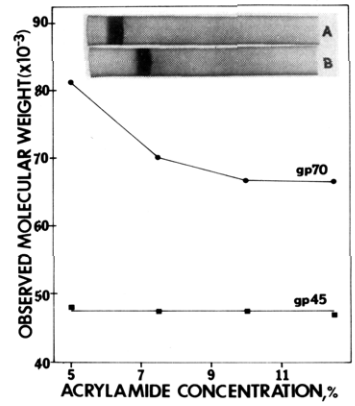

FIGURE 3: Determination of the molecular weight of gp70 and gp45 by sodium dodecyl sulfate gel electrophoresis on polyacrylamide gels of varying concentrations of acrylamide. Observed molecular weights of both glycoproteins were determined by comparing their electrophoretic mobilities with those of standard proteins of known molecular weight. (●—●) gp70; (■—■) gp45. Gels A and B correspond to gp70 and gp45, respectively. Approximately 9 μg of each glycoprotein was electrophoresed on 10% acrylamide gels and stained with Coomassie blue. Samples were dissolved at concentrations of 1 μg/μl in 1 mM sodium phosphate buffer (pH 7.0), containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 8 M urea for 1 h at 37 °C and 2 min at 100 °C. Acrylamide gels (5, 7.5, 10, 12.5%) containing 4% methylenebisacrylamide were used. After electrophoresis, the gels were washed in 50% methanol and 5% acetic acid overnight, stained with Coomassie brilliant blue, and destained in 5% methanol and 7.5% acetic acid.

described above. A typical chromatogram is presented in the lower panel of Figure 1. gp45 was observed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the leading edge of the gp70 peak as indicated with a bar.

RLV gp70 extracted with 3.5 M KBr showed extreme charge heterogeneity. This resulted in an elevated baseline throughout the effluent from phosphocellulose columns. A portion of gp70 was eluted near the end of the gradient, and the gp70 remaining on the column was eluted by increasing the KCl concentration of the effluent to 1.5 M. The major portion of gp70 in the KBr-E eluted from phosphocellulose in the 40–120-mL fraction (lower panel of Figure 1). The recovery of gp70 in this fraction was about 70% of the total input gp70 as measured by spectrophotometric scanning of periodic acid–Schiff stained sodium dodecyl sulfate–polyacrylamide gels and plotting areas of glycoprotein peaks as a function of known amounts of purified gp70.

Gel Permeation Chromatography on Bio-Gel A-1.5m. The phosphocellulose purified gp70 fraction (Figure 1A) was chromatographed on a Bio-Gel A-1.5m column. A typical elution pattern is illustrated in the upper panel of Figure 2. gp70 eluted as the major peak and was separated from small amounts of aggregated gp70, gp45, and small molecular weight proteins.

The phosphocellulose purified gp70 and gp45 pool (lower panel of Figure 1, 40–120-mL fraction) was subjected to gel permeation chromatography on Bio-Gel A-1.5m. The lower panel of Figure 2 shows a representative chromatogram. Three distinct fractions were obtained: one containing gp70, one gp45, and one an unidentified low molecular weight protein. The tailing edge of the gp45 fraction showed two minor components with apparent molecular weights of 43 500 and 35 000 on 10% sodium dodecyl sulfate–polyacrylamide gels. Both proteins reacted with anti-gp70 antibodies in immunodiffusion studies. A rechromatography of the major portion of the gp45 fraction on Bio-Gel A-1.5m resulted in isolation of highly purified gp45.

Table I presents data with regard to the amount of recovered gp70 and gp45 after phosphocellulose and gel permeation chromatography during each of the three sequential extraction steps from one representative RLV preparation.

Physicochemical Characterization of Isolated gp70 and gp45. Size Homogeneity. Figure 3 shows the electrophoretic protein patterns of isolated gp70 and gp45 on 10% sodium dodecyl sulfate–polyacrylamide gels. The stained gels indicate a high degree of homogeneity for both purified glycoproteins. The patterns depicted are representative of most preparations obtained. In a few instances, some lighter material with an apparent molecular weight of 43 500 was observed in the gp45 preparations; however, this accounted for less than 5% of the total protein.

