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Characterization of Rauscher Murine Leukemia Virus Envelope Glycoprotein Receptor in Membranes from Murine Fibroblasts

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Plasma membrane preparations from KA31 (mouse) cells contained receptors for the binding of Rauscher murine leukemia virus (R-MuLV) envelope glycoprotein, gp70. This binding was demonstrated by gel filtration of a mixture of the microsomal fraction of the cells and ^{125}I-labeled gp70. A rapid and convenient assay was developed to measure the complex formation between the membrane receptors and gp70 involving specific precipitation of the complex by 3 to 4% polyethylene glycol. The complex formation was responsive to the concentrations of both the receptor and gp70 and also to changes in temperature and pH. The gp70 binding was a noncooperative, saturable process, and an association constant of 3.5×10^8 M^{-1} was estimated from the binding data. The complex formation was reversible and a near-total exchange of ^{125}I-labeled gp70 in the complex was achieved by incubation with excess of unlabeled gp70. The complex formation was inhibited by protein denaturing agents, guanidine-hydrochloride and urea. Pretreatment of the membrane fractions with either chymotrypsin or phospholipase C led to a loss of the membrane-associated receptor activity, indicating that a lipoprotein structure was important for the receptor function, consistent with the observation that nonionic detergents strongly inhibited the complex formation.

Interaction between a cell and a virus particle at the surface level is one of the essential initial steps in viral infection of a cell. Therefore, cells susceptible to retroviral infection must have specific surface receptors to mediate the adsorption and penetration of the virus particle. Such specific binding of enveloped viruses on cell surfaces has been visualized by electron microscopy (6, 20). Several lines of evidence argue that this cell-virus interaction involves the glycoprotein component of the virus envelope. Thus, Rous sarcoma virus stocks that lack the envelope glycoprotein as a result of genetic defect (7) do not adsorb to cells and are therefore noninfectious. This defect can be overcome by preparing pseudotypes of these viruses containing the envelope glycoprotein of another virus with the proper specificity. Enzymatic removal of the envelope glycoprotein also results in the loss of virus infectivity (23). The plasma membrane is known to be involved in the early stages of infection by many enveloped viruses (5, 18), but information on the molecular aspects of the specificity of RNA tumor virus interaction with cell membranes is extremely limited. Cultures of some chicken embryos have been shown to be selectively resistant to infection with certain strains of Rous sarcoma virus. This has been explained as a result of these cells lacking the receptor for the particular virus strains (9, 30). Two viruses bearing related envelope glycoproteins interfere with each other in the infection of a susceptible cell. The first virus that infects appears to block the receptor for the second virus (31). Normal murine cells have been shown to specifically bind purified envelope glycoprotein, gp70 of Rauscher murine leukemia virus (R-MuLV) (3, 8, 10; K. Ganguly, V. S. Kalyanaraman, R. C. Gallo, and M. G. Sarngadharan, unpublished results). The present study was initiated to analyze the nature of the murine cell surface receptor for R-MuLV. Our results demonstrate fractionation of these receptors and a partial characterization of their chemical nature.

MATERIALS AND METHODS

Cells and viruses. A Kirsten murine sarcoma virus-transformed A31 clone of BALB/c-3T3 (KA31) cells was grown in monolayer in RPMI-1640 medium supplemented with 5% fetal calf serum, provided by Biotech Research Laboratories, Rockville, Md. R-MuLV, Moloney-MuLV, AKR virus, Gross-MuLV,

simian sarcoma virus (SSV), and baboon endogenous virus (M7) were provided by the Virus Cancer Program, National Cancer Institute.

Enzymes. Neuraminidase (*Vibrio cholerae*) was purchased from Calbiochem. Trypsin (code: TRTPCK) and phospholipases A and C were obtained from Worthington. α-Galactosidase, β-galactosidase, α-glucosidase, α-L-fucosidase, and *N*-acetyl-β-glucosaminidase were from Boehringer-Mannheim. Papain and α-chymotrypsin were Sigma Chemical Co. products.

Chemicals and reagents. Lentil lectin bound to agarose was either purchased from P. L. Biochemicals or prepared in our laboratory. In the latter instance, the lectin was purified from lentil extracts by an adaptation of published procedures (13, 14) and attached to CNBr-activated Sepharose 4B. Carrier-free $Na^{125}I$ was obtained from New England Nuclear. Phenylmethylsulfonylfluoride (PMSF) was purchased from Sigma. All other chemicals used were of the highest purity commercially available.

Preparation of the membrane fractions. KA31 cells (2 g) were suspended in 20 ml of 10 mM Tris-hydrochloride buffer, pH 7.5, containing 10 mM NaCl, 0.1 mM PMSF, and 1 mM $MgCl_2$ and were allowed to swell for 20 min. The cells were broken by manual Dounce homogenization (20 to 40 strokes), and the nuclei and mitochondria were removed from the homogenate by successive 10-min centrifugations at 1,000 × *g* and 10,000 × *g*. The 10,000-×-*g* supernatant was centrifuged at 100,000 × *g* in a Spinco ultracentrifuge, and the pellet obtained was collected and suspended at a protein concentration of 2 to 3 mg/ml in 10 mM Tris buffer, pH 7.5, containing 10% glycerol, 1 mM EDTA, and 0.1 mM PMSF.

The majority of the receptor activity was observed in the 1,000-×-*g* supernatant fraction from the cell lysate. When this supernatant fraction was further fractionated into a 10,000-×-*g* pellet and a 100,000-×-*g* pellet, the receptor activity was fairly evenly distributed between the two fractions. There was no activity seen in the 100,000-×-*g* supernatant. In most experiments, we used the 100,000-×-*g* pellet as the receptor source because of the higher specific activity of this fraction compared with the 10,000-×-*g* pellet. However, we used the 10,000-×-*g* pellet in the experiments with digestive enzymes because of the convenience in isolating the treated membranes by relatively low-speed centrifugations.

When the 1,000-×-*g* supernatant of the cell lysate was centrifuged through a 25 to 65% (wt/vol) sucrose gradient, according to a published procedure (22), the receptor activity was recovered in the plasma membrane fraction (data not shown).

Purification of R-MuLV gp70. We have used two different procedures for purifying the viral envelope glycoprotein, gp70, and both have yielded satisfactory preparations with identical receptor binding properties.

