JOURNAL OF VIROLOGY, Dec. 1992, p. 7270–7281
0022-538X/92/127270-12$02.00/0

Vol. 66, No. 12

Characterization of Lymphocytic Choriomeningitis Virus-Binding Protein(s): a Candidate Cellular Receptor for the Virus

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Received 30 June 1992/Accepted 15 September 1992

The attachment of lymphocytic choriomeningitis virus (LCMV) to murine and primate cell lines was quantitated by a fluorescence-activated cell sorter assay in which binding of biotinylated virus was detected with streptavidin-fluorescein isothiocyanate. Cell lines that were readily infected by LCMV (e.g., MC57, Rin, BHK, Vero, and HeLa) bound virus in a dose-dependent manner, whereas no significant binding was observed to lymphocytic cell lines (e.g., RMA and WIL 2) that were not readily infected. Binding was specific and competitively blocked by nonbiotinylated LCMV. It was also blocked by LCMV-specific antiserum and a neutralizing monoclonal antibody to the virus glycoprotein GP-1 but not by antibodies specific for GP-2, indicating that attachment was likely mediated by GP-1. Treatment of cells with any of several proteases abolished LCMV binding, whereas phospholipases including phosphatidylinositol-specific phospholipase C had no effect, indicating that one or more membrane proteins were involved in virus attachment. These proteins were characterized with a virus overlay protein blot assay. Virus bound to protein(s) with a molecular mass of 120 to 140 kDa in membranes from cell lines permissive for LCMV but not from nonpermissive cell lines. Binding was specific, since unlabeled LCMV, but not the unrelated enveloped virus herpes simplex virus type 1, competed with ^{125}I-labeled LCMV for binding to the 120- to 140-kDa band. The proteinaceous nature of the LCMV-binding substance was confirmed by the lack of virus binding to proteinase K-treated membrane components. By contrast, glycosidase treatment of membranes did not abolish virus binding. However, in membranes treated with endoglycosidase F/N-glycosidase F, and/or neuraminidase and in membranes from cells grown in tunicamycin, the molecular mass of the LCMV-binding entity was reduced. Hence, LCMV attachment to rodent fibroblastic cell lines is mediated by a glycoprotein(s) with a molecular mass of 120 to 140 kDa, with complex N-linked sugars that are not involved in virus binding.

The first step in virus infection of a host cell is attachment to the cell surface. One or more virion proteins, termed viral attachment proteins (VAPs), interact with components of the cell's plasma membrane to mediate this productive viral binding, and these membrane components constitute cellular receptors for the virus. Diverse constituents of host cell membranes can act as viral receptors. For example, sialyloligosaccharides are receptors for influenza virus (45) and phosphatidylserine and phosphatidylinositol are receptors for vesicular stomatitis virus (VSV) (38). Some well-characterized examples of protein virus receptors include the CD4 molecule for human immunodeficiency virus type 1 (17, 35), the C3d receptor CR2 for Epstein-Barr virus (26, 32, 40, 59), the acetylcholine receptor for rabies virus (37), the intercellular adhesion molecule-1 (ICAM-1) for the major subgroup of human rhinoviruses (29, 49, 54), another member of the immunoglobulin superfamily for poliovirus (39), a basic amino acid transporter for ecotropic murine leukemia virus (4, 34, 57), a phosphate transporter which is the receptor shared by gibbon ape leukemia virus and feline leukemia virus subgroup B (41, 51, 56), and a member of the carcinoembryonic antigen family of proteins for the coronavirus mouse hepatitis virus (22). These are among the few cellular receptors for viruses that have been identified; for most viruses, little is known about the nature of the host cell receptor or which virion protein acts as the VAP. In addition, a single virus may use distinct receptors to infect different cell types or host species.

The importance of defining the host cell receptors for viruses lies in at least two areas. First, receptor expression is a major factor in controlling the host range and tropism of a virus and hence plays a key role in determining virus pathogenicity. Second, the characterization of virus receptors facilitates the design of antiviral agents that can intervene early in viral infection. To date, no host cell receptors are known for the arenaviruses, a family including the human pathogens Lassa, Junin, and Machupo viruses. We have been studying the cellular receptors used by lymphocytic choriomeningitis virus (LCMV), the prototypic arenavirus, which is a natural pathogen in mice and infects many other species including humans (reviewed in reference 13). LCMV has a bisegmented, single-stranded, ambisense RNA genome. The viral RNA is complexed with the virus's nucleoprotein (NP) and is enclosed by a lipid envelope with which the two viral glycoproteins, GP-1 and GP-2, are associated. As recently shown (14), GP-2 is an integral membrane protein, whereas GP-1 is a more peripheral membrane protein noncovalently associated with GP-2. Both glycoproteins are homotetramers. Here we provide evidence that GP-1 rather than GP-2 serves as the VAP.

To study the attachment of LCMV to rodent and primate cell lines, we used a fluorescence-activated cell sorter (FACS) assay in which binding of biotinylated virus to cells was detected with streptavidin-fluorescein isothiocyanate (FITC). The results show that the cell surface LCMV-binding sites are proteinaceous in nature. We then went on to define the nature of the LCMV-binding protein(s) on rodent fibroblastic cell lines, using a virus overlay protein blot assay (VOPBA). LCMV is shown to bind to a 120- to

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140-kDa membrane glycoprotein(s) with complex N-linked sugars.

MATERIALS AND METHODS

Virus growth and purification. The ARM 53b strain of LCMV is a triple-plaque-purified clone from ARM CA 1371 (21), subsequently maintained by passage in baby hamster kidney (BHK-21) cells. Clone 13 is a triple-plaque-purified variant of this strain derived from spleen cells of an adult BALB/WEHI mouse persistently infected from birth with ARM 53b (3). Both viruses bound to and infected the cell types used here at similar levels. The FACS assay and VOPBA experiments described were carried out with clone 13.

LCMV from the culture fluid of infected cells was enriched by precipitation with 6.5% polyethylene glycol (without NaCl) followed by centrifugation at 35,000 rpm in a Beckman SW41 rotor for 75 min on a discontinuous renografin gradient (12, 14). The titer of the resulting virus preparation (determined by plaque assay on Vero cells; 21) was 5×10^9 to 2×10^{10} PFU/ml; and the protein concentration was 5 to 15 mg/ml, as determined by Bradford assay with bovine serum albumin (BSA) as the standard (9). Much of the protein was cellular components that copurified with the virus, since fluid from uninfected cell cultures processed in the same way yielded similar protein concentrations.

Virus stocks were free of mycoplasma contamination as judged by Hoechst staining of cells growing in antibiotic-free medium at 48 h after virus infection.

VSV used for FACS assay competition experiments was the Mudd-Summers strain of the Indiana serotype (a gift from J. Holland, University of California–San Diego, La Jolla, Calif.). This virus was grown in BHK-21 cells and enriched by pelleting culture fluids from infected cells and then banding them on a 5 to 40% continuous sucrose gradient (19). The resulting virus preparation had a titer of 10^{11} PFU/ml and a total protein concentration of 750 μg/ml.

Herpes simplex virus type 1 (HSV-1) used for VOPBA competition experiments was a recombinant, $Cgal^+$, expressing β-galactosidase (31). It was grown and assayed as described by Field and Wildy (25) and had a titer of 5×10^8 PFU/ml.

Origin and maintenance of cell lines. The origin and morphologic description of the majority of the cell lines used are given in Table 1. MC57, RMA, RIE, Rin, and WIL 2 cells were maintained in RPMI medium supplemented with penicillin, streptomycin, and 1% glutamine plus 3.5% fetal bovine serum (FBS) and 3.5% calf serum (both heat activated at 56°C for 30 min), as were EL-4 cells, a lymphoma line which expresses the Thy 1.2 antigen (47). BALB C1 7, Vero, and HeLa S3 cells were grown in minimal essential medium plus penicillin, streptomycin, 1% glutamine, 0.075% sodium bicarbonate, and 7% FBS. BHK-21 cells were grown in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, 1% glutamine, 0.7% glucose, 5% 2× tryptose phosphate broth, and 3.5% FBS plus 3.5% calf serum.