Molecular Weight. The molecular weights of gp70 and gp45 were determined on sodium dodecyl sulfate–polyacrylamide gels of varying acrylamide concentrations by comparing their electrophoretic mobilities with those of standard proteins of known molecular weight. The apparent molecular weights of gp70 and gp45 were extrapolated from the appropriate standard curves and plotted against acrylamide concentration, as shown in Figure 3. In each example the apparent molecular weight of gp70 decreased asymptotically with increasing gel concentrations giving a minimal molecular weight of 67 500.

The apparent molecular weight of gp45 was found to be independent of the acrylamide concentration. An average molecular weight of 47 500 ± 1500 was calculated as the arithmetical mean of the molecular weight values obtained from the different percentages of acrylamide.

Charge Heterogeneity. Electrophoretic analyses of isolated gp70 was made in acidic (Reisfeld et al., 1962) and basic (Davis, 1964) multiphasic electrophoretic systems in the presence of urea. gp70 migrated as a broad band in both systems at different pore sizes (5 and 7.5% acrylamide gels). Some material was detected in the upper stacking gel, suggesting substantial charge heterogeneity.

Considerable charge homogeneity of gp70 was, however, achieved after treatment of gp70 with neuraminidase, as described in Materials and Methods. The electrophoretic pattern of neuraminidase treated gp70 is shown in Figure 4A. A prominent band that migrated at pH 4.3 toward the cathode was observed and was followed by two extremely faint bands. Some stained protein was detected at the interface of the upper stacking gel and lower running gel possibly due to aggregation. Upon reelectrophoresis, the prominent gp70 band consisted of a single electrophoretic component that exhibited specific antigenic activity in immunodiffusion analysis. Figure 4C shows the electrophoretic pattern of gp45. The highly purified gp45 appeared homogenous and migrated with slightly lesser mobility than neuraminidase treated gp70. Coelectrophoresis

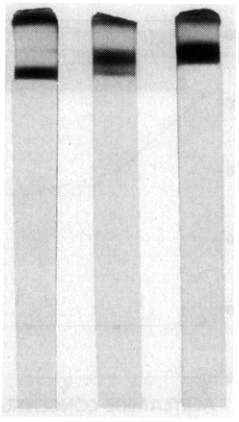

FIGURE 4: Comparison between electrophoretic patterns of neuraminidase treated gp70 (A) and untreated gp45 (C) resolved by disc polyacrylamide gel electrophoresis at pH 4.3 in the presence of 2 M urea using 2.5% upper gel and a 7.5% lower gel. Lyophilized samples were reconstituted in diluted upper gel buffer (1:8) containing 8 M urea. After the electrophoretic fractionation, the polyacrylamide gels were fixed, stained, and destained according to methods previously published (Chrambach et al., 1967). Approximately 6 μg of each glycoprotein was electrophoresed on 7.5% acrylamide gels and stained with Coomassie blue. (B) Coelectrophoresis of 3 μg of gp45 (untreated) and 3 μg of gp70 treated with neuraminidase as described in Materials and Methods. The cathode is at the bottom.

TABLE II: Amino Acid Composition of RLV gp70.

Amino Acid	Amount (μmol/100 mg of gp70)	Residue Weight (mg/100 mg of gp70)	Residues[a] (No./mol[b] of gp70)
Asp	63.05	7.26	43.2 (43)
Thr[c]	67.00	6.77	45.9 (46)
Ser[c]	51.05	4.45	34.9 (35)
Glu	42.57	5.50	29.1 (29)
Pro	75.76	7.36	51.9 (52)
Gly	57.32	3.27	39.2 (39)
Ala	36.38	2.58	24.9 (25)
½-Cystine[e]	28.38	2.93	19.4 (19)
Val[d]	40.97	4.06	28.0 (28)
Met[f]	3.04	0.40	2.1 (2)
Ile[d]	13.36	1.51	9.2 (9)
Leu[d]	59.06	6.68	40.4 (40)
Tyr	22.70	3.70	15.5 (16)
Phe	8.58	1.26	5.9 (6)
Lys	21.81	2.80	14.9 (15)
His	14.23	1.95	9.7 (10)
Arg	26.17	4.09	17.9 (18)
Trp[g]	7.20	1.34	4.9 (5)
Total		67.91	437

[a] Residues per peptide. Values are rounded off to the nearest whole number. [b] Values are calculated on the basis of a molecular weight of 68 000 for the gp70 molecule. [c] Estimated by extrapolation to zero time hydrolysis from the 24 and 72 h hydrolysis data. [d] Values are taken from the analysis of 72 h hydrolysates. [e] Determined as cysteic acid after performic acid oxidation. [f] Average of two determinations after hydrolysis for 24 h. [g] Determined after alkaline hydrolysis.

of gp70 treated with neuraminidase and gp45 is depicted in Figure 4B.

