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Cell Surface Binding Proteins for the Major Envelope Glycoprotein of Murine Leukemia Virus (40670)[1]

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The initial step in establishing a productive oncornavirus infection requires the presence of specific "receptors" on the surface of the host cell (1). Secondary factors, however, subsequent to adsorption have also been shown to influence host susceptibility, i.e., Fv-1 restriction (2, 3). The 71,000 M_r major envelope glycoprotein (gp71) of murine leukemia virus (MuLV) is required for virus–receptor interaction and subsequent infection. Interference studies utilizing purified gp71 indicate that N and B tropic viruses share the same family of receptors, whereas mouse xenotropic viruses use a different family of receptors than do ecotropic viruses (4–6). To our knowledge however, no information is presently available identifying those cell surface component(s) involved in structuring the functional receptor for oncornaviruses.

The spleen and thymus are target tissues for murine leukemia virus and viral specific sequences were detected in the DNA of these tissues in leukemic mice infected at birth with virus (7). We have previously described an *in vitro* system for studying the binding characteristics of Rauscher murine leukemia virus (R-MuLV) gp71 to cells derived from susceptible murine target tissues (8). The binding of gp71 to mouse fibroblasts in culture has also been recently described (9). In this study we have used a cleavable bifunctional chemical reagent to reversibly link R-MuLV gp71 bound to thymocyte surface polypeptides in the vicinity of the gp71 binding site. Analysis of immune precipitates of covalently linked "gp71-receptor" conplexes after reductive cleavage has been used to identify polypeptides either comprising or "nearest neighbors" to the binding site "receptor" for R-MuLV gp71.

Materials and methods. Thymic cells from 6-week-old BALB/c mice were prepared as previously described (8) and were more than 90% viable as determined by trypan dye exclusion. Cells ($1–5 \times 10^7$/ml of medium) were labeled overnight at 37° in a 5% CO_2 atmosphere on a rocker platform in leucine-free RPMI 1640 media containing 200 m*M* glutamine, [^{3}H]leucine (0.5 mCi/ml [22 Ci/mmole]) and 10% dialyzed fetal calf serum. The major envelope glycoprotein gp71 was purified as previously described (10) from freeze–thaw preparations of double-banded R-MuLV produced in JLS-V9 cells (10^{12} virus particles/ml; 2×10^8 focus-forming units/ml). The purified glycoprotein (Schiff's positive) was labeled with ^{125}I ($2–5 \times 10^4$ cpm/ng of protein) using the chloramine-T method (11) and migrated as a single band on SDS linear 7.5–25% gradient polyacrylamide gel electrophoresis (PAGE) with an estimated molecular weight of 71,000 M_r. Binding reactions contain 1×10^7 [^{3}H]leucine cells/ml and were incubated at 37° in the presence of ^{125}I-labeled gp71 in RPMI 1640 containing 1% BSA. The [^{3}H]leucine cell surface polypeptide–^{125}I-labeled gp71 bound complex was solubilized in a solution containing 0.25% NP40. Cross-linking with methyl 4-merceptobutyrimidate (MMB) was a modification of the procedure as described by Traut *et al.* (12). NP40-solubilized ^{125}I-labeled gp71 [^{3}H]leucine thymocyte surface polypeptides were reacted with MMB (3 mg/ml) for 6 min at 22° and were quenched by the addition of ethanolamine-HCl (pH 8.0) (0.14 *M* final concentration) and incubated for another 10 min. Disulfide bond formation between MMB-introduced thiol groups was initiated by the addition of hydrogen peroxide (40 m*M*) followed by a 30-min incubation at 24°. Samples were first counted in an ultra-

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rack gamma counter and then solubilized and recounted in a Searle liquid scintillation counter utilizing a computer-directed program to determine spillover. Appropriate standards of ^{125}I, ^{3}H, and $^{125}I + {}^{3}H$ were included and ^{3}H activity then calculated. Protein was determined using the Bramhall assay (13).

Results. BALB/c thymocytes incorporated 145,000 cpm of [^{3}H]leucine per 10^{6} cells (0.7 pmole/μg protein) when incubated for 10 hr at 37° in the presence of RPMI 1640 supplemented with excess glutamine (200 m*M*) and 10% fetal calf serum. The binding of gp71 was initially linear with 50% maximum binding (2.5–3.0 ng of ^{125}I-labeled gp71/10^{6} cells) occurring within 30 min at 37° (8). Antibody to purified R-MuLV gp71 prevented binding and the addition of cold gp71 competitively inhibited the binding of the ^{125}I-labeled gp71 to thymocytes.