Infection of cells with LCMV and quantitation of infected cells by immunofluorescence and infectious center assays. Cells were infected with LCMV at a multiplicity of infection of 3 and grown overnight (16 to 20 h) at 37°C in 5% CO_2. The number of cells expressing the viral NP was then quantitated by immunofluorescence. Cells were resuspended at 2×10^6/ml in tissue culture medium, 25-μl drops of which were then spotted onto Cel-Line 10-175-coated slides (Cel-Line, Newfield, N.J.), air dried, and fixed in acetone. Staining was achieved by successive incubations for 45 min at room temperature with mouse monoclonal antibody 1-1.3 to the viral NP (11) (undiluted hybridoma tissue culture fluid) and a goat anti-mouse FITC-labeled second antibody (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) (diluted 1:50 in phosphate-buffered saline (PBS) plus 2% BSA), with three washes in PBS plus 2% BSA after each addition of antibody. The slides were mounted, and the percentage of NP-positive cells was determined with an Olympus BH-2 fluorescence microscope. The number of cells productively infected with LCMV was also quantitated, using an infectious center assay as described by Doyle and Oldstone (20).

TABLE 1. Origin and susceptibility to LCMV infection of cell lines used to study LCMV binding[a]

Cell line	Species of origin	Description	% Expressing NP	% Infectious centers
MC57	Mouse	Fibroblast	>90	>90
BALB Cl 7	Mouse	Fibroblast	>90	>90
RMA	Mouse	T-lymphocyte line	0.1	0.5
Rin	Rat	Insulinoma	>90	>90
BHK-21	Hamster	Kidney fibroblast	>90	>90
Vero	Monkey	Kidney	>90	>90
HeLa S3	Human	Epithelioid cervical carcinoma	>90	>90
WIL 2	Human	B-lymphocyte line	0.1	0.4

[a] Cells were infected with LCMV ARM 53b at a multiplicity of infection of 3 PFU per cell; 16 to 20 h later, aliquots of cells were fixed and the proportion expressing the viral nucleoprotein (NP) was determined by immunofluorescence (percent expressing NP). The number of cells scoring as infectious centers was also analyzed (percent infectious centers).

Analysis of LCMV binding to cells by FACS assay. To measure the binding of virus to cells, we modified the procedure described by Ingharimi et al. (30). Briefly, partially purified virus (see above; diluted to 1 to 5 mg of protein per ml) was mixed in a carbonate buffer (0.1 M $NaHCO_3$, 0.1 M NaCl [pH 8.3 to 9.0]) with *N*-hydroxysuccinimidobiotin (Sigma Chemical Co., St. Louis, Mo.) diluted to 1 mg/ml in dimethylsulfoxide. A 5/1 ratio of virus to biotin was incubated overnight in the dark at 4°C, after which free biotin was removed by extensive dialysis against PBS at 4°C. Virus infectivity was determined by plaque assay, and only biotinylated virus retaining >50% infectivity was used in experiments.

For the binding assay, amounts of biotinylated virus from 0 to 10^8 PFU (approximately 120 μg of total protein) were added to aliquots of 10^6 cells in suspension in a total volume of 500 μl of PBS plus 2% FBS and incubated on ice for 45 min. The cells were then washed twice in PBS plus 2% FBS and resuspended in 200 μl of a 1:75 dilution of streptavidin-FITC (Dakopatts, Carpinteria, Calif.) in PBS plus 2% FBS. After another 30-min incubation on ice, cells were washed once in PBS plus 2% FBS plus 1 μg of propidium iodide (PI) per ml and once in PBS plus 2% FBS only. Finally the percentage positive for fluorescence was determined by FACS analysis. Dead cells labeled with PI were counted as a measure of the viability of the population being tested and then were excluded from the analysis.

Specificity of binding was demonstrated by the ability of unlabeled LCMV but not VSV to block the binding of biotinylated LCMV to cells. For this experiment, cells were incubated on ice for 45 min with different amounts of unbiotinylated LCMV (10^8 to 10^9 PFU, approximately 100 to 1,000 μg of total protein) or unbiotinylated VSV (10^8 to 10^9

PFU, approximately 0.75 to 7.5 μg of total protein) in a final volume of 200 μl of PBS plus 2% FBS. Then, biotinylated LCMV was added (0 to 10^8 PFU, approximately 120 μg of total protein), and the total volume per tube was increased to 500 μl of PBS plus 2% FBS. The cells were incubated at 4°C for another 45 min, and the amount of biotinylated virus bound was determined as above.

FACS assay for EL-4 cell Thy 1 expression. Aliquots of 10^6 EL-4 cells were incubated on ice at 4°C for 45 min in 100 μl of the rat immunoglobulin G2b (IgG2b) monoclonal antibody 30.H12, specific for mouse Thy 1.2 (undiluted hybridoma tissue culture fluid; the hybridoma was obtained from the American Type Culture Collection, Rockville, Md.). The cells were then washed twice in PBS plus 2% FBS and resuspended in 100 μl of a 1:50 dilution in PBS plus 2% FBS of FITC-labeled $F(ab')_2$ fragments of mouse anti-rat IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.). After a 30-min incubation at 4°C, the cells were washed once in PBS plus 2% FBS plus 1 μg of PI per ml and once in PBS plus 2% FBS only. Cell viability and the percentage positive for fluorescence were determined by FACS analysis.

Blocking of virus binding to cells with LCMV-specific antibodies. The antibodies used were human convalescent serum from an LCMV-infected patient, which had a total protein concentration of 44 mg/ml as measured by the Bradford assay with BSA as a standard, an ELISA titer (the highest dilution giving a positive reaction in an enzyme-linked immunosorbent assay [ELISA]) against LCMV ARM (3) of 1:20,000, and a neutralization titer of >1:50. Additionally, we used three mouse monoclonal antibodies specific for LCMV: WE-36.1, an IgG1 antibody specific for GP-1 with a total protein concentration of 2 mg/ml and an ELISA titer against LCMV ARM of 1:10,000; WE-33.6, an IgG2a antibody specific for GP-2 with a total protein concentration of 2.6 mg/ml and an ELISA titer against LCMV ARM of 1:2,500; and 9-7.9, an IgG2a antibody specific for another epitope on GP-2 with a total protein concentration of 2.3 mg/ml and an ELISA titer against LCMV ARM of 1:2,500. The isolation and characterization of these monoclonal antibodies were described by Parekh and Buchmeier (44), and ascitic fluid containing the antibodies was a gift from M. Buchmeier (The Scripps Research Institute, La Jolla, Calif.).

To determine the ability of these antibodies to block the binding of LCMV to cells, biotinylated LCMV (2×10^7 or 4×10^7 PFU) was incubated with increasing dilutions of the antibodies, or with FBS (total protein concentration 83 mg/ml) as a negative control, on ice for 30 min. Binding of this virus and untreated biotinylated LCMV to MC57 cells was then assessed by FACS assay. Binding of the preincubated virus to cells is expressed as a percentage of the binding of a similar quantity of virus that was not preincubated with antibody.