When the coelectrophoresis experiment was performed at pH 8.9, neither gp45 nor neuraminidase treated gp70 migrated into the lower running gel.

TABLE III: Composition of RLV gp45.

Component	Amount (μmol/100 mg of gp45)	Residue Weight (mg/100 mg of gp45)	Residues[a] (No./mol[b] of gp45)
Asp	90.89	10.46	43.2 (43)
Thr[c]	69.74	7.05	33.1 (33)
Ser[c]	83.53	7.27	39.7 (40)
Glu	60.80	7.85	28.9 (29)
Pro	114.32	11.10	54.3 (54)
Gly	90.81	5.18	43.1 (43)
Ala	50.84	3.61	24.1 (24)
½-Cystine[d]	36.38	3.75	17.3 (17)
Val[f]	45.92	4.55	21.8 (22)
Met[e]	3.89	0.51	1.9 (2)
Ile[f]	23.13	2.62	11.0 (11)
Leu[f]	77.01	8.71	36.6 (37)
Tyr	29.64	4.84	14.1 (14)
Phe	13.11	1.93	6.2 (6)
Lys	27.00	3.46	12.8 (13)
His	21.19	2.91	10.1 (10)
Arg	35.99	5.62	17.1 (17)
Trp	ND		
Glucosamine[g]	19.33	3.93	
Galactosamine[g]	9.38	1.91	
Total		97.26	

[a] Residues per peptide. Values are rounded off to the nearest whole number. [b] Values are calculated on the basis of a molecular weight of 47 500 for the gp45 molecule. The results are from one analysis of a 24 h hydrolysate and one 24 h hydrolysate after performic acid oxidation. [c] Corrected for hydrolytic losses. [d] Determined as cysteic acid after performic acid oxidation. [e] Average of two determinations after hydrolysis for 24 h and as methionine sulfone after performic acid oxidation. [f] Not corrected for incomplete hydrolysis. [g] Results were obtained after hydrolysis with 4 N HCl for 6 h at 100 °C and were expressed as *N*-acetyl derivatives. Monosaccharides and neuraminic acid were not determined. ND, not done.

Chemical Characterization of gp70 and gp45. N-Terminal Analysis. Alanine was found to be the amino-terminal amino acid of both gp70 and gp45. Thus, despite the charge heterogeneity of gp70, the protein molecules appeared to be homogenous by N-terminal amino acid analysis.

Amino Acid Composition. Amino acid analyses were performed on gp70 preparations of the three different extracts derived from RLV and on fractions from various regions in the gp70 peak on phosphocellulose. No significant differences were observed. Representative results are given in Table II. The total number of residues of each amino acid found was calculated by assuming that gp70 has a molecular weight of 68 000 and consists of 68% protein. A molecular weight of 46 500 was calculated for the peptide chain of gp70 on the basis of its amino acid composition.

The amino acid analyses of gp45 were performed on one preparation of the KBr-E derived from RLV. The results are presented in Table III. The total number of residues of each amino acid found in the peptide chain was calculated by assuming that gp45 has a molecular weight of 47 500 and contains 91% protein. A molecular weight of 43 500 was calculated for the peptide chain of gp45 on the basis of its amino acid composition. The contribution of tryptophan to the size of the peptide chain of gp45 was not taken into account for this calculation.

