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Characterization of Murine-Specific Leukemia Virus Receptor from L Cells

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The host cell receptor for Moloney murine leukemia virus was solubilized from murine L-cell membranes and characterized. In initial studies designed to identify a receptor-rich cell line, different mouse cells were screened for binding to Moloney gp70, the viral envelope glycoprotein which determines host cell-binding specificity. gp70 binding to murine L cells was specific and saturable, with an apparent affinity constant (K_a) of 4×10^8 M^{-1}, and the number of receptors per cell (6×10^5) was similar to that of other mouse fibroblast cell lines. Characterization of the gp70 receptor with regard to extraction by detergents, protease sensitivity, and heat denaturation suggests that the receptor is an intrinsic membrane protein. Upon extraction of L-cell membranes with 0.2% deoxycholic acid and precipitation with acetone, specific and saturable binding of gp70 could be detected. The solubilized gp70-binding component was eluted upon gel filtration on Sephacryl S-300 into a species with an approximate molecular weight of 110,000.

The study of animal retroviruses provides important information for understanding the onset of leukemogenesis and the spread of pathological agents. In mice, initiation of leukemogenesis is commonly attributed to the action of murine-infecting C-type viruses, although the detailed mechanism of leukemogenesis is not known. Analogous C-type viruses have been found in humans, as well as in felines, primates, and other species (for a review, see reference 34). One approach to determination of the mechanism by which these viruses recognize and enter host cells is through the identification and characterization of receptors for ecotropic murine leukemia viruses (MuLVs).

The initial event in the penetration of host cells by retroviruses appears to be attachment to specific receptors on the cell surface. For example, avian leukosis viruses require a particular surface determinant specified by an autosomal dominant gene for absorption and penetration (10, 32). Binding studies involving viral envelope glycoproteins and host cells, interference and neutralization studies, and pseudotype mixing experiments have all served to define the requirement of MuLVs for specific host cell determinants (3, 7, 11). At least four classes of viral receptors have been identified by interference and binding studies: ecotropic (mouse-infecting), xenotropic (nonmouse-infecting), amphotropic (mouse and non-mouse-infecting) and dualtropic (mink cell focus-forming-type mouse and non-mouse-infecting) (7, 11, 22).

The viral specificity is determined by the envelope glycoprotein gp70. Viruses lacking the envelope glycoprotein through genetic or enzymatic means will not infect normally permissive cells (6, 23). Somatic cell hybrids between mouse and Chinese hamster cells have been used to localize the receptor for murine ecotropic viruses to mouse chromosome 5 (15, 19, 21, 25) and the receptor for dualtropic mink cell focus-forming viruses from C3H mice to chromosome 1 (14). These results indicate that there are distinct populations of viral receptors in mouse cells which can be distinguished by their ability to bind envelope glycoproteins from the respective viral classes.

The best characterized viral receptor is that of the ecotropic viruses. Early binding studies with purified gp70 demonstrated specific and saturable binding of gp70 to murine fibroblasts and lymphocytes (4, 5, 7, 8, 13). The ecotropic MuLV receptor is presumed to be a protein, since trypsin and chymotrypsin treatment abolish binding activity (4, 13). Studies based upon cross-linking (3), affinity chromatography (16), or immunoprecipitation (24, 27) have identified several putative gp70 binding proteins ranging in molecular weight from 10,000 to 190,000. The relationship of these different proteins to each other or to the receptor for MuLV is not known. The major problem with these studies is the lack of an adequate means of verifying the identity of a putative membrane-bound viral receptor. Thus, specific, saturable binding of gp70 has been demonstrated in intact cells and isolated membrane preparations but not in solubilized systems.

We now report detergent solubilization and partial characterization of a saturable and stable gp70-binding component. A filtration assay was developed for solubilized receptor, which enables the quantitation of gp70-binding activity in dilute protein solutions. Following gel filtration chromatography, the gp70-binding activity eluted in the molecular weight range of 67,000 to 150,000.

MATERIALS AND METHODS

Materials. DEAE-cellulose DE52 was obtained from Whatman, Inc., Clifton, N.J.; lentil lectin and molecular weight marker proteins (bovine serum albumin [BSA], ovalbumin, and ribonuclease) were obtained from Pharmacia, Inc., Piscataway, N.J.; octyl-β-D-glucoside and ([3-cholamidopropyo]-dimethylaminonio)-1-propane sulfonate (CHAPS) were obtained from Behring Diagnostics; Triton X-100 was obtained from Rohm & Haas Co., Philadelphia, Pa.; deoxycholic acid (DOC) was recrystallized from 70% ethanol (28); BSA for binding assays, phenylmethylsulfonyl

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fluoride, and molecular weight marker proteins (apoferritin, β-amylase, and alcohol dehydrogenase) were obtained from Sigma Chemical Co., St. Louis, Mo.; egg lecithin was obtained from Avanti Polar Lipid, Inc.; sodium [^{125}I]iodide (11 to 17 mCi/μg) was obtained from Amersham Corp., Arlington Heights, Ill.; Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) was obtained from Pierce Chemical Co., Rockford, Ill.; Nuflow cellulose acetate membrane filters (pore size, 0.45 μm; diameter, 25 mm) were obtained from Oxoid Ltd., Basingstoke, England.

Source of cells and virus. The following cell lines were used: mouse L cells (26), NIH 3T3 cells (12), and Chinese hamster E36 cells (9). Moloney murine leukemia virus (MoLV) clone 1-infected NIH 3T3 cells were used as a source of virus. Cells in monolayer culture were maintained at 37°C in Dulbecco modified Eagles medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). L cells grown in suspension were maintained in RPMI 1640 (GIBCO) supplemented with 5% calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (0.025 μg/ml). For harvesting virus, MoLV-infected cells were grown in Dulbecco modified Eagle medium supplemented with 5% calf serum. Cells used for binding studies were plated 48 h in advance at 1×10^5 to 2×10^5 cells per well (2 cm^2 per well) in 24-well dishes or 1.5×10^4 to 3×10^4 cells per well (0.28 cm^2 per well) in 96-well dishes.