Enzyme treatment of cells. To determine the nature of the cell surface component to which LCMV binds, aliquots of 10^6 MC57 or HeLa S3 cells in a total volume of 200 μl were treated with the enzymes or lectins listed below in PBS at pH 7.5 or the buffer specified for 1 h at 37°C and then were washed twice in PBS plus 2% FBS to remove the enzymes. Virus binding to the treated cells was analyzed by FACS assay. Enzymes were used at concentrations that did not significantly affect cell viability (measured by PI exclusion during the FACS analysis); these are detailed in the Results section. The enzymes used were as follows. Proteases: trypsin (EC 3.4.21.4) from bovine pancreas, 8,600 U/mg (solid) (Sigma, St. Louis, Mo.); α-chymotrypsin (EC 3.4.21.1) type 1-S from bovine pancreas, 53 U/mg (solid) (Sigma, St. Louis, Mo.); proteinase K (EC 3.4.21.14) from *Tritirachium album*, 20 U/mg (solid) (Boehringer Mannheim, Indianapolis, Ind.); subtilisin, protease type VIII from *Bacillus licheniformins*, 10 U/mg (solid) (Sigma, St. Louis, Mo.); pronase E, protease type XXV from *Streptomyces griseus*, 5 U/mg (solid) (Sigma, St. Louis, Mo.); papain (EC 3.4.22.2) from papaya latex, 14 U/mg (solid) (Sigma, St. Louis, Mo.), used in sodium phosphate buffer, pH 6.0; bromelain (EC 3.4.22.4) from pineapple stem, 3.6 U/mg (solid) (Sigma), used in sodium phosphate buffer, pH 6.0. Phospholipases: phospholipase A_2 (PLA_2) (EC 3.1.1.4) from bee venom, 1,020 U/mg (solid) (Sigma, St. Louis, Mo.); phospholipase C (PLC) from *Bacillus cereus*, 2 mg/ml (suspension) (Boehringer Mannheim, Indianapolis, Ind.); phospholipase D (PLD) from cabbage (solid) (Boehringer Mannheim, Indianapolis, Ind.), used in sodium phosphate buffer, pH 6.0; phosphatidylinositol-specific phospholipase C (PI-PLC) from *B. cereus*, 5 U/100 μl (Boehringer Mannheim, Indianapolis, Ind.). Glycosidases: recombinant *N*-glycanase, 10 U/40 μl (Genzyme, Cambridge, Mass.); neuraminidase from *Vibrio cholerae*, 1 U/ml (Boehringer Mannheim, Indianapolis, Ind.); *O*-glycanase from *Diplococcus pneumoniae*, 1 mU/μl (Genzyme, Cambridge, Mass.); β-galactosidase from *Escherichia coli* 1,500 U/ml (suspension) (Boehringer Mannheim, Indianapolis, Ind.); α-mannosidase from *Canavalia ensiformis*, 5 mg/ml (suspension) (Boehringer Mannheim, Indianapolis, Ind.); α-fucosidase (EC 3.2.1.51) from bovine epididymis, 2.5 U/mg (Sigma, St. Louis, Mo.). Sodium *m*-periodate was also obtained from Sigma (St. Louis, Mo.). Lectins: wheat germ agglutinin, lectin from *Thermoactinomyces vulgaris*, 31 μg/ml (Sigma, St. Louis, Mo.); concanavalin A type IV from *C. ensiformis*, 64 μg/ml (Sigma, St. Louis, Mo.). *N*-acetyl neuraminic acid release by neuraminidase was determined by the method of Aminoff (5).

Tunicamycin treatment of cells. MC57 or HeLa S3 cells were grown overnight (16 to 20 h) or for 2 to 4 days in medium containing either a mixture of tunicamycin homologs (Sigma, St. Louis, Mo.) or the B_2 homolog of tunicamycin (Boehringer Mannheim, Indianapolis, Ind.) at the concentrations described in the Results section.

Preparation of cell membranes. Membranes were prepared from cells that were subconfluent. Adherent cells were removed from the flask with a cell scraper, then these and nonadherent cells were pelleted by centrifugation at 1,200 rpm for 5 min in an IECPR-7000 centrifuge. Cells were washed once in homogenization buffer (250 mM sucrose, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4], 1 mM EDTA, 100 U of aprotinin per ml) and then resuspended in this buffer and homogenized by three to four passages through a ballbearing homogenizer (Bemi-Tech Engineering, Saratoga, Calif.). This procedure ruptured the plasma membrane but left internal organelles intact. Debris, including nuclei, was pelleted by centrifugation at 1500 rpm for 10 min in an IECPR-7000 centrifuge, membranes were pelleted from the remaining supernatant by a further spin at 32,500 rpm for 1 h at 4°C in a Beckman SW41 rotor, membrane pellets were resuspended in 0.01 M Tris HCl buffer, pH 7.5, and their protein concentrations were determined by the Bradford assay with BSA as a standard (9). Typically, 100 to 1,000 μg of protein (depending on the cell type used) was obtained from each T175 flask of cells. The membrane proteins were adjusted to 10 mg/ml, aliquoted, and stored at −70°C.

Separation of proteins by SDS-PAGE under reducing and

nonreducing conditions. Membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Sambrook et al. (46). Most experiments were performed with a Biorad (Richmond, Calif.) miniprotean II apparatus (7.2 by 10 cm plates), but some employed 18-cm^2 glass plates. Six percent separating and 3% stacking gels were used, and 50 to 100 μg of membrane proteins was run per well. Most experiments were carried out under reducing conditions: the proteins in a 20-μl volume were boiled for 3 min with 10 μl of a 3× reducing sample buffer (0.125 M Tris buffer [pH 6.8], 5% SDS, 1.8 M 2-mercaptoethanol, 0.25% glycerol, plus a small amount of bromophenol blue) before loading. For comparison, some gels were also run under nonreducing conditions: the proteins in a 20-μl volume were mixed with 10 μl of a 3× nonreducing sample buffer (0.125 M Tris buffer [pH 6.8], 5% SDS, 0.25% glycerol, and a small amount of bromophenol blue, but without 2-mercaptoethanol) and loaded without boiling. Gels were run at 15 V/cm until the dye front reached the bottom of the gel. Prestained high-molecular-weight SDS-PAGE protein standards (Biorad, Richmond, Calif.) were run on all gels. When 18-cm^2 gels were run to more accurately determine the size of the receptor protein band, unstained high-molecular-weight SDS-PAGE protein standards (from both Biorad, Richmond, Calif., and Pharmacia, Alameda, Calif.) were used. The lanes in which they were run were excised from the gel and stained with Coomassie blue (46) to locate the marker proteins.

VOPBA. Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose (0.45-μm-pore-size, Schleicher & Schuell, Keene, N.H.) using a Biorad transfer apparatus (46). The nitrocellulose was soaked overnight at 4°C in a milk buffer (2% Carnation nonfat milk, 0.2% Tween 20, in PBS) to block nonspecific binding sites, rinsed three times in PBS, and incubated successively with (i) LCMV concentrated and partly purified by precipitation with polyethylene glycol and renografin density gradient centrifugation, at approximately 10^{10} PFU/ml, diluted 1:10 in PBS plus 0.3% BSA; (ii) monoclonal antibodies specific for the LCMV glycoproteins (GP) (19), undiluted tissue culture fluid from the hybridomas WE-36.1 (GP-1 specific) and WE-33.1 (GP-2 specific) mixed 1:1; (iii) rabbit anti-mouse immunoglobulins (Dakopatts, Carpinteria, Calif.) diluted 1:5,000 in milk buffer; and (iv) ^{125}I-labeled protein A (Amersham, Arlington Heights, Ill.; 100 μCi/ml; specific activity, 30 mCi/mg of total protein A) diluted 1:100 in milk buffer. The first incubation lasted 1.5 h, and the others lasted 1 h each, all at 4°C on a rocking platform. After each incubation, the nitrocellulose was washed by being rocked in three changes of milk buffer for 10 min each at 4°C. After the final washes, the nitrocellulose was rocked overnight at 4°C in milk buffer, then blotted with Whatman 3MM paper, and exposed to Kodak XAR5 film, usually for 24 h.

In control experiments to determine the specificity of binding, the first layer was replaced with proteins from the culture fluids of uninfected cells which had been concentrated and purified in the same way as the virus or with PBS plus 0.3% BSA only. Alternatively, the second layer was replaced with undiluted tissue culture fluid from an irrelevant mouse monoclonal antibody (B22/249 or 15-5-5, each specific for different mouse major histocompatibility complex class I molecules) or with tissue culture medium only. In other experiments, the third layer was replaced with milk buffer only.

Preparation of ^{125}I-labeled LCMV for VOPBA and competitive binding experiments. Concentrated and purified LCMV was ^{125}I-labeled with the IODO-GEN reagent (Pierce, Rockford, Ill.). Briefly, 1 ml of virus (10^{10} PFU, or approximately 5 mg of total protein) was placed in an IODO-GEN-coated glass vial with 5 mCi of $Na^{125}I$ (Amersham, Arlington Heights, Ill.; 109 mCi/ml; specific activity, 17.0 mCi/μg of iodine) and left for 15 min at room temperature. The virus was then separated from unreacted ^{125}I by using a disposable excellulose desalting column (Pierce, Rockford, Ill.). The first 5 ml (1 column volume) of eluate contained the virus and had a specific activity of 1×10^5 to 5×10^5 cpm/μg of protein.

Binding of ^{125}I-LCMV to membrane protein(s) was evaluated with a modified VOPBA technique. The nitrocellulose blot was incubated for 1.5 h at 4°C on a rocking platform with iodinated material from the column in which BSA was dissolved to give a 0.3% solution, which reduced nonspecific binding of iodinated proteins. The nitrocellulose was then washed in many changes of milk buffer (with rocking, at 4°C) for 5 to 8 h, blotted dry, and exposed to film.