Carbohydrate Composition. Hexosamines were quantitated on the amino acid analyzer. The glucosamine and galactosamine contents for gp45 were 3.93 and 1.91% (w/w), respec-

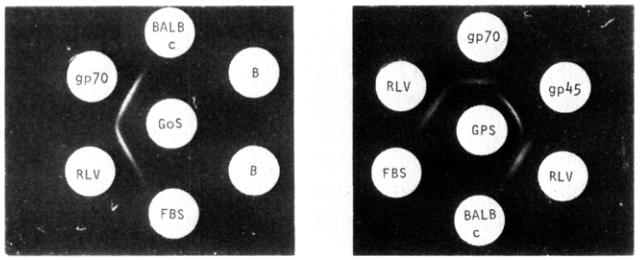

FIGURE 5: Immunodiffusion analyses of highly purified gp70 and gp45 from RLV with goat anti-RLV gp70 (GoS) and guinea pig anti-RLV gp70 (GPS). gp70 (2.8 μg) and gp45 (2.1 μg) were tested with diluted GoS (1:8) and concentrated GPS (twofold). Triton X-100 solubilized RLV was tested at a protein concentration of 1.5 mg/ml with GoS and at a concentration of 3.0 mg/ml with GPS. The antisera did not react with fetal bovine serum (FBS) or Triton X-100 solubilized BALB/c cells (BALB c), which were used as virus-shedding cell line. (B) Empty well.

TABLE IV: Carbohydrate Composition of RLV gp70.

Monosaccharide	Residue Weight (mg/100 mg of gp70)
Glucosamine[a]	8.3
Galactosamine[a]	1.8
Mannose	7.7
Galactose	8.8
Fucose	1.3
Neuraminic acid[b]	4.1
Total	32.0

[a] Average of duplicate analysis after hydrolysis with 4 N HCl for 6 h at 100 °C and expressed as *N*-acetyl derivatives. [b] Calculated as *N*-acetylneuraminic acid.

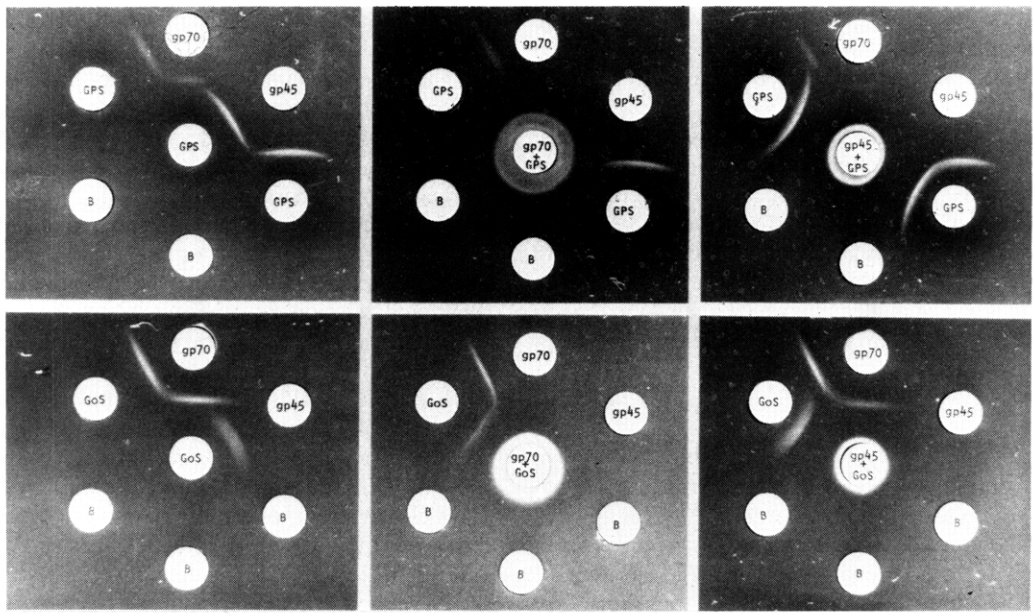

FIGURE 6: Immunological characterization of gp70 and gp45 by absorption-in-gel technique with goat (GoS) and guinea pig (GPS) sera to RLV gp70. The absorption experiments were performed by adding 10 μl of gp70 (1.4 μg of protein) and 10 μl of gp45 (2.1 μg of protein) to the center wells on separate gel diffusion plates. After complete diffusion of the absorbing antigens into the agarose gel, 10 μl of diluted GPS or GoS (1:4, v/v) was added. The absorbed antisera were then tested by immunodiffusion analyses using 10 μl of gp70 (1.4 μg of protein) and 10 μl of gp45 (1.1 μg of protein) in separate wells as antigens.

tively (Table III). Other sugars of gp45 were not determined. The amino acid and hexosamine contents accounted for 97.3% of the total gp45 molecule, based on a molecular weight of 47 500 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Complete carbohydrate analyses of gp70 were done on preparations isolated from the sodium chloride extract. The results are presented in Table IV and are expressed as residue weight in percent. Only small amounts of fucose and galactosamine were present. The major components were clearly mannose, galactose, glucosamine, and neuraminic acid. The total carbohydrate content was approximately 32%.