Procedure 1: Density-banded R-MuLV (20 mg) was lysed with a mixture of Triton X-100 (0.5%) and NaCl (0.8 M). The lysate was treated with 0.1 mM PMSF, and diluted to reduce the NaCl concentration to 0.25 M, and chromatographed on a column (15 ml) of DEAE-cellulose equilibrated with 0.25 M NaCl in 50 mM Tris-hydrochloride buffer, pH 7.9, containing 1 mM dithiothreitol to remove the nucleic acids. The flow-through fractions and the 0.25 M NaCl wash containing the unadsorbed proteins were combined and dialyzed against buffer A [10 mM *N,N*-bis(2-hydroxyethyl)-2-aminomethane sulfonic acid (BES) buffer, pH 6.5, containing 1 mM EDTA] and chromatographed on a 15-ml column of phosphocellulose equilibrated with buffer A, according to the method of Strand and August (27). After a thorough wash, the adsorbed proteins were eluted from the column by a linear 0 to 1 M NaCl gradient (150 ml) in buffer A. Aliquots from the column fractions were analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis, according to the method of Laemmli (17), and the fractions corresponding to the 70,000-molecular-weight glycoprotein were pooled. After concentration by lyophilization, the protein was further purified by gel filtration on a Sephadex G-150 column equilibrated with 10 mM sodium phosphate buffer containing 0.3 M NaCl and 0.5 mM PMSF.

Procedure 2: In this alternative procedure, the unadsorbed proteins from the DEAE-cellulose step described in procedure 1 were dialyzed against buffer B (10 mM sodium phosphate [pH 6.5], 0.2 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM $MnCl_2$) and applied to a column (5 ml) of lentil lectin attached to Sepharose 4B, equilibrated with buffer B. After washing with buffer B, the column was developed with 0.1 M α-methylmannopyranoside in buffer B. The glycoprotein eluted by this step was, in most cases, nearly homogeneous, as indicated by a single protein band when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When necessary, this material was further purified by gel filtration on Sephadex G-150 as described in procedure 1.

Purified gp70 from either procedure was dialyzed against 10 mM sodium phosphate containing 0.1 mM PMSF and stored in small portions at −70°C. Samples were labeled with ^{125}I, according to the procedure of Greenwood et al. (11), using chloramine T as the oxidant. The specific radioactivity of the iodinated gp70 varied between 5,000 and 10,000 cpm/ng of protein. A representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of an iodinated (^{125}I) sample of purified gp70 is shown in Fig. 1.

Assay for receptor-gp70 complex. A sample of the membrane suspension (10 to 20 μg of protein/20 μl) was incubated at 24°C with 200 μl of ^{125}I-labeled R-MuLV gp70 (ca. 50,000 cpm) in Dulbecco modified Eagle medium containing 50 mM BES buffer, pH 6.5, and 1 mg of bovine serum albumin per ml. After 1 h, 0.5 ml of a 0.5-mg/ml concentration of bovine γ-globulin and 0.5 ml of 9.75% polyethylene glycol 6000 (Fisher Chemicals) were added, and the mixture was kept in ice for 10 min. The precipitated complex was centrifuged for 5 min in an Eppendorf centrifuge. The pellet was resuspended in 4% polyethylene glycol and recentrifuged, and the radioactivity in the washed pellet was determined in an LKB Ultrogamma counter. Nonspecific background binding (4,000 to 7,000 cpm), determined by including, in addition to radiolabeled gp70, 450 ng of unlabeled R-MuLV gp70 in the assay mixture, was subtracted from each experiment.

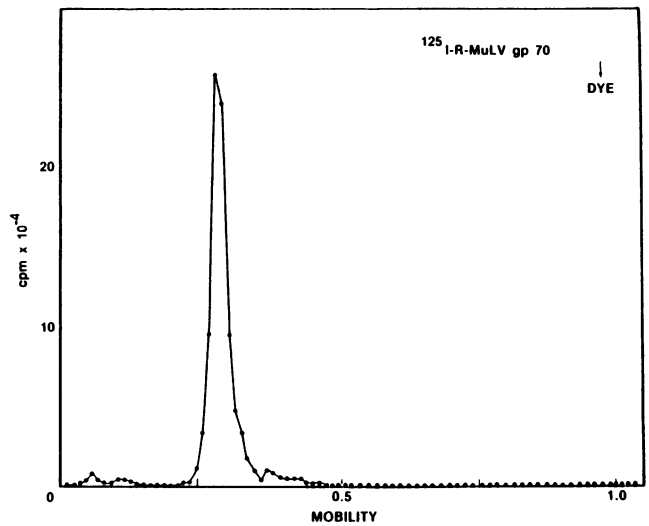


FIG. 1. *Electrophoretic profile of ^{125}I-labeled R-MuLV glycoprotein, gp70, purified by procedure 1 and iodinated using the chloramine T procedure. Electrophoresis was performed in the presence of sodium dodecyl sulfate on 10% polyacrylamide gels, according to the method of Laemmli (17). The gels were divided in 1-mm slices, and the radioactivity was determined by a gamma counter. A molecular weight value of 70,000 was determined for the radioactivity peak. The standards used for molecular weight calibration were phosphorylase B (93,000), bovine serum albumin (68,000), ovalbumin (43,000), and chymotrypsinogen (25,500).*

Protein concentrations were determined by the method of Lowry et al. (19).

RESULTS

Purification of R-MuLV gp70. The envelope glycoprotein (gp70) of R-MuLV used in the present study was purified from concentrated virus preparations by either ion-exchange and gel filtration chromatographies, according to the procedure of Strand and August (27), or by affinity chromatography on a lectin-Sepharose column as described above. Both procedures have consistently yielded nearly homogeneous preparations. A representative electrophoretic profile of a purified preparation of the glycoprotein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis is given in Fig. 1. The ^{125}I-labeled gp70 used in the following experiments was almost completely precipitable by trichloroacetic acid and was greater than 90% precipitable by an antiserum directed against R-MuLV gp70.

Specific precipitation of the receptor-gp70 complex by polyethylene glycol. Many different approaches have been used to monitor complex formation between proteins and their specific receptors. Selective precipitation with ammonium sulfate (24) or polyethylene glycol (4) have been employed in the case of complexes involving soluble receptors. For membrane-bound receptors, filtration through nitrocellulose disks has been used to determine the complex formation (28). In our experience, the filter method gave extremely high background values when applied to the binding of ^{125}I-labeled gp70 to membrane-associated receptors. We were interested in developing an assay that would be immediately applicable to membrane-associated receptors but would also be capable of assaying for the receptor molecule when we have it solubilized and fractionated. The method we described above has been very satisfactory for the assay of membrane-bound receptors and is also adaptable to assay of soluble receptors.

Figure 2 shows the effect of polyethylene glycol on the precipitation of the complex between ^{125}I-labeled gp70 and the cell membrane fraction. The specific precipitation is optimum at 3 to 4% polyethylene glycol. Above 6% polyethylene glycol precipitation of ^{125}I-labeled gp70 starts to increase rapidly, and the specificity for the receptor-gp70 complex is lost.