To demonstrate the specificity of ^{125}I-labeled LCMV binding, a competition experiment was performed. First, three nitrocellulose blots were incubated for 1.5 h at 4°C with 3 ml of PBS plus 0.3% BSA containing (i) no virus, (ii) 3×10^8 PFU/ml of unlabeled LCMV, or (iii) 1.5×10^8 PFU of HSV-1 per ml. Second, ^{125}I-labeled LCMV (1/3 of the material from one column, diluted to 2 ml and with BSA added to 0.3%), plus competing virus as in the first step, was added for an additional 1.5-h incubation at 4°C. After extensive washing in milk buffer, the three blots were exposed to film.

Enzyme treatment of membrane proteins. In a series of experiments, membrane protein preparations from MC57 and BHK cells were treated with different proteolytic or glycosidic enzymes before being separated by SDS-PAGE and VOPBA. Membrane proteins were adjusted to 10 mg/ml, and enzyme digests were performed on 9-μl aliquots, i.e., 90 μg of total protein.

(i) **Proteases.** One microliter of a 10-mg/ml solution of protease was added before overnight incubation at 37°C. The proteases used were proteinase K, α-chymotrypsin, subtilisin, pronase E, and papain.

(ii) **Endoglycosidase H.** Fifteen microliters of 2× endoglycosidase H buffer (50 mmol/liter of citrate buffer, pH 5.0, with 2.5% SDS, 0.2 mol/liter of 2-mercaptoethanol, and 0.5 mmol/liter of phenylmethylsulfonyl fluoride) was added, and the tube was boiled for 2 min. After being cooled on ice, 2.5 mU (5 μl) of endoglycosidase H (of *Streptomyces plicatus* from a recombinant *E. coli*; EC 3.2.1.96; 1 U/ml; Boehringer Mannheim, Indianapolis, Ind.) was added before overnight incubation at 37°C.

(iii) **Endoglycosidase F.** One microliter of 10% SDS was added, and the tube was boiled for 2 min. After being cooled on ice, 90 μl of endoglycosidase F buffer (20 mM sodium phosphate buffer, pH 7.6, with 10 mM sodium azide, 50 mM EDTA, and 0.5% [wt/vol] *n*-octyl-β-D-glucopyranoside [NOG]) was added. The tube was boiled again for 2 min and cooled on ice, then 10 μl, equal to 0.5 U of endoglycosidase F/N-glycosidase F (from *Flavobacterium meningosepticum*, 50 U/ml, Boehringer Mannheim, Indianapolis, Ind.), was added for overnight incubation at 37°C.

(iv) **Neuraminidases.** One microliter of 10% SDS was added, and the tube was boiled for 2 min. After being cooled on ice, 15 μl of 2× neuraminidase buffer (20 mM sodium phosphate buffer, pH 6.0, with 20 mM magnesium acetate, 2 mM phenylmethylsulfonyl fluoride, and 1.2% [wt/vol] NOG) was added. Then, 5 μl of neuraminidase was added, and the

tube was incubated overnight at 37°C. Neuraminidases (EC 3.2.1.18) were from *Clostridium perfringens* (1.1 U/mg [solid], dissolved to be 167 mU/5 μl; Sigma, St. Louis, Mo.); *Salmonella typhimurium* (16 U/mg of protein, 32 U/ml, i.e., 161 mU/5 μl; Sigma, St. Louis, Mo.); *V. cholerae* (12 U/mg of protein, 0.14 mg of protein per ml, i.e., 8 mU/5 μl; Sigma, St. Louis, Mo.), and *Arthrobacter ureafaciens* (81.6 U/mg of enzyme, dissolved to be 167 mU/5 μl; Calbiochem, La Jolla, Calif.).

(v) ***O*-glycosidase.** This enzyme was used after digestion of proteins with neuraminidase. Membrane proteins were treated with doses of neuraminidase from 3 to 167 mU as described above for 1 h at 37°C, then 3 μl, equal to 3 mU *O*-glycanase (from *D. pneumoniae*; EC 3.2.1.97; 1 mU/μl; Genzyme, Cambridge, Mass.), was added, followed by overnight incubation at 37°C.

(vi) Multiple enzyme digests involving the use of neuraminidase (with or without *O*-glycanase) and endoglycosidase F. Membrane proteins were boiled with 1 μl of 10% SDS for 2 min and cooled, then 90 μl of all-enzyme buffer was added (20 mM sodium phosphate buffer, pH 7.0, with 10 mM sodium azide, 50 mM EDTA, 6% [wt/vol] NOG, and 1 mM phenylmethylsulfonyl fluoride). Sequential digests with the enzymes (as described above) were then performed in the order (i) endoglycosidase F/N glycosidase F (1 h), (ii) neuraminidase (1 h or overnight), and (iii) *O*-glycanase (overnight) at 37°C.

For these experiments, control incubations were always performed, in which MilliQ water was substituted for the test enzyme. These controls, the enzyme-digested samples, and completely untreated membranes were analyzed in parallel by SDS-PAGE and VOPBA.

Experiments examining kinetics of return of the LCMV-binding protein to the cell surface after removal by proteases. Subconfluent MC57 cells in T75 flasks were rinsed two times in PBS and then rocked gently at 37°C for 1 h with 2 ml of proteinase K at 20 μg/ml in PBS or with PBS alone (untreated control flask). Twenty milliliters of complete RPMI medium (containing 7% serum, see above) plus 100 U of aprotinin (Sigma, St. Louis, Mo.) per ml was then added to each flask, and membranes were prepared from the untreated control cells and one of the protease-treated samples (time zero). The remaining protease-treated flasks of cells were incubated at 37°C in 5% CO_2, and after the specified lengths (15, 30, and 45 min and 1, 1.5, 2, and 3 h) of incubation, membranes were prepared from one of these flasks. The pelleted membranes from each flask of cells were resuspended in 30 μl of 0.01 M Tris (pH 7.5), and 10 μl of each sample was analyzed by SDS-PAGE and VOPBA.

To determine whether binding protein return to the cell surface required new protein synthesis, four T75 flasks of MC57 cells were preincubated for 5 h at 37°C in 5% CO_2 with complete RPMI medium plus 100 μg of cycloheximide (Sigma, St. Louis, Mo.) per ml. They were then rinsed two times in PBS, and three flasks were treated with 20 μg of proteinase K per ml in PBS plus 100 μg of cycloheximide per ml, and one flask was treated with PBS plus 100 μg of cycloheximide per ml for 1 h at 37°C. Twenty milliliters of complete RPMI medium plus 100 U of aprotinin per ml and 100 μg of cycloheximide per ml was added to each flask, and membranes were prepared from the non-protease-treated cells and one of the protease-treated samples (time zero). The other two flasks were incubated for 3 and 6 h at 37°C in 5% CO_2 before membrane preparation. Four other flasks of cells were simultaneously treated in an identical manner, but in the absence of cycloheximide. Membrane samples from all eight flasks were analyzed by SDS-PAGE and VOPBA.

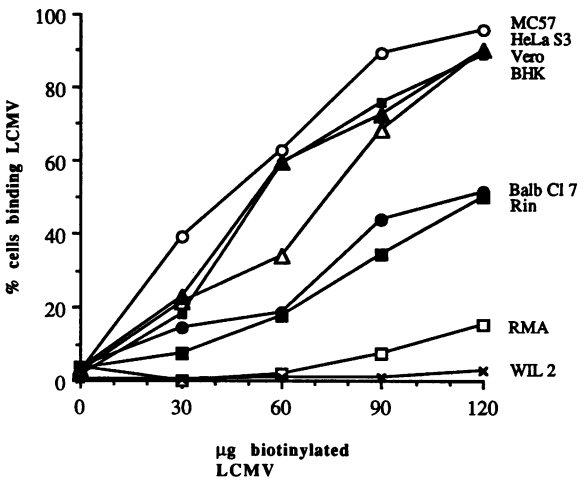

FIG. 1. LCMV binding to several cell types as assessed by FACS assay. Aliquots of 10^6 cells of each type were incubated with increasing amounts of biotinylated LCMV and were processed as described in Materials and Methods. The percentage of FITC-positive cells, i.e., cells that bound the virus, is shown. These results were confirmed in at least five separate experiments.