Soluble ^{125}I-labeled gp71 bound [^{3}H]leucine cell surface polypeptide complexes stripped from washed thymocytes with 0.25% NP40 were immediately introduced into reaction mixtures containing the cleavable cross-linking reagent MMB. The kinetics of the cross-linking reaction for gp71 are rapid and maximum extent of ^{125}I-labeled gp71 oligomer formation was observed after 6 min of incubation at 10 m*M* MMB. SDS-PAGE (Fig. 1) under nonreducing conditions (no β-mercaptoethanol) of the products of these reaction mixtures demonstrated the formation of high-molecular-weight oligomers containing disulfide-linked ^{125}I-labeled gp71 and [^{3}H]leucine thymocyte polypeptides migrating in the nonlinear region of the gel (fractions 5–10). Gel slices corresponding to these fractions contained a total of approximately 7000 cpm of ^{125}I and 35,000 cpm of [^{3}H]-leucine as determined by differential counting (Materials and Methods). These oligomers were completely cleaved by extensive reduction with 3% β-mercaptoethanol to monomers migrating in the region of gp71 (fractions 60–70). Data on the immunoprecipitation with various antisera of either NP40 stripped, MMB cross-linked ^{125}I-labeled gp71 preincubated with thymocytes and ^{125}I-labeled gp71 samples which were not preincubated is presented in Table I. Experiments in which unlabeled gp71 was used in

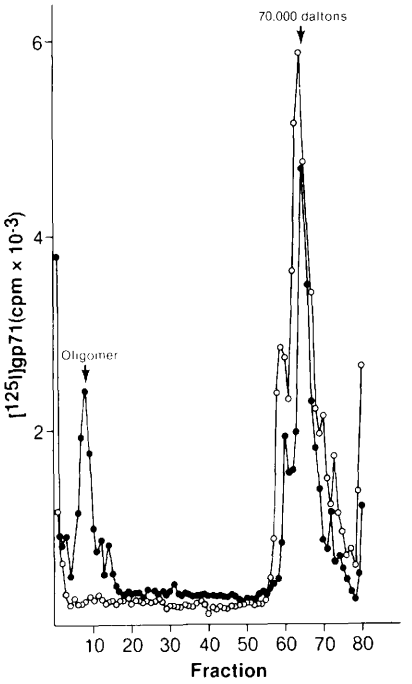

FIG. 1. SDS–Polyacrylamide gel electrophoresis of MMB-cross-linked ^{125}I-labeled gp71 oligomers under reducing (○) and nonreducing conditions (●). Samples for reductive cleavage were removed from MMB-cross-linking reactions and extensively dialyzed against 3% β-mercaptoethanol at 4° for 25 hr followed by dialysis against SPB buffer (50 m*M* Tris–Cl, pH 6.7, 1% SDS, 5% β-mercaptoethanol 10% glycerol and 0.005% bromophenol blue) and electrophoresed in 7.5–20% gradient SDS–polyacrylamide tube gels as previously described (31). Gels were cut in 1.2-mm slices and counted directly in an LKB 1207 Rackgamma M Counter.

the initial linking reaction also demonstrated the cross-linked-dependent immunoprecipitation of [^{3}H]leucine thymocyte surface proteins with gp71 antiserum. Antiserum to gp71 was preadsorbed with thymocytes (five cycles) and demonstrated to be highly specific. Postlinked ^{125}I-labeled gp71 [^{3}H]leucine thymocyte polypeptide oligomers were precipitable with gp71 antiserum (>70%) and the cross-linking reaction had little or no effect on the affinity of ^{125}I-labeled gp71 for homologous antibody. Although antisera containing H-2^{d} and Ia reactivity had no specificity for ^{125}I-labeled gp71 (<0.4%), the MMB cross-linking reaction did covalently bind cell surface polypeptides with determinants which were now reactive with antisera (>9% of input ^{125}I-labeled gp71). Similarly, antisera