Preparation of MoLV clone 1. MoLV was harvested from 20 to 40 liters of clarified medium (12,000 × *g*, 10 min) as follows: the virus was banded on a 250-ml 15 to 50% sucrose gradient in buffer A (50 mM Tris hydrochloride [pH 7.8], 10 mM NaCl, 1 mM EDTA) for 1 h at 25,000 rpm in a CF-32 rotor and a model L5-50 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The banded virus was pumped out with 60% sucrose (wt/wt) in buffer A, slowly diluted threefold with buffer A, and pelleted at ca. 100,000 × *g* for 1 h. The pellets were resuspended in a small volume of buffer A and banded on four to six sucrose gradients (31 ml each; 25 to 45% sucrose) at 20,000 rpm overnight in an SW 28 Beckman rotor. The viral bands were removed, slowly diluted with buffer A, and repelleted. The viral pellets were frozen at −80°C until use. The yield of viral protein from 20 liters of media was approximately 100 to 200 mg, corresponding to 166 to 333 mg of virus.

Purification of the viral glycoprotein gp70. Banded, frozen MoLV was thawed and solubilized in Buffer A containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 0.14 TIU of trasylol per ml at 4°C. The virus was homogenized in a Dounce homogenizer and incubated on ice for 30 min. Particulate material was removed by centrifugation at 25,000 × *g* for 20 min. The supernatant fraction, which contained the gp70, was dialyzed overnight against 10 mM Tris hydrochloride (pH 7.3)–140 mM NaCl containing 0.5% Triton X-100 (20). $MnCl_2$ (1 mM) was added to the dialyzed viral protein (20 mg), and the dialysate was applied to a lentil lectin column (9 ml) equilibrated in the same buffer. gp70 eluted with 0.2 M α-methyl mannoside and 1% octyglucoside. If further purification was required, the lentil lectin column was eluted with the above-mentioned buffer containing 0.5% Triton X-100 as well as α-methyl mannoside, and the eluate was dialyzed overnight against 0.1 mM EDTA–0.5% Triton X-100–10 mM Tris hydrochloride (pH 7.8) at 4°C. The dialysate was applied to a DE-52–cellulose column (1.5 by 5 cm) and eluted with a linear gradient of 0 to 300 mM NaCl in Tris buffer containing 1% octylglucoside. The final gp70 preparation was dialyzed against phosphate-buffered saline overnight and stored at −80°C prior to use. This procedure yields gp70 as a single band when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. gp70 prepared by chromatography on a P-11 column (16) had comparable activity.

Labeling of gp70 with ^{125}I-iodide. Purified gp70 was ^{125}I-iodide labeled with the iodinating reagent chloroglycoluril described by Markwell and Fox (18). The labeled proteins were 90% precipitable with 10% trichloroacetic acid, and the final specific activity was 0.3×10^4 to 5×10^4 cpm/ng of protein.

Preparation of membranes and solubilization of the gp70 receptor. L-cell membranes were prepared by the $ZnCl_2$ hypotonic lysis method developed by Warren et al. (33) and modified by Bardin and Johnstone (1). Briefly, the cells were homogenized in the hypotonic buffer, the cell debris and nuclei were removed by low-speed centrifugation, and the membrane fraction was purified on a step 30 to 60% sucrose gradient. The membrane bands were extracted, diluted in water (1.5-fold), mixed with EDTA to remove the zinc, and then centrifuged at 10,000 × *g* for 30 min. The membrane pellets can be stored at −80°C for 1 month without loss of activity.

To solubilize membranes, the frozen membrane pellets were thawed, washed by centrifugation once in 100 mM NaCl–20 mM Tris hydrochloride (pH 7.8)–1 mM phenylmethylsulfonyl fluoride (buffer B), and resuspended in the same buffer. DOC (0.4%) in the same buffer was added to a final concentration of 0.2% with an approximate membrane protein concentration of 2 mg/ml. The membranes in detergent buffer were incubated for 15 min at 4°C. The resultant suspension was centrifuged at 100,000 × *g* for 1 h. The clear supernatant fraction containing the solubilized receptor was assayed directly following precipitation or was subjected to further purification.

Precipitation of solubilized receptors. Before the gp70 binding to solubilized receptor fractions was assayed, the receptors were precipitated. To assay maximal specific activity, the solubilized membrane fractions were diluted fivefold in buffer B and incubated for 10 min at 4°C, and the resultant precipitate was pelleted by centrifugation at 100,000 × *g* for 1 h. To increase the yield of precipitated receptor (routinely 30 to 50%), although slightly decreasing the specific activity, cold acetone was routinely added at a final concentration of 37.5% (29), and then the mixture was centrifuged at 20,000 × *g* for 20 min at 4°C. The pellet was then suspended in 100 mM NaCl–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) (pH 7.0) (buffer C). For column-purified receptor fractions, approximately 2 mg of L-α-lecithin per ml in 30 mM Tris hydrochloride (pH 7.0), prepared by the method of Schneider et al. (29), was added before precipitation. In all cases, the buffer was adjusted to 0.1 to 0.3 M NaCl prior to precipitation.

Binding assays for gp70. (i) Binding of gp70 to cell monolayers. For characterization of ^{125}I-labeled gp70 binding to the intact cells, the following assay was developed. Confluent monolayers of viable cells were propagated for 24 to 48 h in 24- (or 96-) well dishes. Cells were then rinsed once with reaction buffer 1 (Dulbecco modified Eagle medium, 0.1% BSA, 50 mM HEPES [pH 7]) at 25°C. The washed cells were incubated at 37°C for 60 min with 1 to 150 ng of ^{125}I-labeled gp70 in 0.2 ml (or 0.03 ml) of reaction buffer. The supernatant was then removed, and the cell monolayers were rinsed twice with cold reaction buffer. Cells were dispersed with 0.5 ml (or 0.05 ml) of 0.2 N NaOH for 20 min at 70°C and washed with HCl (or swabbed with Q-tips [Johnson and Johnson, Inc.]), and the solution (or Q-tips) was transferred to vials

for determination of radioactivity. Nonspecific binding, determined by binding to cells in the presence of 100-fold-excess gp70 or to receptor-minus or virus-infected cells, was subtracted from the value obtained. The maximum amount of added radiolabeled gp 70 which binds to cells was 10%. Standard deviation mean values from triplicate or duplicate determinations was less that 15%.