Demonstration of the binding of R-MuLV gp70 to cell membrane fractions. To ascertain that the results obtained with the assay system described earlier in the paper truly reflected specific binding of purified gp70 to the membrane receptors, the following experiment was conducted. A portion of the microsomal preparation was mixed with ^{125}I-labeled gp70, and the mixture was chromatographed on a column of Sepharose CL-6B (Pharmacia). Fractions of 1.5 ml were collected, and samples were assayed for radioactivity. Two peaks of radio-

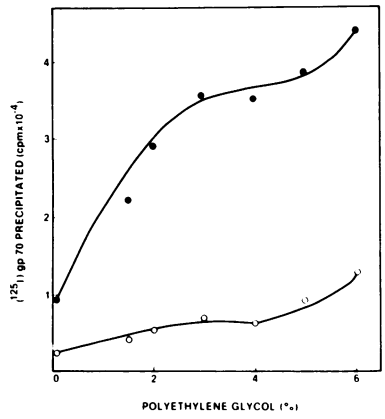


FIG. 2. *Effect of polyethylene glycol 6000 concentration on the precipitation of ^{125}I-labeled R-MuLV gp70-membrane receptor complex. The assays were performed using 85,000 cpm of ^{125}I-labeled gp70 as described in the text, except that the final concentration of polyethylene glycol used for precipitation was varied from 0 to 6%. ●, precipitation of ^{125}I-labeled gp70 in the presence of the membrane fraction with no added nonradioactive gp70; ○, precipitation of ^{125}I-labeled gp70 in the presence of membrane fraction when nonradioactive gp70 was also added to the reaction mixture. The latter represents the nonspecific binding of ^{125}I-labeled gp70 to the membranes.*

activity were obtained, one corresponding to the void volume of the column (fractions 23 to 27) that represented ^{125}I-labeled gp70 bound to the membranes, and the other corresponding to the peak of free ^{125}I-labeled gp70 (fractions 44 to 60) (Fig. 3). When the microsomal fraction was preincubated with nonradioactive gp70 before addition of ^{125}I-labeled gp70, most of the labeled gp70 was recovered in the second peak in the chromatogram, and very little was found as part of the complex (Fig. 3). These results demonstrate that the microsome membrane fractions contained true saturable receptor sites for R-MuLV gp70.

When assayed as described above, the amount of gp70 that was bound increased as a function of the concentration of the membrane fraction in the incubation. At a fixed concentration of ^{125}I-labeled gp70, the binding of the protein was linear at the lower concentrations of the membrane fraction, but it approached a plateau at higher concentrations (Fig. 4).

Effect of temperature and pH on receptor activity. Figure 5 shows the kinetics of formation of the receptor-gp70 complex at 4, 24, and 37°C. The complex formation was very rapid at 37°C, reaching the maximum point in about 25 min. With longer incubation there was a substantial decrease in the complex yield, presumably due to degradation of ^{125}I-labeled gp70. At 24°C, although the reaction rate was slower, the same maximum binding was reached as at 37°C, and there was no detectable decline in the complex yield with prolonged incubation. The rate of complex formation at 4°C was extremely slow and required overnight incubation to reach the same plateau as at 24°C (data not shown). For the gp70-receptor binding assays, we regularly use incubation at 24°C.

The receptor-gp70 complex formation was also affected by pH. The maximum binding was observed at pH 6.5. Relative to the binding at pH 6.5, the binding at the pH values of 6, 7, 8, and 9 was 84.4, 89.1, 83, and 58.3%, respectively. These results are substantially similar to those observed in the binding of R-MuLV gp70 to intact murine cells (8; K. Ganguly, unpublished data).

Requirements of Ca^{2+} for gp70-receptor complex formation. The metal ion requirement for the complex formation between gp70

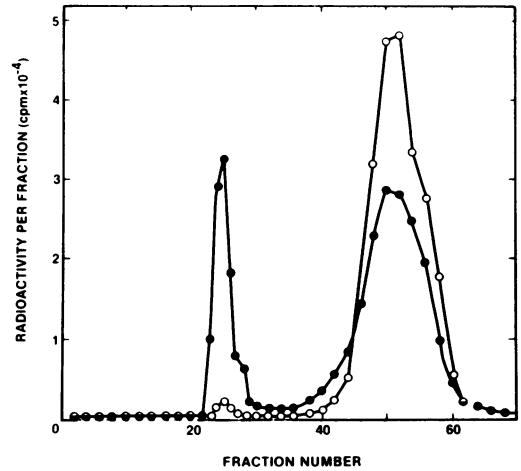


FIG. 3. *Gel filtration of ^{125}I-labeled R-MuLV gp70-membrane complex. ^{125}I-labeled R-MuLV gp70 (670,000 cpm) was incubated with the membrane fraction (370 μg of protein), prepared as described in the text, in 1.1 ml of Dulbecco modified Eagle medium buffered with 50 mM BES (pH 6.5) and containing 1 mg of bovine serum albumin per ml (binding medium) for 1 h at 24°C. The mixture was then applied to a column (1.2 × 50 cm) of Sepharose CL-6B equilibrated with the binding medium. The column was developed with the same medium, and fractions of 1.5 ml were collected and their radioactivity was determined in a gamma counter. As a control, an identical experiment was done by including 5 μg of unlabeled R-MuLV gp70 in the initial reaction mixture in addition to ^{125}I-labeled gp70. Total radioactivity in the fractions from the experiment without (●) and with (○) unlabeled gp70 is represented in the profile. The total recovery of radioactivity in both experiments was similar.*

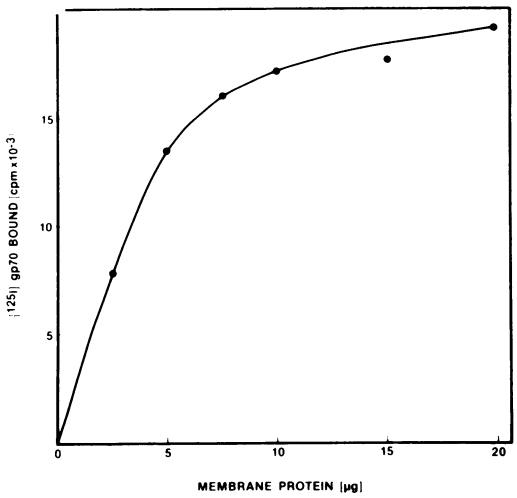


FIG. 4. *Receptor activity as a function of membrane protein concentration. Increasing amounts of membrane fraction were incubated with a constant amount (54,000 cpm) of ^{125}I-labeled R-MuLV gp70 under standard assay conditions, and the amount of bound radioactivity was then determined by precipitation with polyethylene glycol. The nonspecific background binding was 7,600 cpm, which was subtracted from each point.*

and the membrane receptor was analyzed by using a modification of the standard assay system. The mixture of ^{125}I-labeled gp70 and receptor preparation was incubated in the absence of Dulbecco modified Eagle medium but in the presence of various divalent metal salts or EDTA (Table 1). The maximum binding of gp70 was obtained in the presence of Ca^{2+}, Mg^{2+}, Zn^{2+}, or Co^{2+} could not effectively replace Ca^{2+} for the binding activity. Mg^{2+} when added with Ca^{2+} had no stimulatory effect. Mn^{2+} partially substitutes for Ca^{2+}. A low activity observed in the absence of any added divalent cation may be due to trace amounts of metal ions present in either the membrane fractions or in one of the assay components because this activity is completely eliminated when EDTA is added to the binding mixture. The requirement of Ca^{2+} and/or Mn^{2+} for the complex formation is typical of interactions involving glycoproteins (1, 15).