Antigenic Relatedness of gp70 and gp45. Immunodiffusion Analysis. Two antisera to purified gp70 were available for studies of possible relationship between gp70 and gp45: one produced in goats (GoS) and the other in guinea pigs (GPS). Both sera gave single precipitin lines in gel diffusion when tested with whole virus or purified gp70. As expected identity reactions resulted from side-by-side comparisons of gp70 and whole virus disrupted by detergent (Figure 5). These antisera have not reacted with other major virion proteins, e.g., p30, p15, p12, and p10 in either immunodiffusion or radioimmunoassay (data not shown). As shown for the guinea pig antiserum in Figure 5 and the goat reagent in Figure 6, both sera also reacted with purified gp45. In a number of experiments side-by-side patterns did not give clear resolution (e.g., Figure 6, right panel), although indications of partial identity were seen on occasion. The gp45 reaction was never seen to cross the gp70 line but seemed to blur and loose definition in its vicinity. Other experiments with iodinated gp70 and gp45 showed both antisera capable of quantitatively precipitating labeled gp45 (Marquardt and Charman, unpublished observations). Absorption experiments gave further evidence of partial identity between gp70 and gp45. In Figure 6 (center panel), gp70 in excess added to the center well before addition of antiserum resulted in complete removal of reactivity to both gp70 and gp45. A similar experiment with gp45 as the absorbing antigen (right hand panel) gave only a reduction in the intensity of the

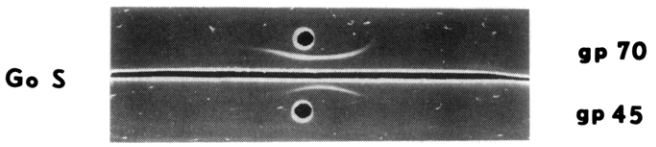

FIGURE 7: Immunoelectrophoresis of isolated RLV gp70 (gp70) and RLV gp45 (gp45). The concentrations of gp70 and of gp45 were 500 and 450 μg/ml, respectively. The antiserum used to develop precipitin arcs was goat anti-RLV gp70 antiserum (GoS, 1:4 diluted). Electrophoresis was performed in 1% agarose in sodium borate buffer (pH 8.6), ionic strength 0.1, for 90 min at 5 V/cm. The anode was to the left.

precipitin line as seen most clearly with the guinea pig antiserum. A clear indication of antigen excess is evidenced by precipitation reactions obtained with antisera opposite the center well. Another interesting feature of these patterns is the relative sharpness of the precipitin line between GPS and gp45 and the diffuse reaction of GoS with the same preparation. The relative curvature difference of the precipitin lines of gp70 and gp45 is another indication that the cross-reactivity observed is not simply based on presence of gp70 in the gp45 preparation.

Immunoelectrophoresis. The immunoelectrophoretic analysis of isolated gp70 and gp45 is shown in Figure 7. The electrophoresis was performed in sodium borate buffer, pH 8.6, since the resolution of gp70 in this buffer was clearly superior to that obtained in sodium barbital buffer at the same pH. Both glycoproteins migrated slightly toward the cathode. The asymmetric arc shape of gp70 compared with gp45 in the immunoelectropherogram clearly demonstrated the charge heterogeneity of gp70.

Discussion

In the present study chaotropic agents which destabilize membrane structure by weakening both electrostatic and hydrophobic interactions (Marquardt et al., 1973; Hatefi and Hanstein, 1974) have been added to previous procedures in order to solubilize both glycoproteins from the envelope of Rauscher murine leukemia virus. While gp70 has previously been isolated from several murine viruses (Nowinski et al., 1972; Ihle et al., 1973; Strand and August, 1973; Moennig et al., 1974; Hino et al., 1976), to date no clear definition of gp45 has been obtained. The differential release of these glycoproteins from intact virions by chaotropes of increasing potency suggests the participation of different stabilizing forces or perhaps heterogeneity in the virion population.