(ii) Binding of gp70 to membranes or reconstituted receptor. Membranes or precipitated receptor fractions were suspended in buffer C, and a portion was added to the incubation mixture containing ^{125}I-labeled gp70 in reaction buffer 2 (100 mM NaCl, 20 mM HEPES [pH 7.0], 1% BSA, 2 mM $CaCl_2$) or reaction buffer 1 containing 1% BSA but without sodium bicarbonate. To assay the receptor-containing fractions, two methods were used: centrifugation and filtration. The centrifugation method minimizes nonspecific assay background binding; the advantage of the filtration assay is to collect 2 to 3 times more activity associated with the precipitated receptor and to rapidly assay multiple samples. Background due to the gp70 alone was 0.5% of the added ^{125}I-labeled gp70 in both assay systems. ^{125}I-labeled gp70 was centrifuged at 12,000 × *g* before use in binding studies. Of the radioactivity and protein, 80% remained in the supernatant fraction. Nonspecific binding was determined by incubating the labeled gp70 in the presence of 5 to 10 mM EDTA, in a 100-fold excess of unlabeled gp70, or with heat-treated (100°C, 10 min) receptor fractions. Specific binding was calculated by subtracting the value for nonspecific binding from the value for total binding.

For the centrifugation assay, following the incubation of membranes (30 to 50 μg of protein) with 100 μl of radiolabeled gp70 (ca. 1 ng/μl) in reaction buffer 2 at room temperature, a small amount was removed (3 to 25 μl). The membranes were centrifuged through 400 μl of fetal calf serum at 12,000 × *g* for 5 min. The tips containing the pellet were cut off, and the radioactivity was determined by counting.

For the filtration assay, samples were routinely incubated in 100 μl of reaction buffer 2, as described for the centrifugation assay. The filters (cellulose acetate; pore size, 0.45 μm) were soaked in 100% fetal calf serum for at least 30 min before assay and then placed on the filtration apparatus (Hoefer Scientific Instruments). The ice-cold reaction buffer (5 ml) was added to the filters without suction, and then a 25-μl portion of the reaction mixture was added. Suction was applied, and the filter was washed once with 10 ml of ice-cold buffer prior to transfer to a plastic tube for determination of radioactivity.

Determination of protein. Protein was assayed by the method of Lowry et al. (17) as modified by Bensadoun and Weinstein (2). Standard curves were the same whether octyglucoside, CHAPS, or DOC (10 to 20 μg) was added. To obtain maximal protein determination, the precipitate was solubilized with approximately 3% octylglucoside (final concentration) before addition of DOC and trichloroacetic acid precipitation (29). Standard curves were generated by using BSA in the same buffer as the samples.

Sephacryl S-300 chromatography of detergent-solubilized L-cell membrane protein. L-cell membranes (4 mg) were solubilized as described above at the given DOC concentrations. The solubilized membranes (850 μg of protein in 1 ml) were applied to a column of Sephacryl S-300 (1 by 49 cm) equilibrated in 100 mM NaCl–20 mM Tris hydrochloride (pH 8.0) at the given DOC concentration. The column was eluted at a flow rate of 0.3 ml/min. The fraction size was 0.5 ml. Aliquots were removed, lipid was added, and the mixture was precipitated with ice-cold acetone as described above. Binding of ^{125}I-labeled gp70 to the precipitate was measured as described above. The void volume (V_0) was determined by the elution position of blue dextran; the included volume (V_i) was the elution position of [^{3}H]*N*-acetylglucosamine (3 μCi). The K_{av} for the protein markers are plotted against the logarithm of their molecular weights. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume for a protein and V_t is the total bed volume.

Enzyme and urea treatment of L-cell membranes. For enzyme treatment of L-cell membranes, 50 μg of membrane protein in phosphate-buffered saline (100 μl) was used for each incubation condition. For neuraminidase treatment, 0.1% BSA was included. All incubations were at room temperature, except for those with pronase and neuraminidase, which were at 37°C. After 30 min of incubation, 20 μg of ice-cold soybean trypsin inhibitor was added, and the samples were placed on ice. The samples were diluted with 1 ml of ice-cold phosphate-buffered saline, mixed, and centrifuged at 12,000 × *g* for 10 min. Incubation of membrane residues (approximately 4 μg per 25-μl aliquot) with ^{125}I-labeled gp70 (10 ng/25 μl) is described above (specific activity of ^{125}I-labeled gp70, 10^4 cpm/ng of gp70.

For urea treatment of L-cell membranes, 1.2 mg of membrane protein per ml of 100 mM NaCl–20 mM HEPES (pH 7.0) with or without 4 M urea was incubated for the designated time 4°C. An aliquot was removed, diluted fivefold in NaCl-HEPES buffer, and centrifuged at 12,000 × *g* for 10 min. Incubation of membrane residues (approximately 1 μg of membrane protein per 25-μl aliquot) with ^{125}I-labeled gp70 (13 ng/25 μl) is described above (specific activity of ^{125}I-labeled gp70, 5×10^3 ng of gp70 per mg of protein).

RESULTS

Characterization of MoLV gp70 binding to cells. To find a receptor-rich cell line, mouse cells were characterized with respect to the affinity of binding (K_a) and the number of receptors per cell. A comparison of MoLV gp70 binding to the Chinese hamster cell line E36, the mouse fibroblast cell line NIH 3T3, and the mouse L-cell line (Fig. 1A) shows specific and saturable binding to confluent monolayers of the mouse cells, with highest binding to L cells. It should be noted that studies with proteases confirmed that gp70 remains bound on the cell surface and is not internalized under these conditions (data not shown). To ensure that the gp70-binding component in L cells was a functional receptor, we infected L cells with MoLV and could demonstrate complete loss of MoLV gp70 binding, a characteristic of ecotropic virus-infected cells, after 2 weeks (data not shown). Nonspecific binding to mouse L cells, determined with an excess of unlabeled gp70, with MoLV-infected cells, or with Chinese hamster E36 cells, was less than 10% of the total gp70 bound (Fig. 1A).

Scatchard analysis of gp70 binding to mouse L cells showed a linear plot indicative of a single class of receptor sites (Fig. 1B). The number of receptors per cell was 6×10^5; the apparent affinity constant (K_a) was 4×10^8 M^{-1}. NIH 3T3 cells had approximately 4×10^5 receptors per cell with similar affinity (4, 7). Since L cells in monolayer occupy less than 10% of the area of an equivalent number of fibroblast cells, they yield more receptors per volume of packed cells. Further, L cells can also be grown in suspension to facilitate membrane preparation. Since L cells show specific and saturable binding and are a good source of gp70 receptor, they were used for further purification studies.