Characteristics of the binding of R-MuLV gp70 to cell membrane fraction. The extent of binding of gp70 by the cell surface receptor increased as a function of the concentration of gp70. This saturation process was studied by using the precipitation assay described above. In these experiments, a constant amount of the membrane fraction containing the receptors was incubated with a constant amount of ^{125}I-labeled gp70 and increasing amounts of nonradioactive gp70. Because of the steady decrease in specific radioactivity in successive experiments, there was also a steady decrease in the radioactivity recovered in the complex. However, as shown in Fig. 6A, the amount of gp70 bound to the receptor showed a hyperbolic increase in response to

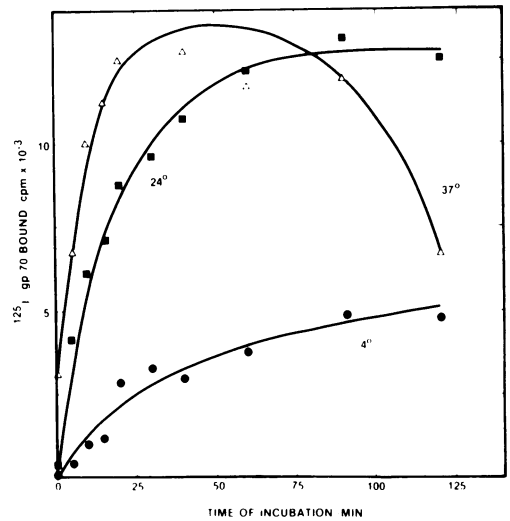


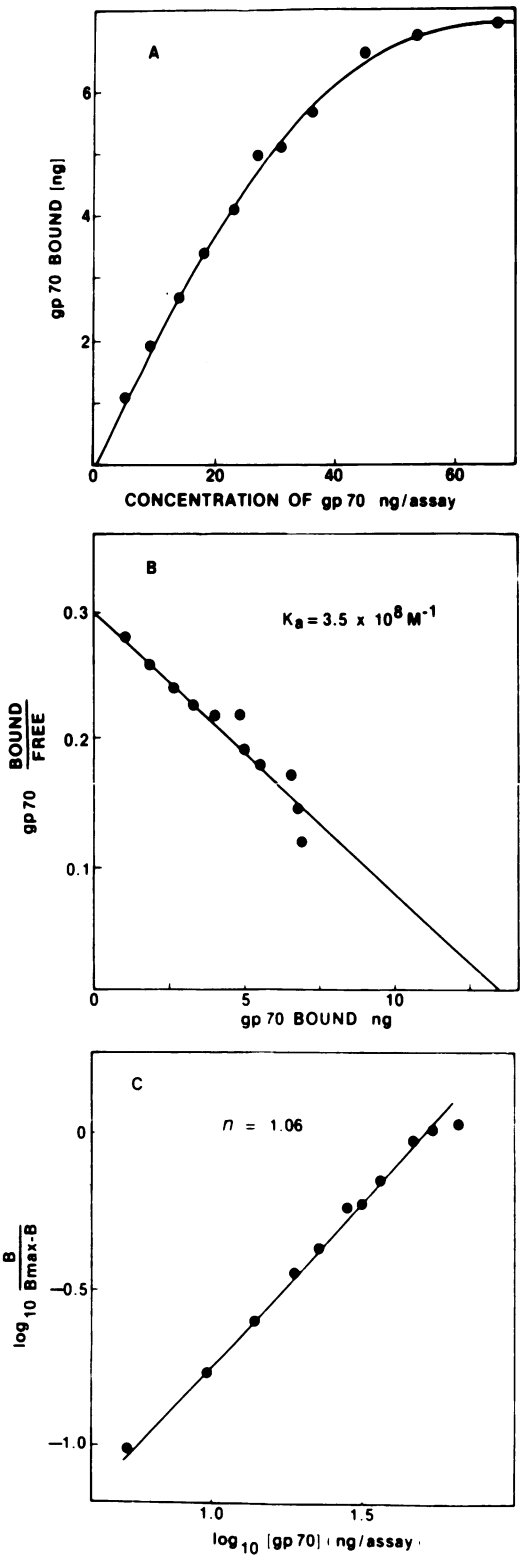
FIG. 5. *Time course and effect of temperature on the velocity of binding of R-MuLV gp70 to the membrane receptor. The standard assay mixture was incubated for varying lengths of time at 4, 24, and 37°C, and membrane-bound radioactivity was determined. The input radioactivity was 42,000 cpm for the 37°C experiment and 43,000 cpm each for the 4 and 24°C experiments. The nonspecific background binding subtracted from each point was 4,260 cpm at 37°C, 6,300 cpm at 24°C, and 6,700 cpm at 4°C.*

TABLE 1. *Requirement of Ca^{2+} for the gp70-receptor complex formation*[a]

Addition	Concn (mM)	^{125}I-labeled gp70 bound[b] (%)
$CaCl_2$	5	100
None		24
EDTA	5	0
$MgCl_2$	5	12
$CaCl_2$ plus $MgCl_2$	5 plus 5	92
$MnCl_2$	1	60
$ZnCl_2$	5	17
$Co(OAc)_2$	5	26

[a] The assay was done as described in the text except that 50 mM BES buffer, pH 6.5, containing 0.1 M NaCl and 1 mg of bovine serum albumin per ml in the presence of Dulbecco modified Eagle medium was used, and it was supplemented with the components listed.