RESULTS

The nature of the cell surface component(s) to which LCMV attaches was investigated by using a virus-binding assay, in which cells were incubated at 4°C with biotinylated LCMV and then with streptavidin-FITC, and the amount of virus bound to the cell surface was quantitated by FACS analysis. This assay was carried out on a panel of rodent and primate cell lines, whose origin and susceptibility to infection by LCMV are detailed in Table 1. As shown by these cells' expression of LCMV NP and scores for infectious centers (indicating productive infection) 16 h after exposure to LCMV, only RMA and WIL 2 cells were resistant to LCMV. All other cell lines tested were productively infected.

The results of a FACS assay comparing the ability of various cells to bind LCMV are illustrated in Fig. 1 and represent one of several repeated observations. Cell lines readily infected by LCMV bound virus in a dose-dependent manner but, by contrast, the infection-resistant lymphocyte lines RMA and WIL 2 failed to bind significant amounts of virus. To confirm that the binding observed in this assay was specific, we carried out a competition experiment. Unbiotinylated LCMV successfully competed in a dose-dependent manner with the biotinylated virus for binding sites on MC57 cells (Fig. 2). However, the unrelated enveloped virus VSV was unable to do so.

Next, anti-LCMV antiserum and monoclonal antibodies specific for LCMV proteins were tested as inhibitors of virus binding to ensure that the virus-cell surface interaction being measured was both specific and biologically relevant to the infection process. Preincubation of biotinylated LCMV with either of the two antibodies having neutralizing activity, human convalescent serum or the murine monoclonal antibody WE-36.1 against GP-1, blocked virus binding. In contrast, two nonneutralizing monoclonal antibodies against epitopes on GP-2, WE-33.6 and 9-7.9, and the FBS control

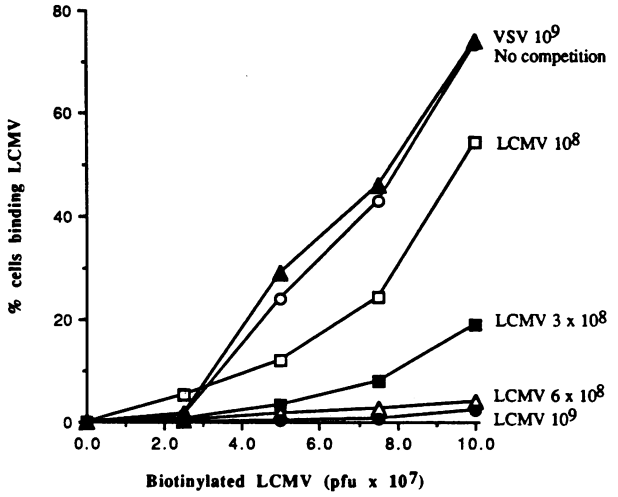

FIG. 2. Competition experiments illustrate that the binding of LCMV to MC57 cells is specific. Groups of 10^6 MC57 cells were incubated on ice for 45 min with either no virus, doses of unbiotinylated LCMV from 10^8 to 10^9 PFU, or 10^9 PFU of unbiotinylated VSV. The amounts of biotinylated LCMV indicated on the bottom axis were then added to the cells in each group, and a further 45-min incubation on ice was carried out. After the cells were washed and incubated on ice for 30 min with streptavidin-FITC, the percentage of FITC-positive cells, i.e., cells with bound biotinylated LCMV, was quantitated by FACS assay.

did not reduce virus binding (Fig. 3). The difference in blocking ability of the three monoclonal antibodies was not due to a different quantity of antiviral antibody in the ascites used, because the ELISA titers of the three ascites preparations against LCMV were similar (WE-36.1, 1:10,000;

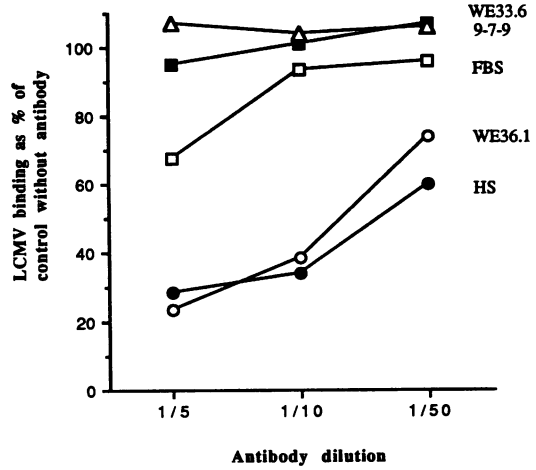

FIG. 3. LCMV-specific antibodies block virus binding to cells. Biotinylated LCMV was incubated on ice for 30 min with the indicated dilutions of the following: human serum from a convalescent LCMV-infected patient (HS); the LCMV GP-1-specific monoclonal antibody WE-36.1; two monoclonal antibodies, WE-33.6 and 9-7.9, specific for different epitopes on LCMV GP-2; or FBS as a control. The binding of preincubated virus and untreated biotinylated LCMV to MC57 cells was assessed by FACS assay. The binding of the preincubated virus to cells is expressed as a percentage of the binding of the untreated virus measured in the same experiment.

TABLE 2. Effect of protease treatment of MC57 cells on the ability to bind LCMV

Protease	Concn	Binding as % of untreated control	Cell viability (%)
Trypsin	0.1 μg/ml	102.8	98
	1 μg/ml	59.9	98
	10 μg/ml	13.1	98
Chymotrypsin	0.1 μg/ml	59.3	98
	1 μg/ml	54.5	97
	10 μg/ml	6.3	98
Proteinase K	0.1 μg/ml	44.4	96
	1 μg/ml	22.4	90
	10 μg/ml	1.5	76
Subtilisin	2 mU/ml	15.9	87
	10 mU/ml	12.3	85
	50 mU/ml	2.2	94
Pronase E	10 μg/ml	4.7	88
	100 μg/ml	0.1	96
Papain	2 mU/ml	64.1	95
	10 mU/ml	14.9	93
	50 mU/ml	4.0	93
Bromelain	2 mU/ml	30.2	94
	20 mU/ml	16.9	94
	200 mU/ml	7.7	94

WE33.6 and 9-7.9, 1:2,500). Since monoclonal antibodies specific for GP-1 but not the other candidate VAP, GP-2, blocked virus binding, these results suggest that the VAP of LCMV is GP-1.

To determine the biochemical nature (e.g., protein, lipid, or carbohydrate) of the LCMV-binding component on the cell surface, we used the FACS assay to test cells pretreated in ways that destroyed individual membrane components for the ability to bind LCMV. The results of representative experiments (all treatments were tested at least twice) with the murine fibroblast cell line MC57 are shown in Tables 2 through 4. Results shown are the virus binding of treated cells expressed as a percentage of that of appropriate control cells analyzed in the same experiment. All cells were labeled with PI preceding FACS analysis so that the effect of each treatment on cell viability could be assessed; the percentage of live cells in control and treated samples is also given in the tables.

Table 2 shows the effect on LCMV binding of pretreating 10^6 MC57 cells in a 1-h incubation at 37°C with each of seven proteases at the indicated concentrations. All proteases used here, which included both serine and thiol proteases, ablated LCMV binding in a dose-dependent manner, indicating that the LCMV-binding substance was either a protein or a protein-bound entity.

In agreement with this result, treatment of cells with PLA_2, PLC, or PLD did not significantly affect the ability to bind LCMV (Table 3). Table 4 shows the results of experiments with PI-PLC, which removes glycosyl-phosphatidylinositol-linked moieties from the cell surface. As a control, the effect of PI-PLC on cell surface levels of Thy 1, a glycosyl-phosphatidylinositol-linked, pronase E-resistant protein on the EL-4 thymoma cell line, was assessed in parallel with its effect on LCMV binding to MC57 cells. FACS assay showed that PI-PLC treatment drastically reduced Thy 1 expression on the EL-4 cell line but did not significantly affect LCMV binding to MC57 cells, indicating that the membrane protein(s) on which LCMV binding is dependent is not attached by glycosyl-phosphatidylinositol linkage.