Characterization of gp70 binding to isolated cell mem-

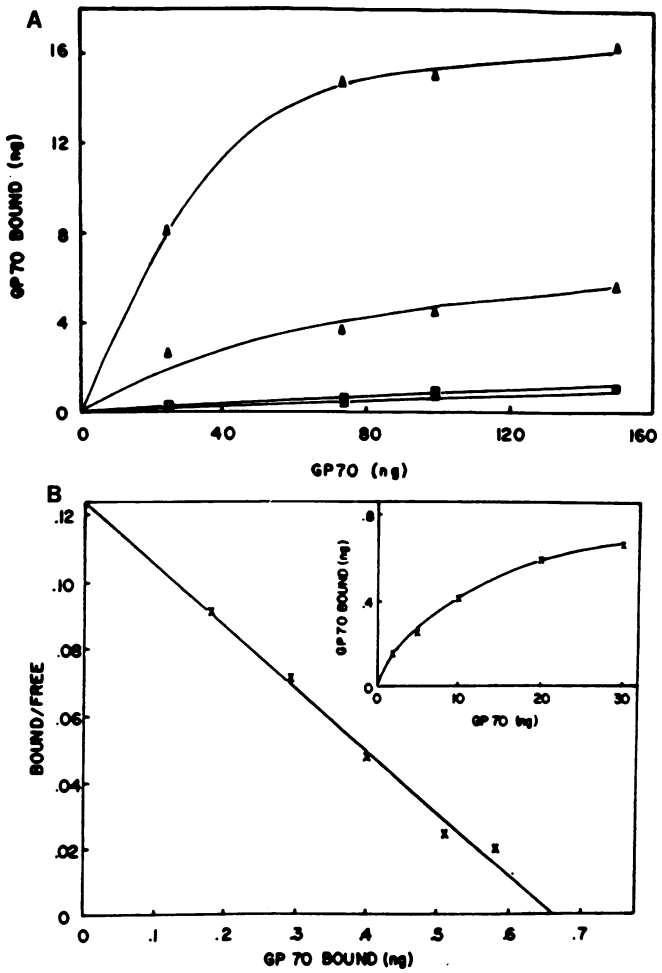

FIG. 1. Saturable binding of ^{125}I-labeled gp70 to different cell lines. Confluent monolayers of cells were incubated in reaction buffer containing 30 μl of ^{125}I-labeled gp70 as described in Materials and Methods and (specific activity of ^{125}I-labeled gp70, 5.5×10^2 cpm/ng) Each point is the average of duplicate determinations. Symbols: △, L cells; ▲, NIH 3T3 cells; □, E36 cells; ■, L cells plus 4 μg of gp 70. (B) Scatchard analysis of ^{125}I-labeled gp70 binding to L cells. L cells (10^4 cells per well) were incubated with ^{125}I-labeled gp70 as for panel A (specific activity of ^{125}I-labeled gp70, 2.4×10^4 cpm/ng). From analysis of the plot of ^{125}I-labeled gp70-specific binding to L cells (inset), a Scatchard plot was generated. Bound/free refers to the amount of ^{125}I-labeled gp70 bound (ng/30 μl) divided by the amount of unbound gp70 in the reaction mixture.

branes. Since the mouse cell gp70-binding component appeared to be localized to the cell surface, we isolated L-cell membranes and characterized the gp70-binding activity (see Materials and Methods). To obtain a value for nonspecific binding, a number of criteria were tested. Heat treatment (100°C for 10 min at pH 7) of membranes, addition of 5 mM EDTA, or addition of excess unlabeled gp70 gave similar levels of nonspecific binding of gp70 to membranes (Fig. 2A). gp70 binding to L-cell membranes was proportional up to 25 μg of membrane protein (Fig. 3A), and equilibrium was reached after 15 min at room temperature (Fig. 4A). Binding was specific and saturable, with properties similar to those of the intact cell (Fig. 5). The apparent K_a, 1.0×10^8 M^{-1}, was within the same order of magnitude as that for intact cells. To further confirm the specificity of the binding reaction, studies were also conducted with human HeLa cell membranes which do not contain the gp70 receptor and can also be isolated from cells grown in suspension. No specific binding of labeled gp70 to HeLa membranes was observed (data not shown).

Several lines of evidence suggest that the gp70 receptor is an intrinsic membrane protein. gp70-binding activity was lost following treatment with 4 M urea, consistent with the receptor's being a protein (Table 1). Limited freeze-thawing of the membranes and heat treatment resulted in loss of over 50% of the gp70-binding activity. Further, treatment of membranes with a variety of enzymes, including neuraminidase, chymotrypsin, trypsin, and pronase, confirmed previ-

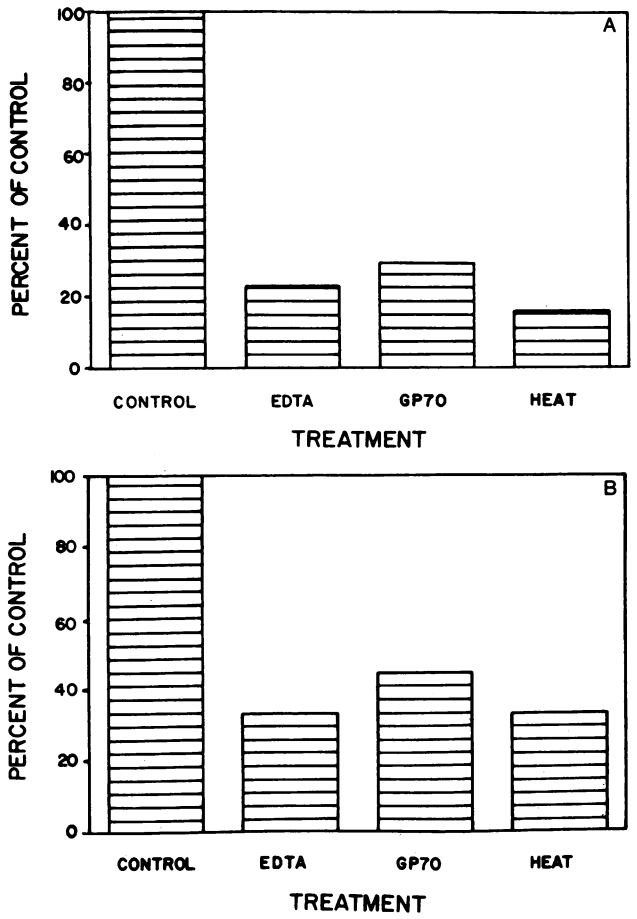

FIG. 2. Specific ^{125}I-labeled gp70 binding to (A) L-cell membranes and (B) detergent-solubilized membrane protein. (A) Membranes (8 μg of L-cell membrane protein per 25 μl) were incubated with ^{125}I-labeled gp70 (14 ng/25 μl) in the presence or absence of excess gp70. Samples were centrifuged and analyzed as described in Materials and Methods. The values given are the mean of triplicate determinations (specific activity of ^{125}I-labeled gp70, 9.5×10^3 cpm/ng). The samples were treated as follows. Control: untreated; EDTA: 5 mM EDTA added; gp70: 100-fold excess of unlabeled gp70 added; heat: samples heated at 100°C for 10 min prior to assay. (B) Extracts from 0.2% DOC-solubilized L-cell membranes were precipitated with acetone as described in Materials and Methods. The precipitate (40 μg/20 μl) was incubated with ^{125}I-labeled gp70 (11 ng/20 μl) in the presence or absence of excess unlabeled gp70 and filtered as described in Materials and Methods. The values given are mean of triplicate determinations (specific activity of ^{125}I-labeled gp70, 1.6×10^4 cpm/ng). Samples were the same as in panel A, except that the concentration of EDTA was 10 nM.