[b] The binding obtained in the presence of 5 mM $CaCl_2$ was used as 100%. This value was similar to the binding observed in the standard assay system.



the increase in gp70 concentration in the reaction mixture. The association constant for the gp70-receptor complex was determined from the above data by plotting the ratio of gp70 bound/gp70 free versus (gp70 bound), according to Scatchard (26). The plot gave a single straight line (Fig. 6B), indicating that no cooperative interactions were involved in the binding and that there was only a single affinity class of receptors present. The association constant (K_a) calculated from the slope of the Scatchard plot was 3.5×10^8 M^{-1}. This value is very similar to that found for the binding of R-MuLV gp70 to intact murine cells (K. Ganguly et al., unpublished data). Further evidence that noncooperative interactions are involved in the complex formation was provided by the Hill coefficient (1.06) determined from the slope of the plot (Fig. 6C) of $\log_{10}$ ($B/B_{max} - B$) versus $\log_{10}$[gp70], where B is the amount of gp70 bound at a given concentration of gp70, and B_{max} is the maximum amount of gp70 bound at infinite concentration of gp70. B_{max} is calculated from the intercept on the abscissa in Fig. 6B.

Reversibility of the receptor-gp70 complex formation. To examine whether the complex formation between the receptor and gp70 was reversible, a preformed complex containing 176 μg of the receptor preparation and 2×10^6 cpm of ^{125}I-labeled gp70 was suspended in the binding medium, as described above, with and without nonradioactive gp70 and was incubated either at 4 or 37°C. Fractions were withdrawn at various times and assayed for the amount of ^{125}I-labeled gp70 still remaining in the complex, as described above. No significant dissociation was observed at 4°C; about 95% of ^{125}I-labeled gp70 still remained in the complex at the end of 180

FIG. 6. *Binding of ^{125}I-labeled gp70 to the membrane receptor as a function of concentration of gp70. The binding was measured by using the standard assay system (220-μl volume) described in the text with 35 μg of the membrane fraction and increasing amounts of R-MuLV gp70 composed of a mixture of a constant amount of ^{125}I-labeled gp70 (5.1 ng, 8200 cpm/ng) and varying amounts of nonradioactive gp70. The specific radioactivity of gp70 is calculated for each concentration of gp70 used in the experiment. (A) Plot of gp70 bound as a function of the amount of gp70 used per assay. (B) Scatchard plot of the data in (A). Binding constant (K_a) calculated from the slope was 3.5×10^8 M^{-1}, and the maximum binding capacity (B_{max}) determined from the intercept on the abscissa was 13.5 ng of gp70. (C) Hill plot of the same data where B is the amount of gp70 bound at a given concentration of gp70, and B_{max} is the maximum possible binding at infinite concentration of gp70 as determined from (B).*

min of incubation with and without added nonradioactive gp70. On the other hand, there was a substantial dissociation of ^{125}I-labeled gp70 at 37°C, with about 50% dissociation being observed in 3 h in the absence of added nonradioactive gp70. When an excess of nonradioactive gp70 was present during incubation, there was nearly complete exchange of the labeled gp70 in 3 h. These results indicate that the complex formation between gp70 and the cell membrane receptor is reversible.

Specificity of the binding of gp70 to the membrane receptor. The specificity of the receptor binding was examined by measuring the competition exerted by different viral proteins in the binding of ^{125}I-labeled R-MuLV gp70. In this experiment, the membrane fraction was preincubated with the competing protein before addition of the labeled R-MuLV gp70 (Table 2). As expected, nonradioactive R-MuLV gp70 completely prevented the binding of ^{125}I-labeled gp70. No competition was observed by high concentrations of R-MuLV p30 or gp70 of a distantly related virus, SSV. The competition was further examined by using lysates of different type C viruses. The only competition obtained was with ecotropic murine viruses, R-MuLV, Moloney-MuLV, Gross-MuLV, and AKR. The xenotropic murine virus BALB virus 2 showed no competition even at a considerably higher concentration. Feline leukemia virus, baboon endogenous virus, and SSV were also without any detectable effect on the binding of R-MuLV gp70 to the cell membrane receptor. These data confirm the specificity of the receptor for R-MuLV gp70.

Effect of detergents and protein denaturants on complex formation. Nonionic detergents, Nonidet P-40 and Triton X-100 were inhibitory at 0.01% when added to the receptor binding assay. Lithium diiodosalicylate was also inhibitory at 0.05 M. So far we have not been able to solubilize active receptor molecules from the membrane preparations by treatment with the above detergents. Difficulty in removing all of the detergents from the solubilized fractions might account for a part of this problem. In addition, there is also a possibility that the precipitation assay that has been optimized for the complex between membrane-bound receptors and gp70 may not be suitable for complexes between detergent-solubilized receptors and gp70. Recently, Moldow et al. (21) have reported the solubilization of a putative receptor for avian retroviruses by treatment of chicken embryo fibroblasts with lithium diiodosalicylate. This material interfered with the binding of intact virus to plasma membrane preparations and therefore was assumed to contain solubilized receptors.

TABLE 2. *Competitive inhibition of the binding of ^{125}I-labeled R-MuLV gp70 to the receptor by viral proteins*[a]

Competing protein	Amt used (μg)	Relative binding of ^{125}I-labeled gp70 to receptor
None		100[b]
Purified proteins[c]		
R-MuLV gp70	0.6	0
R-MuLV p30	10	100
SSV gp70	1.0	91
Solubilized viruses[d]		
R-MuLV	0.5	69
	2.0	27
Moloney-MuLV	0.5	53
	2.0	18
AKR-MuLV	0.5	81
	2.0	40
Gross-MuLV	1.0	83
	2.0	47
Xenotropic-MuLV (BALB virus 2)	10	107
FeLV	10	105
BaEV (M7)	10	99
SSV	10	100

[a] The assay was performed as follows. The membrane fraction (25 μg) was preincubated at 24°C with the purified protein or solubilized virus in a volume of 115 μl of Dulbecco modified Eagle medium containing 50 mM BES buffer, pH 6.5, and 1 mg of bovine serum albumin per ml. After 30 min, 110 μl of ^{125}I-labeled gp70 of R-MuLV (153,000 cpm) was added, and incubation was continued for an additional 60 min. The radioactivity bound to the receptor was determined as described in the text.

[b] 100 represents 45,300 cpm specifically bound to the membrane in the absence of other proteins out of 153,000 cpm added. Background binding obtained in the presence of 600 ng of unlabeled R-MuLV gp70 was 8,100 cpm and was subtracted from all experimental values.

[c] gp70 of R-MuLV and SSV were purified using procedure 1 described in the text. p30 was purified according to the method of Strand and August (27).

[d] Virus suspensions (approximately 1 mg/ml) were disrupted by manual homogenization in the presence of 0.1 M lithium diiodosalicylate in 10 mM Tris-hydrochloride, pH 7.5, containing 0.5 mM PMSF. The homogenate was left to stand at room temperature for 30 min and centrifuged at 35,000 rpm in a Spinco type 65 rotor. The supernatant fraction was collected and dialyzed against 10 mM sodium phosphate, pH 7.5, and used in the competition assay. FeLV, Feline leukemia virus; BaEV, baboon endogenous virus.