The major findings of this study relate to the apparent high degree of similarity of the protein portion of gp70 and gp45, the principal differences being carbohydrate content. gp70 was found to contain approximately 32% carbohydrate. This value was derived from multiple analyses of one gp70 preparation isolated from the sodium chloride extract. The carbohydrate content of different gp70 preparations and the absolute number of individual carbohydrate residues may vary and be dependent on the solubilization of RLV and the isolation procedure of gp70 from soluble extracts. Evidence for the microheterogeneity in the carbohydrate moiety of gp70 was obtained by comparing the ratios of glucosamine and galactosamine in gp70 preparations isolated from the sodium chloride and potassium bromide extracts (Marquardt, unpublished data). The sugar constituents were characteristic of glycoproteins. Analyses of the total neuraminic acid fraction of gp70 has indicated the presence of *N*-acetylneuraminic acid only. The release of neuraminic acid by neuraminidase suggested the peripheral location of this carbohydrate moiety on the gp70 molecule. gp45 contained only about 6–9% carbohydrate.

The molecular weights of gp70 and gp45 were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The method relies on the assumption that the electrophoretic mobility of a protein is a unique function of the molecular weight, which only occurs when the charge per unit of mass is approximately constant (Reynolds and Tanford, 1970a) and the hydrodynamic properties are a function of the chain length only (Reynolds and Tanford, 1970b). Glycoproteins containing more than 10% carbohydrate bind less sodium dodecyl sulfate per unit of mass (Segrest et al., 1971) compared with standard proteins, which result in a decrease in charge per unit of mass, a decreased mobility in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and thus a higher apparent molecular weight.

The observed molecular weight of gp70 was strongly dependent on the acrylamide concentration. An asymptotic minimal molecular weight of 67 500 was estimated for gp70—equal to its molecular weight calculated from compositional data.

The observed molecular weight of gp45 was found to be independent of the acrylamide concentration. An average molecular weight of 47 500 was calculated as the arithmetical mean of the molecular weight values obtained for the different percentages of acrylamide.

Further indication of the high degree of similarity of gp70 and gp45 was obtained by amino acid analyses. Despite some clear differences in the contents of threonine and valine, which bear confirmation, no other major differences were seen. In addition, immunologic analysis provided clear evidence of cross-reactivity. Anti-gp70 produced a single precipitin line with purified gp45 which was completely removed by absorption with gp70. The curvature of the precipitin lines of gp45 and gp70 was markedly different indicating distinct physical differences among molecules with similar antigenicity. This clearly excludes contamination of gp45 with gp70. The cross-reactivity is supported by results of radioimmunoassays (Marquardt and Charman, unpublished data) in which labeled gp45 was completely precipitated by anti-gp70 serum and the reaction was completely inhibited by gp70 free of gp45. Complete identity was not obtained in any of the immunologic tests performed. The results thus indicate antigenic sites unique to gp70. These could result from contribution of carbohydrate related determinants or conformational antibodies.

At present the significance of two glycoproteins with seemingly highly related polypeptide chains in virions is not clear. If, indeed, the only difference is in the degree of glycosylation, gp70 and gp45 may exist on different virion populations; i.e., gp45 may derive from incomplete noninfectious virions certainly present in large amounts in most type C viral preparations. If both are present in the same envelope, the evidence would suggest that gp70 occupies a more peripheral location since a percentage of the gp70 content is released simply by osmotic shock while gp45 remains tightly associated with the virion.

Natural history studies indicate the presence of antibody to gp70, gp45, and a third virion surface component, p15E, in sera of certain mouse strains (Ihle et al., 1974). These mouse sera show type-specificity for AKR gp70 and cross-reactivity among MuLV's based on group reactivity of p15E (Ihle et al., 1976). Based on our results, we would predict that AKR gp45 would exhibit type-specific reactivity with natural mouse antibodies. This seems to be the case as indicated by the failure of mouse sera with natural antibody to AKR virus to precipitate either RLV gp70 (Ihle et al., 1976) or gp45 (Charman,

personal communication) in highly sensitive radioimmunoassays.

Added in Proof

We have recently prepared antibodies to gp45 in guinea pigs. The antibody gives reactions of identity with gp70 and gp45 in immunodiffusion and neutralizes virus efficiently in in vitro assays.

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