ous studies (4, 13) indicating that the gp70 receptor-binding activity is sensitive to protease treatment and resistant to sialic acid removel. Finally, extraction with 0.9 M sodium iodide, a chaotropic ion which releases extrinsic membrane proteins, resulted in the retention of 83% of the original membrane-associated receptor activity (data not shown). These findings, together with denaturation and protease studies, suggest that the cell surface receptor for gp70 is an intrinsic membrane protein and should be released by detergents.

Solubilization of the gp70 receptor. The conditions for receptor release were determined by treatment of L-cell membranes with a variety of detergents, including octylglucoside, CHAPS, and DOC. Under these conditions, approximately 7 to 30% of the original gp70-binding activity remained associated with the detergent-insoluble residue. Routinely, L-cell membranes were solubilized with 0.2 to 0.4% DOC, which consistently extracted 20 to 40% of the membrane protein.

To monitor the release of gp70-binding activity following detergent solubilization, a precipitation assay was developed for the gp70 receptor. The details of the reconstitution assay and binding studies are presented in Materials and Methods. Following detergent extraction of L-cell membranes, the supernatant fraction was precipitated with 30 to 40% (vol/vol) acetone to retain maximal activity (Fig. 6A). If the

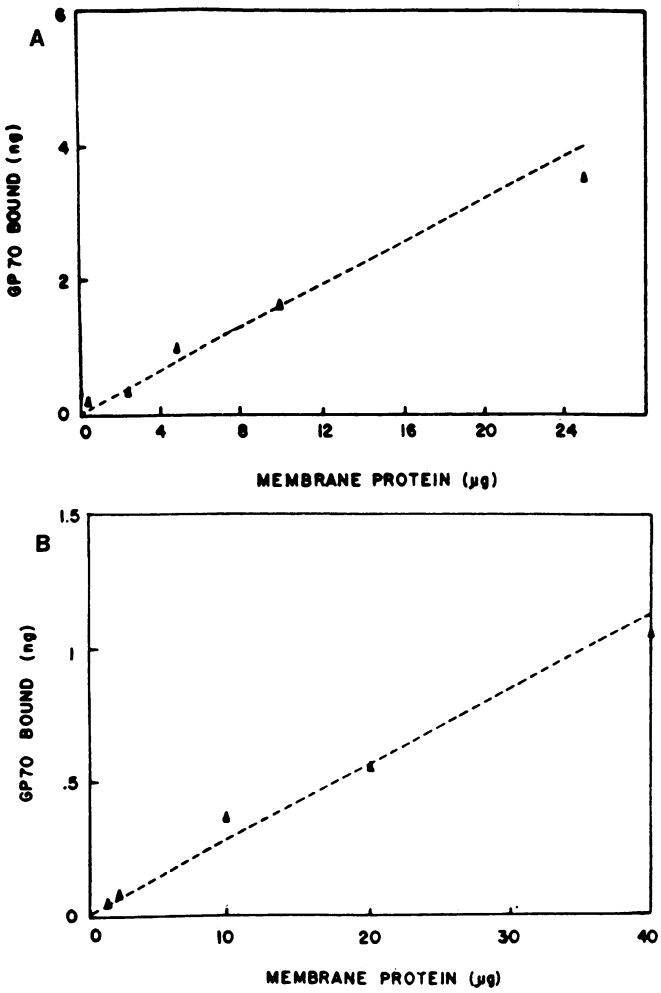

FIG. 3. ^{125}I-labeled gp70 binding is linear with increasing protein concentration. (A) Membranes. The various concentrations of membranes (0.5 to 25 µg/50 µl) were incubated with ^{125}I-labeled gp70 (40 ng/50 µl) and analyzed after centrifugation as described in Materials and Methods. The ^{125}I-labeled gp70 bound to the heat-treated membranes (see Materials and Methods) was subtracted from the total ^{125}I-labeled gp70 binding to yield specific binding. The results are the average of duplicate determinations (specific activity of ^{125}I-labeled gp70, 7.2 × 10^3 cpm/ng). (B) Detergent-solubilized and acetone-precipitated membrane protein. L-cell membrane protein (ca. 4 mg/ml) was solubilized in 0.2% DOC and acetone precipitated (see Materials and Methods). The acetone-precipitated protein (ranging from 0.5 to 80 µg/25 µl) was incubated with ^{125}I-labeled gp70 (22 ng/25 µl) and filtered as described in Materials and Methods. Each point is the mean of triplicate determinations (specific activity of ^{125}I-labeled gp70, 2 × 10^4 cpm/ng). Specific binding was determined by subtracting the background binding to heat-treated membranes or precipitated protein from the total binding.