Protein-denaturing agents produce a significant inhibition on the binding of gp70 to the membrane receptor (Fig. 7). In this experiment, the membrane preparations were preincubated with varying concentrations of guanidine-hydrochloride or urea for 30 min before addition of

labeled gp70. Data in Fig. 7 show that a total inhibition is obtained at 1 M guanidine-hydrochloride, whereas only a 50% inhibition is produced by 2.5 M urea. To understand the nature of this inhibition, the following experiment was done. The membrane preparation and the purified gp70 were separately treated at room temperature for 30 min with 1.5 M guanidine-hydrochloride. The guanidine-hydrochloride was subsequently removed by dialysis against phosphate-buffered saline. The receptor activity of the treated membrane fraction was determined by using untreated ^{125}I-labeled gp70. Likewise, treated gp70 was used in a binding experiment with untreated membranes. No decrease in binding was observed in either experiment compared to a control in which neither gp70 nor the membranes was treated with guanidine-hydrochloride. These results indicated that the protein-denaturing agents do not in short-term incubations cause irreversible changes in the membranes and gp70 that affect their interaction but have a direct dissociating effect on the binding phenomenon when present in the reaction mixture.

Effects of proteases, glycosidases, and lipases on receptor activity. To determine the chemical environment of the receptor site, the membrane fractions isolated from murine cells were treated with various proteases, glycosidases, and lipases, and the receptor activity remaining in the fractions determined. We used the 10,000-×-*g* pellet fraction, which had a substantial proportion of the receptor activity of the cell, as the source for the receptor in these experiments because of the convenience in separating the membranes from the incubation mixture containing the degradative enzymes by low-speed centrifugations. After a 30-min incubation at room temperature, the mixture was centrifuged at 10,000 × *g*. The pellet was washed repeatedly and resuspended in the binding medium (described above), and portions were removed to assay for the receptor activity.

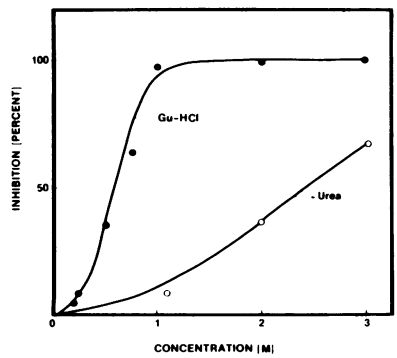


FIG. 7. *Effect of urea and guanidine-hydrochloride on the membrane receptor activity. Urea or guanidine-hydrochloride was added to the standard assay mixtures at the indicated final concentrations, and the bound radioactivity was measured under standard assay conditions.*

TABLE 3. *Effect of proteases, glycosidases, neuraminidase, and lipases on the gp70 receptor activity of cell membrane fractions*

Enzyme[a]	Receptor activity[b] (% of control)
Trypsin	46
Papain	71
α-Chymotrypsin	0
Glycosidases[c]	102
Neuraminidase (10 U/ml)	91
Neuraminidase (20 U/ml)	86
Phospholipase A	100
Phospholipase C	0

[a] All enzymes were used at 20 μg/ml unless otherwise stated.

[b] A suspension of the 10,000-×-*g* pellet from the cytoplasmic fraction of KA31 cells (176 μg of protein) in 100 μl of 10 mM Tris-hydrochloride, pH 7.5, was incubated at room temperature with the indicated enzymes in a total volume of 0.5 ml. All enzymes were used at a concentration of 20 μg/ml except neuraminidase, which was used as indicated. In addition, the following components were included in the incubation mixtures: 1 mM $CaCl_2$ for the trypsin reaction, 2 mM cysteine for the papain reaction, and 5 mM $CaCl_2$ for the neuraminidase reaction. After 30 min, the mixture was centrifuged and the pellet washed three times with 10 mM Tris-hydrochloride, pH 7.5, containing 0.1 mM PMSF, 10% glycerol, and 1 mM EDTA. The washed pellet was suspended in 100 μl of the binding medium and 20-μl portions were assayed for the gp70 binding as described in the text. A sample treated identically, but in the absence of the enzymes, was used as control, and the binding observed with this sample was taken as 100%.

[c] The mixture of glycosidases contained α-galactosidase, β-galactosidase, α-glucosidase, α-L-fucosidase, and *N*-acetyl-β-glucosaminidase, each at 20 μg/ml.

Table 3 shows the effects of various enzymes on the receptor activity of the membranes. Among the proteases tested, trypsin and papain were only partially effective (at 20 μg/ml) in destroying the receptor activity. The receptors, however, were very sensitive to treatment with α-chymotrypsin. The concentration dependence of this effect of chymotrypsin is described in Fig. 8B. Less than 3 μg of the protease was able to produce a 50% drop in the receptor activity under the preincubation conditions. Preincubations with neuraminidase or a mixture of glycosidases (α-galactosidase, β-galactosidase, α-glucosidase, α-fucosidase, and *N*-acetyl-β-glucosaminidase) were without any effect on the receptor activity of the membranes. The conclusions

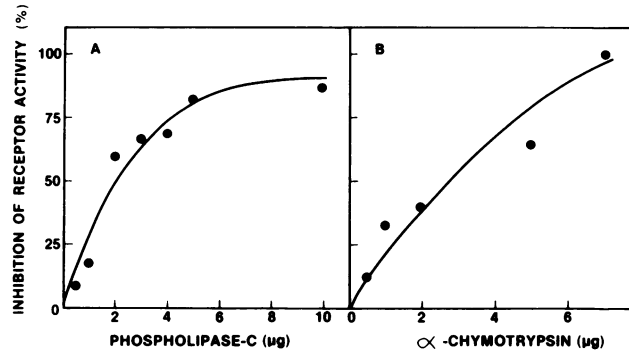


FIG. 8. *Effect of preincubation with chymotrypsin or phospholipase C on the gp 70 receptor activity of the membranes. In this experiment, the 10,000-×-g pellet fraction was used as a source of membrane receptor. (A) Membrane fraction containing 200 µg of protein was incubated with indicated amounts of phospholipase C for 30 min at 24°C in 0.5 ml of 10 mM Tris-hydrochloride, pH 7.5. The membranes were pelleted by centrifugation at 10,000 × g, washed thrice with 10 mM Tris-hydrochloride, pH 7.5, and resuspended in 150 µl of the binding medium (see legend to Fig. 3). The receptor activity in 25 µl of the resuspended membrane was determined under the standard assay conditions. (B) Treatment with α-chymotrypsin. Details of the experiment were as in (A) except that the wash buffer was supplemented with 0.1 mM PMSF to inactivate the protease. The washed pellet was resuspended in 100 µl of the binding medium, and 20-µl samples were used to determine residual receptor activity.*

regarding the carbohydrate involvement at the receptor site could be limited because we may not have used glycosidases with the proper specificity for the sugar residues and glycosidic linkages involved. Observations regarding the sensitivity of the receptors to lipases seem interesting. Whereas phospholipase A showed no effect, the receptor activity was extremely sensitive to treatment with phospholipase C. A total loss of activity is noticed with 20 µg of this enzyme per ml. Figure 8A represents the results of an experiment in which the membrane fractions were preincubated with increasing concentrations of phospholipase C. Throughout the range of concentrations tested only inhibition was observed, and there was no activation similar to the effect of this enzyme on the gonadotropin receptors on ovarian membranes (12). The results, shown in Table 3 and Fig. 8, indicate that an intact lipid protein structure on the plasma membrane is essential for the receptor activity.