TABLE 3. Effect of phospholipase treatment of MC57 cells on the ability to bind LCMV

Phospholipase	Concn (μg/ml)	Binding as % of untreated control	Cell viability (%)
PLA_2	0.1	100.7	95
	1	98.8	95
PLC	10	87.3	96
	100	86.3	88
PLD	10	81.9	88
	100	93.7	88

A final series of FACS experiments (not shown) determined the role of carbohydrates in LCMV binding to MC57 cells. Treatment for an hour at 37°C with a panel of glycosidases did not affect the cells' subsequent ability to bind LCMV. The glycosidases used included *N*-glycanase and *O*-glycanase, which remove N- and O-linked sugars, respectively, from proteins, and four enzymes that remove terminal carbohydrates from sugars. In addition, treatment of cells with periodate, which oxidizes cell surface sialic acid, had no effect on LCMV binding. These enzymes were all used at concentrations known to reduce the binding of other viruses to cells (27, 36, 50). That neuraminidase did remove sialic acid from these cells was also demonstrated by the release of *N*-acetyl neuraminic acid into the supernatant fluid (5; data not shown). Incubation of cells with the lectin concanavalin A (which binds to α-linked terminal mannose residues on N-linked high mannose or hybrid glycans) or wheat germ agglutinin (which recognizes $(GlcNAc\beta1\text{-}4)_n$ on N-linked glycans) for an hour at 37°C prior to assessment of virus binding also did not decrease LCMV binding to the cells. Last, MC57 cells grown overnight in a mixture of tunicamycin homologs or the B_2 homolog, which inhibits N-linked glycosylation but has little effect on protein synthesis, bound LCMV effectively, indicating that glycosylation was not required for virus binding and/or that the LCMV-binding protein had a long half-life.

We then went on to characterize the membrane protein(s) to which LCMV binds, utilizing a VOPBA. In this technique, membrane proteins are separated by SDS-PAGE and transferred onto nitrocellulose that is then incubated successively with virus, mouse monoclonal antibodies specific for the viral glycoproteins GP-1 and GP-2, a rabbit anti-mouse antibody, and ^{125}I-labeled protein A. A typical VOPBA of membranes from six rodent cell lines is shown in Fig. 4. Figure 4A illustrates the binding of LCMV to a 120- to 140-kDa band in membranes from permissive BALB C1 7, MC57, Rin, and BHK cells but not in membranes from the lymphoid cell lines RMA and RIE, which are relatively

TABLE 4. Comparison of the effect of PI-PLC treatment on EL-4 cell Thy 1 expression and the ability of MC57 cells to bind LCMV

Cell line	PI-PLC concn (U/ml)	Thy 1 expression as % of untreated control	LCMV binding as % of untreated control	Cell viability (%)
EL-4	1	11.6		96
	2	3.7		96
	4	5.8		96
MC57	1		80.0	85
	2		86.0	88

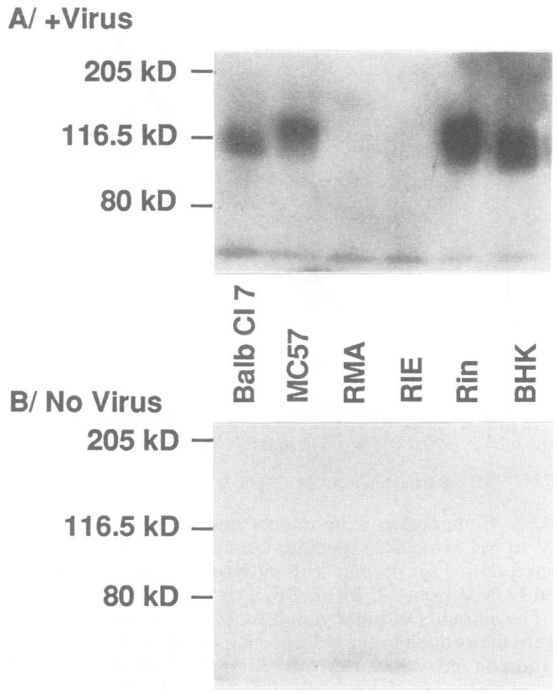

FIG. 4. Analysis of LCMV-binding proteins by VOPBA. Membrane proteins (50 to 100 μg) from four murine cell lines (BALB Cl 7, MC57, RMA, and RIE), a rat cell line (Rin), and a hamster cell line (BHK) were run on two 7.2 by 10 cm SDS-PAGE gels, transferred onto nitrocellulose, and analyzed by VOPBA. The upper blot was treated with all the VOPBA layers. In the lower blot, virus was omitted from the first incubation to control for nonspecific binding.

resistant to LCMV infection. Equal quantities of membrane proteins from each cell type were run on these gels. When LCMV was omitted or when polyethylene glycol-precipitated, renografin-banded proteins from the culture fluids of uninfected cells were used in place of virus as the first VOPBA layer, the 120- to 140-kDa band was not seen (Fig. 4B), indicating that the band in Fig. 4A does represent the interaction of LCMV with particular membrane components in cell types readily infected by the virus. In experiments using higher-percentage gels, no specific binding of LCMV to lower-molecular-weight proteins was detected (not shown).

Further evidence that binding to the 120- to 140-kDa band represented an LCMV-specific interaction was documented by direct binding of iodinated LCMV to nitrocellulose blots of membrane proteins (Fig. 5). Figure 5 also demonstrates the specificity of the binding reaction, because unlabeled LCMV blocked binding but, in contrast, use of an unrelated enveloped virus, HSV-1, did not.

The LCMV-binding proteins on the four rodent cell lines tested (two mouse cell lines, one rat, and one hamster) appear to vary slightly in molecular size (Fig. 4A). To more accurately determine the size of these proteins, membranes were run on 18-cm^2 gels. The results of several experiments (not shown) allowed the calculation that the LCMV-binding component of the murine fibroblastic cell line MC57 had a size of 116 to 137 kDa, whereas that of Rin cells had a slightly larger size, and those of BALB C1 7 and BHK cells were somewhat smaller. These differences likely reflected posttranslational modifications, such as the extent of each

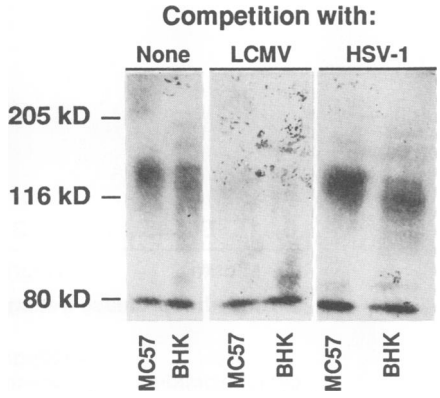

FIG. 5. Competition experiment to show that the direct binding of ^{125}I-labeled LCMV to the 120- to 140-kDa band is specific. LCMV virions were ^{125}I-labeled by the IODO-GEN procedure. The two lanes on the left of this figure show virus binding to membrane proteins from MC57 and BHK cells which had been separated by SDS-PAGE and transferred onto nitrocellulose. The lanes in the center were incubated with unlabeled LCMV prior to and during the treatment with ^{125}I-labeled LCMV, and those on the right were incubated with unlabeled HSV-1 prior to and during treatment with ^{125}I-labeled LCMV.

protein's glycosylation (see below). Further, the appearance of the band was similar under reducing (Fig. 4 and 5) and nonreducing conditions (not shown), indicating that the virus-binding entity did not have disulfide-linked subunits. The virus-binding component could also be solubilized from membranes by detergent treatment (not shown).

To confirm that the LCMV-binding substance was proteinaceous, VOPBAs were performed on MC57 and BHK cell membranes treated with proteinase K prior to SDS-PAGE. As shown in Fig. 6, LCMV failed to react with these membranes; similar results were obtained with other proteases (α-chymotrypsin, subtilisin, pronase E, and papain; data not shown).

The effect of glycosidase treatment of the membrane proteins was also investigated. Endoglycosidase H, which

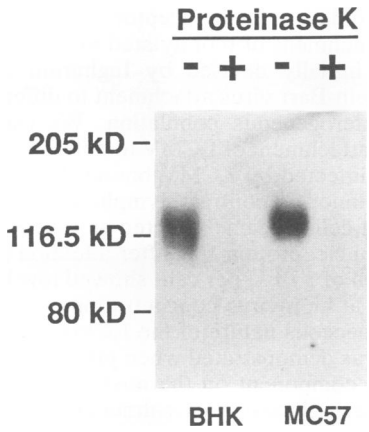

FIG. 6. Effect of proteinase K treatment of membrane components on the entity to which LCMV binds. Ninety-microgram aliquots of membrane proteins from MC57 and BHK cells were incubated overnight at 37°C with (+) or without (−) 10 μg of proteinase K, run on an SDS-PAGE gel, transferred to nitrocellulose, and analyzed by VOPBA for LCMV binding.