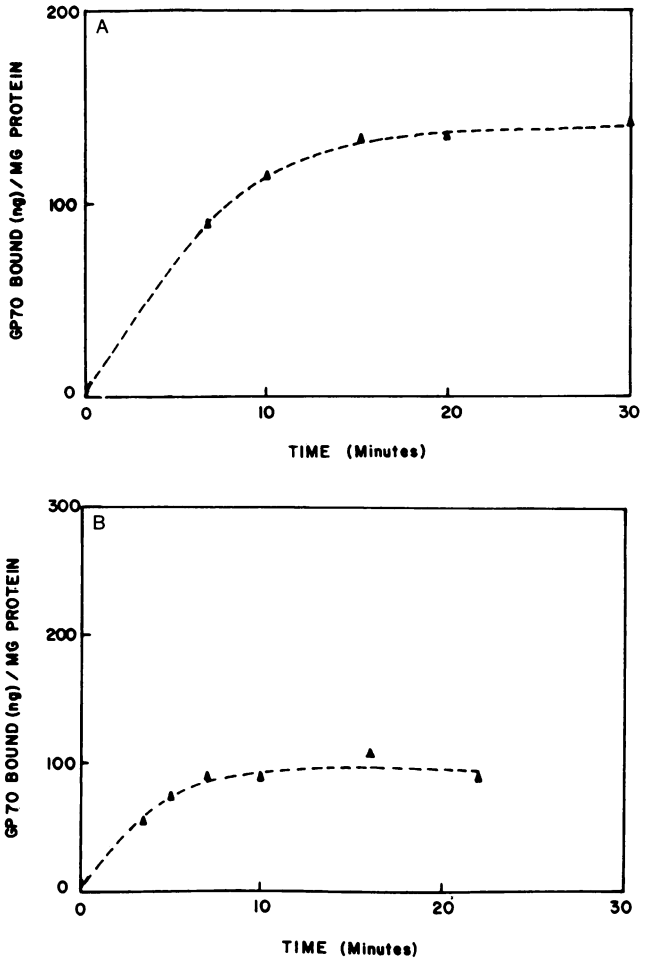

FIG. 4. Time course of ^{125}I-labeled gp70 binding to membranes and detergent-solubilized membrane protein. (A) Membranes (15 µg of membrane protein per 25 µl) were incubated with ^{125}I-labeled gp70 (15 ng/25 µl); aliquots were removed at the designated times, centrifuged, and analyzed as described in Materials and Methods. The results are the mean of duplicate determinants (specific activity of ^{125}I-labeled gp70, 8.3 × 10^3 cpm/ng). (B) Membrane protein was solubilized in 0.2% DOC and acetone precipitated (see Materials and Methods). The precipitate (2 µg of protein per 25 µl) was incubated with ^{125}I-labeled gp70 (10 ng/25 µl) as in panel A, filtered, and analyzed as described in Materials and Methods (specific activity of ^{125}I-labeled gp70, 3.3 × 10^4 cpm/ng). Specific binding was determined by subtracting the background binding to heat-treated membranes or precipitated protein from the total binding.

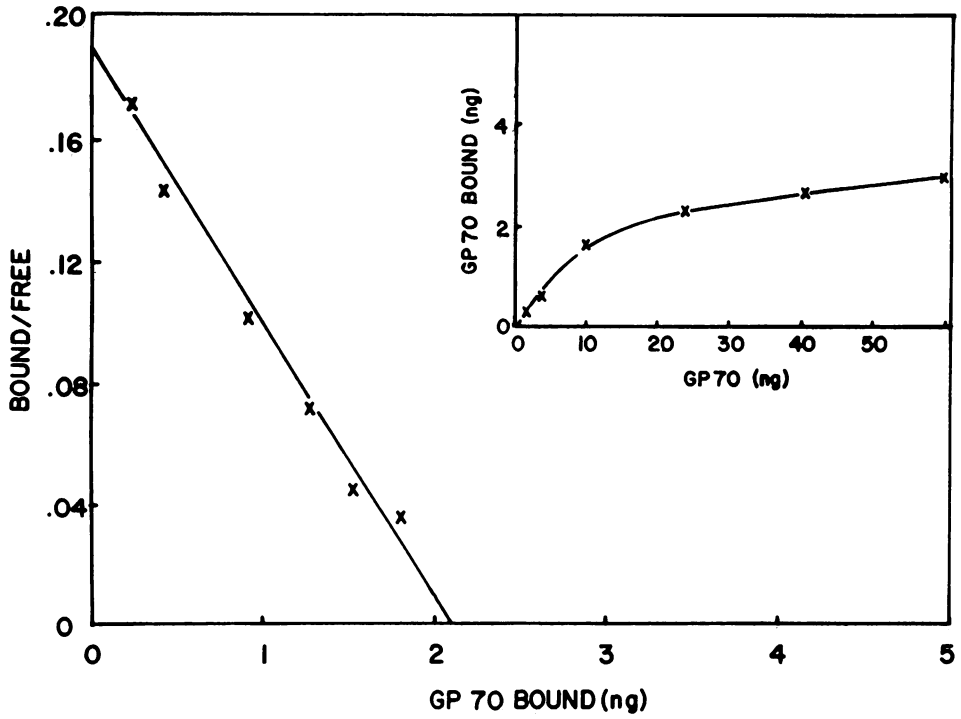

FIG. 5. Scatchard plot of ^{125}I-labeled gp70 binding to L-cell membranes. Membranes (10 μg/25 μl of aliquot) were incubated with ^{125}I-labeled gp70 and then centrifuged as described in Materials and Methods. Each point was determined in triplicate and presented as the mean (specific activity of ^{125}I-labeled gp70, 1×10^4 cpm/ng). To determine specific binding, ^{125}I-labeled gp70 binding to heat-treated membranes was subtracted from the total binding to cell membranes. From analysis of the plot of ^{125}I-labeled gp70-specific binding to L-cell membranes (inset), a Scatchard plot was generated.

acetone precipitation step was omitted, only 10% of the gp70-binding activity remained. If L-α-lecithin was added to the samples up to a lipid-to-protein ratio of 1.5 (Fig. 6B), the gp70-binding activity was retained. No significant specific gp70 binding was observed when liposomes were precipitated alone.

As observed with intact membranes, binding of ^{125}I-labeled gp70 to the precipitated protein was inhibited by exposure to heat, EDTA treatment, and 100-fold excess of unlabeled gp70 (Fig. 2B). Linear dependence of binding upon increasing protein concentration was also observed (Fig. 3B), and the binding reached equilibrium between 10 and 20 min at room temperature (Fig. 4B), similar to that observed with intact membranes. Recovery of the original membrane activity upon solubilization with DOC generally ranged from 60 to 150%; the specific activity increased up to 1.5-fold.

Characterization of the solubilized gp70-binding component indicated that the binding was specific and appeared to be saturable at approximately 30 nM gp70 (Fig. 7). The saturable component of the binding curve was eliminated by heat treatment, 100-fold excess of unlabeled gp70, or 5 mM EDTA. Scatchard analysis of the binding data gave an apparent K_a of 2×10^7 M^{-1} for gp70 binding and a value of 4×10^{12} gp70-binding sites per mg of precipitated membrane protein. A similar value of 2×10^{12} gp70-binding sites per mg of protein was obtained for L-cell membranes (Fig. 5).