DISCUSSION

Most of the known enveloped animal viruses have specific receptors on cells that are susceptible to infection by these viruses. These viruses adsorb to the cells through specific interactions between an anchor molecule on the virus envelope and the receptor molecule on the cell surface. Subsequent to this initial step, several additional events probably precede the penetration and uncoating of the infecting virus particle. Very little is known about the retrovirus-host cell membrane interactions. The only clear evidence available has been that the envelope glycoprotein of the virus is the anchor molecule that interacts with the cell surface and that this glycoprotein possesses the necessary specificity that determines the host range of a particular virus. This conclusion was drawn from observations, mainly in avian and feline virus systems, regarding the host range differences between different subgroups of particular viruses. The approaches that have been used in the past included measurement of interference in infections with different subgroup viruses and study of the host range of pseudotype viruses.

Recently, the interaction between a murine leukemia virus and mouse cells was analyzed by using isolated viral envelope glycoprotein and intact cells. These studies also confirmed earlier indications of specific interactions between susceptible cells and the viral envelope glycoprotein component. The aim of the present study has been to characterize this virus-host interaction and to elucidate the nature of the cell surface receptor, using R-MuLV gp70 and a transformed murine cell line (KA31). These receptors seem to be confined to the external surface of the cell because they are accessible to digestion by phospholipase C (K. Ganguly, V. S. Kalyanaraman, R. C. Gallo, and M. G. Sarngadharan, manuscript in preparation). Therefore, we used membrane preparations to study the receptor-gp 70 interaction.

Complex formation between these membrane fractions and purified gp70 has been demonstrated by gel filtration through Sepharose-CL 6B (Fig. 2). For a convenient and speedy assay of the receptor-gp70 complex formation, we de-

veloped a system similar to the one used for the binding of insulin to its receptor (4). This system has the additional potential usefulness of measuring binding of gp70 to solubilized receptor molecules. The complex formation has been shown to require Ca^{2+} or Mn^{2+} and is inhibited by chelators. These are similar to the general properties of lectin-carbohydrate interactions (1, 15). The receptor-gp70 interaction followed typical kinetic properties such as (i) dependence on pH of the reaction medium, (ii) increased complex yield with increase in membrane concentration (Fig. 4) or gp70 concentration (Fig. 6), and (iii) increased reaction rate with increasing temperature (Fig. 5). The complex between the receptor and gp70 was reversible as evidenced by a time-dependent release of ^{125}I-labeled gp70 from preformed complexes and by the exchangability of the labeled gp70 in the complex with unlabeled gp70.

Detailed analysis of the complex formation revealed not only that the gp70 binding was a saturable process but that only one class of receptors could be detected, having a K_a of $3.5 \times 10^8\ M^{-1}$ (Fig. 6B). This association constant is similar to the one determined for the binding of gp70 to intact cells (K. Ganguly, unpublished results). This implies that receptors functioning in isolated membranes are the same as those that are present on intact cell surfaces and that no new class of receptors is being exposed upon breaking the cell. Our results are inconsistent with the speculation that the cell uses two types of receptors, one on the outside for the adsorption and penetration of the virus and another inside the cell membrane for the assembly and release of the virus (32). If indeed there are two sets of receptors, one on the inside and the other on the outside of the plasma membrane, we do not see any qualitative difference between the two. We propose that there is only a redistribution of these sites during productive infection. That a new set of sites are introduced in the plasma membrane seems unlikely. Further, our binding data give a Hill coefficient of 1 (Fig. 6C), indicating that there is no cooperative interactions involved in the receptor-gp70 complex formation.

The KA31 membrane receptors appeared to be specific to murine viral gp70. For instance, the receptor did not bind the major viral structural protein, p30, nor did this protein affect the binding of gp70 by the receptor. Similarly, gp70 of a distantly related type C virus, SSV, did not compete with R-MuLV gp70 for the receptor sites. Among the viruses examined, ecotropic murine viruses showed competition in the receptor–R-MuLV–gp70 complex formation. The xenotropic murine virus, BALB virus 2 and type C viruses of other species (feline leukemia virus, baboon endogenous virus, and SSV) had no influence on complex formation (Table 2). These results are similar to the observations made in avian (9, 29, 30) and feline (25) viral systems, where viruses belonging to different envelope subgroups did not cause interferences with each other in cellular infection studies because viruses belonging to different envelope subgroups could not bind to the same cellular receptors.

The most useful aspect of the results reported here may be the preliminary determination of the chemical environment of the gp70 receptor. The receptor was fairly rapidly inactivated by the proteolytic enzyme chymotrypsin and somewhat less efficiently by trypsin, indicating that at least a part of the receptor is a protein. The binding was also reversibly inhibited by the protein-denaturing agents urea and guanidine-hydrochloride. The receptor was not sensitive to treatment of the membranes with either neuraminidase or a mixture of glycosidases. Therefore, if sugar residues are involved in the receptor activity, they are not accessible to these enzyme actions. It is possible also that a specific carbohydrate structure not affected by the enzymes we used could be involved in the complex formation. Enzymatic hydrolysis of sugar residues often proceeds in a sequential manner, and a key glycosidic linkage left unhydrolyzed near a terminal region could protect the rest of the sequences (16). The receptor activity of the membrane preparation was very sensitive to phospholipase C. This effect is similar to the action of phospholipase C on immunoglobulin E receptors of rat mast cells (2). The effect of gp70 receptors may be indirect. Phospholipase C might affect the structural integrity of the membrane, which indirectly affects the orientation of the receptor. For instance, phospholipase C activates the insulin receptor, probably by exposing the receptor, which now becomes susceptible to proteolytic digestion. Irrespective of the mechanism involved, phospholipase C is known to influence the activity of receptors for several hormones and regulator molecules (see reference 12). Our tentative conclusion is that the gp70 receptor consists of a lipid-protein structure. An alternative explanation might be that the treatment of the membranes with phospholipase C and chymotrypsin releases the receptor in a soluble form which would be removed during the washing of the treated membranes. This is less likely especially if the receptor has any protein character. The reasons why nonionic detergents are inhibitory to the complex formation between gp70 and the receptor are probably the adverse effects of the detergents on the structural integrity of the lipid-protein complex.