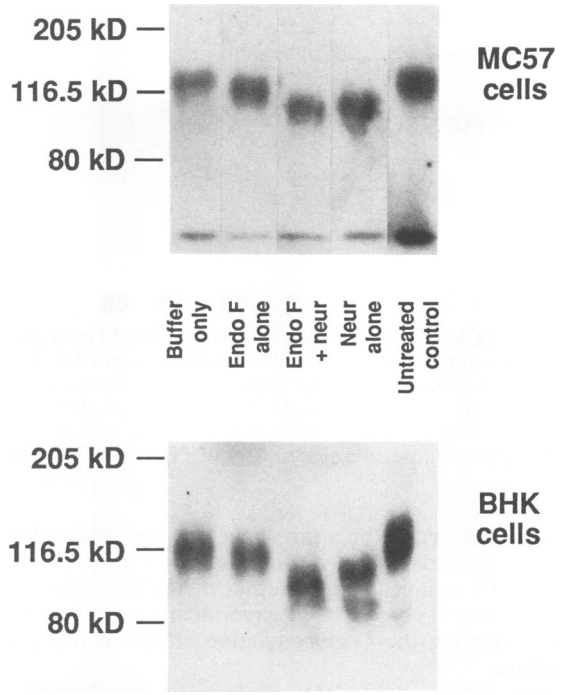

FIG. 7. Effect of neuraminidase and endoglycosidase F/N-glycosidase F treatment of membrane components on the entity to which LCMV binds. Ninety-microgram aliquots of membrane proteins from MC57 cells (upper panel) and BHK cells (lower panel) were denatured and incubated overnight in a pH 7.0 buffer without enzymes (Buffer only), with 0.5 U of endoglycosidase F/N-glycosidase F only (Endo F alone), with 167 mU of neuraminidase from *C. perfringens* only (Neur alone), or for 1 h with neuraminidase and then overnight with endoglycosidase F/N-glycosidase F (Endo F/+ Neur). These samples and untreated control membranes were then run on SDS-PAGE gels, transferred to nitrocellulose, and analyzed by VOPBA for LCMV binding.

removes high-mannose-type carbohydrates from glycoproteins had no effect on the binding ability or size of the membrane component recognized by LCMV (not shown). By contrast, neuraminidases, which remove terminal sialic acid residues from complex glycoproteins, decreased the size of the LCMV-binding protein (Fig. 7). LCMV binding thus occurs to a glycoprotein(s) which bears complex, but not high-mannose-type, carbohydrates. Endoglycosidase F/N-glycosidase F (which removes N-linked sugars from glycoproteins) also decreased the size of the LCMV-binding protein (Fig. 7), indicating that there are N-linked carbohydrates attached to this protein. The neuraminidase used in the experiment shown in Fig. 7 was from *C. perfringens*; this preferentially hydrolyses α(2-3) linkages of sialic acids. Similar results were obtained with two other neuraminidases that also hydrolyse α(2-3) linkages faster than α(2-6) linkages (from *S. typhimurium* and *V. cholerae*) and with neuraminidase from *A. ureafaciens*, which has a higher hydrolysis rate of α(2-6) bonds (not shown).

Neuraminidase produced a larger decrease in the molecular weight of the LCMV-binding membrane component than did endoglycosidase F/N-glycosidase F (Fig. 7), likely reflecting a more efficient reaction because the bonds it hydrolyses are more accessible than those cleaved by endoglycosidase F/N-glycosidase F. Alternatively, the LCMV-binding protein might bear O-linked as well as N-linked

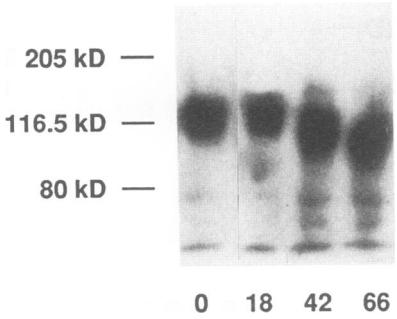

FIG. 8. LCMV binding to membranes from MC57 cells grown in the presence of tunicamycin. Membranes were prepared from MC57 cells grown without tunicamycin (lane 0) or in medium containing 1 μg of tunicamycin per ml overnight (18 h), for 2 days (42 h), or for 3 days (66 h). The membranes were run on SDS-PAGE gels, transferred to nitrocellulose, and analyzed by VOPBA for LCMV binding.

sugars, which would be a target for neuraminidases but not for endoglycosidase F/N-glycosidase F. A large number of experiments to investigate whether the LCMV-binding protein was also O-glycosylated gave inconsistent results (not shown), leaving the O-glycosylation status of this protein uncertain.

Figure 8 records LCMV binding to membranes from MC57 cells grown for different times in the presence of tunicamycin, an inhibitor of N-linked glycosylation of proteins. In agreement with the results of VOPBAs on endoglycosidase F/N-glycosidase F-treated membranes (Fig. 7), the LCMV-binding protein in membranes from cells treated with tunicamycin for approximately 2 or 3 days was smaller than that in membranes from untreated cells, but virus binding was not affected. Interestingly, overnight tunicamycin treatment did not change the molecular mass of the LCMV-binding protein, indicating that the protein likely has a fairly long half-life.

A final series of experiments examined the kinetics of return of the LCMV-binding protein to the cell surface after removal by proteases. Treatment of cells for 1 h at 37°C with 20 μg of proteinase K per ml reduced the LCMV-binding capacity of their membranes to levels undetectable by VOPBA (Fig. 9). After protease removal, the LCMV-binding protein first became detectable again after 45 to 60 min and reached its original levels in 3 to 6 h. Receptor return to the cell surface did not represent cycling of preexisting internal pools of the protein to the cell surface but required new protein synthesis, because it could be blocked by cycloheximide (Fig. 9b).

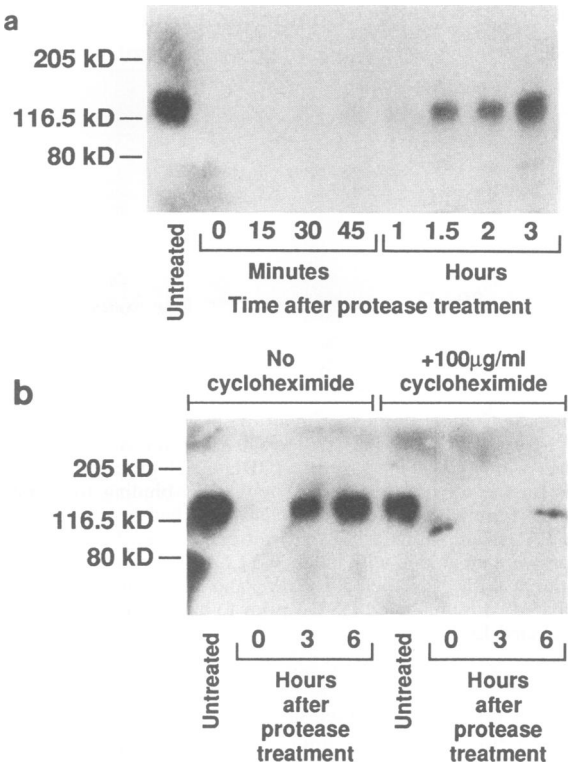

FIG. 9. Time course of LCMV-binding protein return to the cell surface after removal by proteases and cycloheximide block of protein return. MC57 cells were treated for 1 h at 37°C with 20 μg of proteinase K per ml in PBS or with PBS alone (untreated) and then were resuspended in RPMI + 7% serum + 100 U of aprotinin per ml and incubated at 37°C in 5% CO_2 for the indicated times before membrane preparation. An equal proportion of the membranes from the cells in each flask was run on a 7.2 by 10 cm SDS-PAGE gel and analyzed by VOPBA for LCMV binding. (b) Two sets of four flasks of cells were processed in parallel. One set was treated with 100 μg of cycloheximide per ml for 5 h prior to and throughout the experiment, and the other was not (No cycloheximide).

DISCUSSION

We have shown that the arenavirus LCMV binds to 120- to 140-kDa proteins on rodent fibroblastic cell lines. These proteins are heavily glycosylated, with complex N-linked sugars. The presence of O-linked sugars is not clear, but carbohydrate is not involved in virus binding. Variations in glycosylation most likely account for the slight variability in sizes of the LCMV-binding proteins on mouse, rat, and hamster cell lines. Significant binding of LCMV to and the presence of an LCMV-binding protein were not detected on lymphoid cell lines which were not readily infected by the virus. The binding of LCMV to its candidate cell surface receptor protein is likely mediated by the virion's GP-1 molecule.