Sephacryl S-300 chromatography of the solubilized gp70 receptor. To determine the approximate molecular weight of the gp70-binding component, the solubilized L-cell membrane protein was fractionated by Sephacryl S-300 column chromatography. A 0.2% DOC extract of L-cell membranes was centrifuged to remove aggregated species, as described in Materials and Methods, and applied immediately to a Sephacryl S-300 column. To assay the gp70-binding activity, L-α-lecithin was added at a lipid-to-protein ratio of 1 to 2 prior to precipitation with acetone. In the absence of added lipid, no gp70-binding activity was detected in the precipitate (data not shown). In a typical experiment, the gp70-binding activity was included in the column between the two protein markers, alcohol dehydrogenase (150,000) and BSA (67,000), corresponding to an approximate molecular weight of 110,000 (Fig. 8A). In experiments involving membranes solubilized at a higher detergent concentration, other gp70-binding components greater than 150,000 were detected as well; the lowest-molecular-weight species appeared to be 110,000 (Fig. 8B). ^{125}I-labeled gp70 binding to pooled species in the molecular weight range of 67,000 to 310,000 was inhibited by 5 mM EDTA, heat denaturation, or addition of

TABLE 1. Enzyme and urea treatment of L-cell membranes

Treatment	Specific gp70-binding activity[a] (% of control)
Untreated	100
Neuraminidase (20 U/ml)	138
Chymotrypsin (20 μg/ml)	36
Trypsin (20 μg/ml)	39
Pronase (20 μg/ml)	0
Urea (4 M, 8 h)	26

[a] Each point represents the mean of triplicate determinations. The amount of gp70 which bound to untreated membranes was 138 ng of ^{125}I-labeled gp70 per mg of protein.

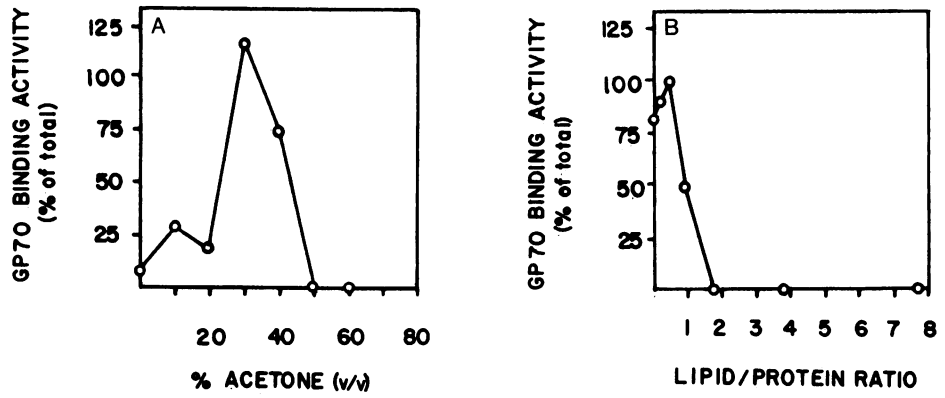

FIG. 6. Precipitation of the solubilized receptor. L-cell membranes (2 mg of protein per ml) were solubilized as described in Materials and Methods. Aliquots (83 μg/200 μl) were incubated with 100 ng of ^{125}I-labeled gp70 and assayed for gp70-binding activity as follows (specific activity of ^{125}I-labeled gp70, 1.2×10^4 cpm/ng of gp70). (A) No L-α-lecithin added. 100 ng of ^{125}I-labeled gp70 was added to each reaction mixture. The gp70-binding activity was precipitated by adding 0 to 300 μl of ice-cold acetone. Aliquots were centrifuged and analyzed as described in Materials and Methods. The amount of gp70 (total) which bound to solubilized protein that was precipitated in the presence of 37.5% acetone and no L-α-lecithin was 24 ng. (B) Various concentrations of L-α-lecithin (20 to 640 μg) were added, and the gp70-binding activity was determined after acetone precipitation and centrifugation as described in Materials and Methods.

a 100-fold excess of unlabeled gp70. These results suggest that the gp70 binding component may be heterogeneous and has a minimal molecular weight of approximately 110,000.

DISCUSSION

With the use of a precipitation-based assay developed for assessing soluble gp70-binding activity, we have achieved solubilization and partial characterization of the MoLV ecotropic receptor from murine L cells. The properties of the soluble gp70-binding component were similar to those of the intact cellular receptor and the isolated membrane receptor with regard to the specificity of binding as determined by sensitivity to EDTA, heat treatment, or the addition of excess unlabeled gp70. Further, the gp70-binding activity in membranes and acetone-precipitated soluble fractions appeared to be saturable, with a similar (although slightly lower) affinity to that of intact cells. Thus, the solubilized gp70-binding activity reflected that of the intact cellular receptor.

It is not surprising that disruption of the native membrane system might result in an alteration in the affinity of the gp70-binding protein for its ligand. One possible explanation is that the apparent affinity of the solubilized gp70-binding protein varies as a function of the concentration of the protein in the liposomes. Such a phenomenon has been observed for immobilized proteins such as enzymes, receptors, and antibodies. Although the actual activity of the enzyme protein per unit volume is unchanged, the apparent affinity of the protein for the ligand increases as the density of the enzyme on the supporting matrix increases. Our observation that the specific activity of the gp70-binding protein decreases at high lipid-to-protein ratios could be explained by a similar density-dependent decrease in apparent affinity. Further experiments are required to resolve the issue.