Although receptors have been identified on the cell surface for several enveloped viruses, no normal physiological function has been attributed to them. Nonetheless, it will be of interest to further characterize them and study their function in the attaching and uncoating of retroviruses.

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LITERATURE CITED

1. **Alter, G. M., E. R. Pandolfino, D. J. Christie, and J. A. Magnuson.** 1977. Calcium-induced cooperativity of manganese binding to concanavalin A. Biochemistry **16**:4031–4038.
2. **Bach, M. K., and J. R. Brashler.** 1973. On the nature of the presumed receptor for IgE on mast cells. I. The effect of sialidase and phospholipase C treatment on the capacity of rat peritoneal cells to participate in IgE-mediated, antigen-induced histamine release *in vitro*. J. Immunol. **110**:1599–1608.
3. **Baird, S., W. Raschke, and I. L. Weissman.** 1977. Evidence that MuLV-induced thymic lymphoma cells possess specific cell membrane binding sites for MuLV. Int. J. Cancer **19**:403–413.
4. **Cuatrecasas, P.** 1972. Isolation of the insulin receptor of liver and fat cell membranes. Proc. Natl. Acad. Sci. U.S.A. **69**:318–322.
5. **Dales, S.** 1973. Early events in cell-animal virus interactions. Bacteriol. Rev. **37**:103–135.
6. **Dales, S., W. Stern, S. B. Weintraub, and T. Huima.** 1976. Genetically controlled surface modifications by poxviruses influencing cell-cell and cell-virus interactions, p. 253–270. *In* R. F. Beers and E. G. Bassett (ed.), Cell membrane receptors for viruses, antigens and antibodies, polypeptide hormones and small molecules. Raven Press, New York.
7. **DeGiuli, C., S. Kawai, S. Dales, and H. Hanafusa.** 1975. Absence of surface projections on some non-infectious forms of RSV. Virology **66**:253–260.
8. **DeLarco, J., and G. J. Todaro.** 1976. Membrane receptor for murine leukemia viruses: characterization using purified viral envelope glycoprotein, gp71. Cell **8**:365–371.
9. **Duff, R. G., and P. K. Vogt.** 1969. Characteristics of two new avian tumor virus subgroups. Virology **39**:18–30.
10. **Fowler, A. K., D. R. Twardzik, C. D. Reed, O. S. Weislow, and A. Hellman.** 1977. Binding characteristics of Rauscher leukemia virus envelope glycoprotein gp71 to murine lymphoid cells. J. Virol. **24**:729–735.
11. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I labeled growth hormone of high specific activity. Biochem. J. **89**:114–123.
12. **Haour, F., and B. B. Saxena.** 1974. Characterization and solubilization of gonadotropin receptor of bovine corpus luteum. J. Biol. Chem. **249**:2195–2205.
13. **Hayman, M. J., and M. J. Crumpton.** 1972. Isolation of glycoproteins from pig lymphocyte plasma membrane using *Lens culinaris* phytohemagglutinin. Biochem. Biophys. Res. Commun. **47**:923–930.
14. **Howard, I. K., H. J. Sage, M. D. Stein, N. M. Young, M. A. Leon, and D. F. Dyckes.** 1971. Studies on a phytohemagglutinin from the lentil. II. Multiple forms of *Lens culinaris* hemagglutinin. J. Biol. Chem. **246**:1590–1595.
15. **Kalb, A. J., and A. Levitzki.** 1968. Metal binding sites of concanavalin A and their role in the binding of α-methyl D-glucopyranoside. Biochem. J. **109**: 669–672.
16. **Koide, N., M. Nose, and T. Muramatsu.** 1977. Recognition of IgG by Fc receptor and complement. Biochem. Biophys. Res. Commun. **75**:838–844.
17. **Laemmli, U. K.** 1970. Cleavage of the structural proteins during the assembly of the bacteriophage T_4. Nature (London) **227**:680–685.
18. **Lonberg-Holm, K., and L. Philipson.** 1974. Early interactions between animal viruses and cells, p. 1–148. *In* J. L. Melnick (ed.), Monographs in virology, vol. 9. S. Karger, Basel.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Miyamoto, K., and R. V. Gilden.** 1971. Electron microscopic studies of tumor viruses. I. Entry of murine leukemia virus into mouse embryo fibroblasts. J. Virol. **7**:395–406.
21. **Moldow, C. F., M. McGrath, and C. Peterson.** 1977. Solubilization of initial attachment site activity for avian tumor viruses by lithium diiodosalicylate. Proc. Soc. Exp. Biol. Med. **154**:201–205.
22. **Perdue, J. F.** 1974. The isolation and characterization of plasma membranes from cultured chick embryo fibroblasts. Methods Enzymol. **31**:162–168.
23. **Rifkin, D. B., and R. W. Compans.** 1971. Identification of spike proteins of Rous sarcoma virus. Virology **46**:485–489.
24. **Rossi, G., S. A. Newman, and H. Metzger.** 1977. Assay and partial characterization of the solubilized cell surface receptor for immunoglobulin E. J. Biol. Chem. **252**:704–711.
25. **Sarma, P. S., and T. Log.** 1971. Viral interference in feline leukemia-sarcoma complex. Virology **44**:352–358.
26. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–674.
27. **Strand, M., and J. T. August.** 1976. Structural proteins of ribonucleic acid tumor viruses. J. Biol. Chem. **251**:559–564.
28. **Tate, R. L., H. I. Schwartz, J. M. Holmes, L. D. Kohn, and R. J. Winand.** 1975. Thyrotropin receptors in thyroid plasma membranes. J. Biol. Chem. **250**: 6509–6515.
29. **Vogt, P. K., and Izhizaki.** 1966. Patterns of viral interference in avian leukosis and sarcoma complex. Virology **30**:368–374.
30. **Weiss, R. A.** 1969. Interference and neutralization studies with Bryan strain Rous sarcoma virus synthesized in the absence of helper virus. J. Gen. Virol. **5**:529–539.
31. **Weiss, R. A.** 1976. Receptors for RNA tumor viruses, p. 237–251. *In* F. Beers and E. G. Bassett (ed.), Cell membrane receptors for viruses, antigens and antibodies, polypeptide hormones and small molecules. Raven Press, New York.
32. **Witte, O. N., A. Tsukamoto-Adey, and I. L. Weissman.** 1977. Cellular maturation of oncornavirus glycoproteins: topological arrangement of precursor and product forms in cellular membranes. Virology **76**:539–553.