Two techniques were used to investigate LCMV interaction with candidate cellular receptors. The first of these, in which the attachment of biotinylated virus to cells is quantitated, was initially devised by Ingharimi et al. (30) to monitor Epstein-Barr virus attachment to different lymphoid cells in a heterogeneous population. We used it here to compare the attachment of LCMV to different cell lines. Cell lines readily infected by LCMV bound the virus in a dose-dependent manner. In contrast, lymphoid cell lines, in which <0.1% of the cells became infected as assessed by expression of viral nucleoprotein 16 h after infection at a multiplicity of infection of 3 PFU per cell, showed low levels of virus binding even at high virus concentrations.

The proteinaceous nature of the LCMV-binding entity on MC57 cells was demonstrated when proteases destroyed the virus-binding component on the host cell surface, whereas phospholipase or glycosidase treatment of cells did not affect virus binding (Tables 2, 3, and 4). The protein binding LCMV was sensitive to all the proteases used, which included serine proteases (chymotrypsin, proteinase K, subtilisin, and trypsin), thiol proteases (bromelain and papain), and pronase, which is a mixture of proteases. By contrast, many other cell surface proteins show differential suscepti-

bilities to proteases, e.g., major histocompatibility complex class I proteins are insensitive to trypsin or chymotrypsin but are cleaved near the cell surface by papain.

Cell membrane proteins either may possess hydrophobic sequences by means of which they are inserted into the lipid bilayer or may be attached to the membrane via covalent linkage to bilayer lipids: a glycosyl-phosphatidylinositol anchor. Bacterial PI-PLC enzymes will cleave most protein-glycosyl-phosphatidylinositol anchors and are commonly used in glycosyl-phosphatidylinositol anchor identification (24). PI-PLC did not remove the LCMV-binding protein from the cell surface, suggesting that it was not glycosyl-phosphatidylinositol linked.

The cell membrane protein(s) to which LCMV was binding was further characterized by using a VOPBA. The VOPBA technique has been used to characterize putative cell receptors for a number of viruses, including Sendai virus (28), mouse hepatitis virus-A59 (8), reovirus (15, 55), human cytomegalovirus (1, 52), Theiler's virus (33), and visna virus (16, 18). With mouse hepatitis virus, the virus-binding protein originally identified by VOPBA was also later shown to act as the virus receptor in vitro and in vivo (22). LCMV binding to a 120- to 140-kDa band was demonstrated by VOPBA. By several criteria, this represented a specific interaction. First, no binding followed omission of any layer in the VOPBA (Fig. 4) or replacement with an irrelevant component, e.g., substitution of proteins from uninfected cells for the virus layer or of monoclonal antibodies specific for antigens other than LCMV for the virus-specific monoclonal antibodies. Second, iodinated LCMV bound directly to the same 120- to 140-kDa band, and unlabeled LCMV, but not an unrelated enveloped virus, HSV-1, inhibited this binding. Third, binding to the 120- to 140-kDa band was found in membranes prepared from four rodent cell lines that LCMV bound to and infected efficiently but not in protein(s) prepared similarly from the membranes of two murine lymphoid cell lines which are much more resistant to LCMV infection (Fig. 4A).

The diffuse appearance of the LCMV-binding protein band under reducing conditions is consistent with the presence of intramolecular disulfide bonds and/or some posttranslational modification, e.g., oligosaccharide processing. Glycosylation of the 120- to 140-kDa protein was shown because neuraminidase, which removes terminal sialic acid residues from complex carbohydrates, and endoglycosidase F/N-glycosidase F, which removes N-linked sugars from glycoproteins, reduced its size. Although the receptor protein was heavily glycosylated, carbohydrates were not involved in virus binding. LCMV bound to the protein itself, since glycosidase treatment of membranes did not alter the efficiency of virus binding, whereas proteinase K treatment of membranes completely destroyed the virus-binding ability. It is of interest that the LCMV-binding protein band still had a diffuse appearance even after treatment with endoglycosidase F/N-glycosidase F (Fig. 7) and on membranes from tunicamycin-treated cells (Fig. 8). This could be because the deglycosylation achieved was incomplete; but an alternative explanation is that the band seen may in fact represent two or more proteins of slightly different molecular weights which are not resolved by the VOPBA technique. Work is currently in progress to investigate this.

Our results suggest that LCMV uses the same proteins for attachment to fibroblastic cell lines from several species (mouse, rat, and hamster). However, whether the same receptor is utilized in all species or in all tissues infected by the virus remains to be determined. In preliminary experiments, we have also demonstrated binding of LCMV to a 120- to 140-kDa protein band in VOPBAs on membrane proteins from two primate cell lines (Vero and 143TK$^-$) that are readily infected by LCMV.

One question of particular interest for understanding the pathogenesis of persistent infection is how viruses infect cells of the immune system. Such an infection appears to be a key factor in the ability of a virus to cause persistence in vivo, since as far as is known, all viruses that cause persistent infections infect cells of the immune system (reviewed in reference 42). In mice persistently infected with LCMV, most tissues carry high levels of virus as detected by in situ hybridization (23, 48), but only 1 to 6% of peripheral blood lymphocytes are infected (2, 43, 53). The idea that lymphocyte infection may be restricted at the level of receptor expression is suggested by our previous findings that only 1 to 2% of T cells of the CD4$^+$ and CD8$^+$ subsets bind the virus (7). In keeping with this hypothesis, we showed here that a 120- to 140-kDa LCMV-binding protein could not be detected on membranes from the murine T-lymphocyte cell lines tested (RMA and RIE). How a small percentage of lymphocytes become infected is not clear but may reflect low levels of expression of the 120- to 140-kDa receptor protein, the presence of a unique receptor of low affinity, or expression restricted to a subpopulation of cells at a particular point in the cell cycle or stage of differentiation or activation.

The data presented here cast light not only on the nature of the host cell receptor bound by LCMV but also on the virion protein that mediates receptor attachment (VAP). Although there is one unconfirmed report of detecting viral NP on the virion surface (10), it is generally accepted that the viral glycoproteins GP-1 and GP-2 are the only proteins exposed on the surfaces of intact virions (reviewed in reference 6; 10a, 42a). Of these two candidate VAPs, GP-1 is the more likely possibility for two reasons. First, it is a peripheral membrane protein, whereas GP-2 is an integral membrane protein (12). Second, Parekh and Buchmeier (44) and Wright and Buchmeier (58) noted that of multiple monoclonal antibodies raised to epitopes on the LCMV glycoproteins, those able to neutralize virus in vitro or mediate protection in vivo were only directed against GP-1, whereas antibodies to GP-2 were nonneutralizing. We found that the GP-1-specific antibody WE-36.1 blocked LCMV binding to MC57 cells, whereas two monoclonal antibodies against different overlapping epitopes on GP-2 failed to affect virus binding (Fig. 3). This finding provides strong evidence that GP-1 mediates LCMV attachment to cells, although direct proof that GP-1 is the VAP awaits evidence that purified GP-1 binds to the cellular receptor for LCMV.

In summary, we have identified a 120- to 140-kDa glycoprotein component of the membrane of cell lines to which LCMV binds which is a candidate cellular receptor for the virus. To definitively document that the 120- to 140-kDa band is the LCMV receptor, we are (i) currently purifying the protein, (ii) raising antibodies to it that will be tested for the ability to block virus attachment both in vivo and in vitro, and (iii) attempting to isolate the gene encoding this protein from bacterial and eukaryotic expression libraries.

ACKNOWLEDGMENTS

The authors thank Michael Buchmeier for LCMV-specific antibodies and helpful discussions; Sandy Schmidt and John Elder for help and advice; Don McQuiddy, Leo Fernandez, and John Jourbran for invaluable expertise with FACS analysis; Victor Vedovato

for technical assistance; and Valerie Felts, Jodi Johnson, and Beatrice Cubitt for preparation of the manuscript and figures.

This work was supported by NIH grants AI09484, NS12428, and AG04342. Persephone Borrow was the recipient of postdoctoral fellowships from the National Multiple Sclerosis Society (FG 822-A-1) and the Multiple Sclerosis Society of Great Britain and Northern Ireland (M/C/S-2/89 [A]).

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