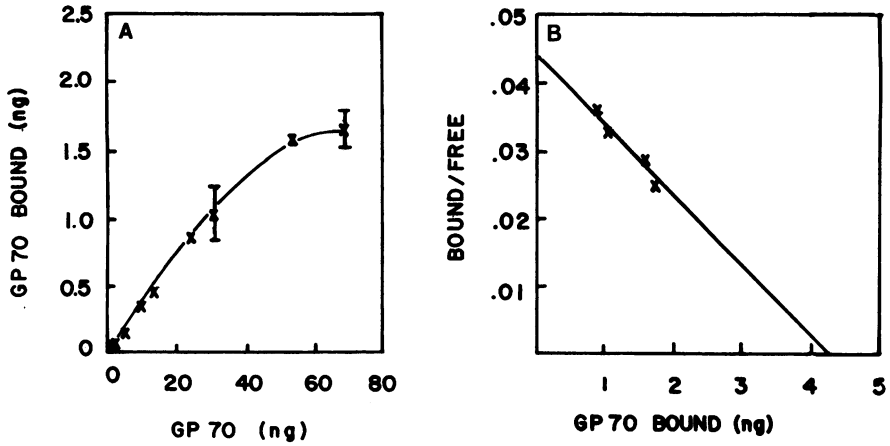

FIG. 7. ^{125}I-labeled gp70 binding to detergent-solubilized membrane protein is saturable. (A) ^{125}I-labeled gp70 binding. DOC (0.2%)-solubilized, acetone-precipitated protein was incubated with ^{125}I-labeled gp70, filtered, and analyzed as described in Materials and Methods. Specific binding to the precipitated membrane protein (7 μg/μl) was determined by subtracting the nonspecific binding to heat-treated precipitate from the total bound amount of ^{125}I-labeled gp70. The nonspecific binding was approximately 50% of the total binding. Each point represents the mean of duplicate determinations (specific activity of ^{125}I-labeled gp70 was 1.3×10^4 cpm/ng). (B) Scatchard analysis. From analysis of the plot of ^{125}I-labeled gp70 specific binding to detergent-solubilized membrane protein in panel A, a Scatchard plot was generated.

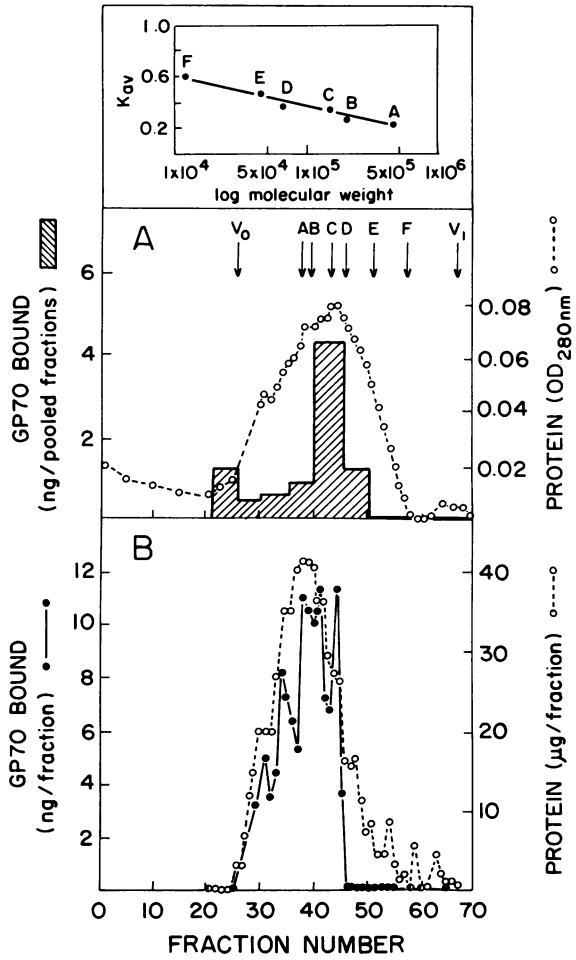

FIG. 8. Sephacryl S-300 chromatography of detergent-solubilized L-cell membrane protein. Inset: estimation of the range of molecular weight of the gp70 receptor as determined by gel filtration. Arrows indicate the elution position of apoferritin, M_r = 443,000 (A); β-amylase, M_r = 200,000 (B); alcohol dehydrogenase, M_r = 150,000 (C); BSA, M_r = 67,000 (D); ovalbumin, M_r = 45,000 (E); ribonuclease, M_r = 12,300 (F). (A) L-cell membranes solubilized and chromatographed in 0.2% DOC. For assay of gp70-binding activity, 250-µl aliquots from every five fractions were pooled, 125 µg of L-α-lecithin was added, and the mixture was precipitated. gp70-binding activity was determined as described in Materials and Methods. ^{125}I-labeled gp70 (10 ng) was added per 25 µl of reaction mixture (specific activity, 1.2×10^4 cpm/ng of gp70). Protein content (○) was determined by optical density at 280 nm. The open bars represent the specific gp70 binding determined by subtracting the nonspecific binding in the presence of a 100-fold excess of unlabeled gp70 from the total gp70 binding. (B) L-cell membranes solubilized and chromatographed in 0.4% DOC. For assay of gp70-binding activity, 100-µl aliquots from each fraction were removed, 100 µl of 100 mM NaCl, 20 mM Tris hydrochloride (pH 8.0), and 10 µg of L-α-lecithin were added, and the mixture was precipitated with ice-cold acetone. gp70-binding activity was determined as described in Materials and Methods. ^{125}I-labeled gp70 (23 ng) was added per 25 µl of reaction mixture (specific activity, 1.2×10^4 cpm/ng of gp70). Symbols: ●, specific gp70-binding activity per fraction as determined by subtracting the gp70 binding to heat-treated fractions from the total gp70 binding; ○, protein content per fraction was determined by assaying 2 to 50-µl aliquots by the method of Lowry et al. (17) as described in Materials and Methods.

Following membrane solubilization in 0.2% DOC, a gp70-binding component was identified that had an approximate molecular weight of 110,000 by gel filtration. This result is consistent with Scatchard analyses of cellular, membrane-bound, and detergent-solubizlied gp70-binding components, which suggest that there is a single class of gp70-binding sites. However, under certain extraction conditions, other higher-molecular-weight gp70-binding species were also detected. Further purification of the detergent-solubilized membrane extract on other chromatographic systems resulted in the aggregation of the gp70-binding species. For instance, the apparent molecular weight of the gp70-binding component eluted from DEAE-cellulose was greater than 10^6 by gel filtration. Thus, the heterogeneity exhibited under certain conditions appears to reflect the aggregation of the gp70-binding species.

The gp70-binding components that we have characterized do not appear to be limited to one viral strain or host cell type. The properties of the MoLV gp70-binding activity in L cells, such as pH profile (unpublished data), calcium dependence (unpublished data), and binding affinity, were similar to those previously described for binding of Rauscher gp70 to other cell lines (4, 13). We have further demonstrated that the gp70-binding component is an intrinsic membrane protein. Treatment with chaotropic agents which release peripheral membrane proteins (30) did not release receptor activity. These results, in combination with protease and denaturation studies, suggest that the gp70 receptor is an integral membrane protein species.

The development of an assay for gp70 binding to detergent-solubilized membrane extracts led to the identification of a gp70-binding component with properties similar to those of the intact viral receptor. Whether this 110,000-molecular-weight gp70-binding species is responsible for mediating MuLV entry into host cells or plays a role in virus binding is not yet known.

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