TABLE I. IMMUNOPRECIPITATION OF MMB-CROSS-LINKED ^{125}I-LABELED GP71 THYMOCYTE SURFACE POLYPEPTIDES

Antiserum to	MMB control[a] ^{125}I-labeled gp71(cpm)			MMB-cross-linked ^{125}I-labeled gp71(cpm)		
	Input	Precipitate	% Precipitation	Input	Precipitate	% Precipitation
gp71	126,900	81,495	64.2	80,022	59,661	74.6
p30	133,098	351	0.3	72,429	12,651	17.5
p15	121,818	285	0.2	81,500	4,808	5.9
H_2d^b	128,331	550	0.4	75,711	6,864	9.1
IgG goat	125,325	234	0.2	74,766	393	0.5
IgG mouse	112,050	105	0.1	75,168	309	0.4

[a] Immune precipitation reaction tubes (final volume of 250 μl) contained 100-μl aliquots of either NP40-stripped, MMB-cross-linked ^{125}I-labeled gp71·thymocyte surface polypeptide oligomers or ^{125}I-labeled gp71 samples which were not preincubated with thymocytes. Antisera stock was diluted with a solution containing 0.05 *M* Tris-Cl, pH 7.2, 0.05 *M* NaCl, 0.001 *M* EDTA (TNE buffer), and 1% bovine serum albumin so that the addition of 100 μl resulted in a final antibody dilution of usually 1/200. Tubes were incubated for 2 hr at 37° and overnight at 4°. Anti-mouse IgG serum produced in goats or pigs was added at a dilution giving maximum precipitation and samples reincubated for 3 hr at 37°. Pellets (500 *g* for 15 min at 5°were washed twice in 3.0 ml of TNE buffer containing 2% BSA, 0.4% Triton X-100 followed by washing twice with phosphate-buffered saline and transferred to clean tubes. The ^{125}I cpm in the final pellet was measured in a LKB 1270 rack gamma counter.

[b] Cell-free ascites fluid served as the source of H-2 and Ia antibody and was prepared by alloimmunization of cogenic strains of mice (generous gift of D. Sachs). Antisera to viral antigens was prepared by hyperimmunization of goats with guanidine-HCl-purified viral protein.

to R-MuLV p30 and p15 precipitated 17.5 and 5.9%, respectively, of the postlinked input radioactivity (cpm). Consistent immunoprecipitation of postlinked ^{125}I-labeled gp71–cell surface polypeptide oligomers with serum containing reactivity to both H-2^d and Ia antigens was observed in four separate experiments utilizing thymocyte preparations derived from pools of 20 mice each (total 80 mice). Minimal precipitation (0.5%) was observed with either goat or mouse anti-IgG. The immune precipitates (gp71 antiserum) were reduced by dialysis against 3% β-mercaptoethanol and the molecular weights of the [^{3}H]leucine polypeptides in postlinked complexes were then estimated by SDS–PAGE. Six major [^{3}H]leucine polypeptides which migrate within the linear range of 7.5% polyacrylamide gels are seen in reduced immune precipitates (gp71 antiserum) of MMB cross-linked ^{125}I-labeled gp71·[^{3}H]leucine thymocyte surface oligomers (Fig. 2). These polypeptides correspond to estimated molecular weights of 26,000, 32,000, 45,000, 65,000, 95,000, and 120,000 M_r. The largest protein (fractions 11–13) did not migrate within the linear range of the gel and is assigned a value greater than 180,000 M_r. Washed immunoprecipitates of NP40-stripped, MMB-linked, thymocyte surface polypeptides to which no ^{125}I-labeled gp71 was added (controls) derived utilizing gp71 antiserum which was preadsorbed with receptor-positive thymocytes contained less than 500–700 cpm of [^{3}H]leucine. SDS–PAGE of these controls after reduction demonstrated low activity in the 70,000 M_r region and probably represents endogenous gp71. ^{125}I-Labeled gp71 recovered from cross-linking reactions which contained no thymocyte proteins migrated as a single band in the region of 70,000 M_r.

Discussion. The identification and characterization of cell surface polypeptides involved in structuring the functional "receptor" complex for oncornaviruses will be instrumental in further expanding our understanding of the horizontal spread of infectious virus progeny to susceptible host cells and also exploring cellular immune mechanism mediated via viral structural components (antigens) which bind to cell surface receptors and both modify and modulate cellular immune responses, i.e., destruction of virus-infected cells by cytotoxic lymphocytes.

In this study we have identified six thymocyte surface polypeptides which appear to be localized on the cell surface in the vicinity of the binding site for R-MuLV gp71. MMB has been utilized in determining the topography of *Escherichia coli* 30 S ribosomal proteins and the quaternary structures of various enzymes (12). The imidate function of the

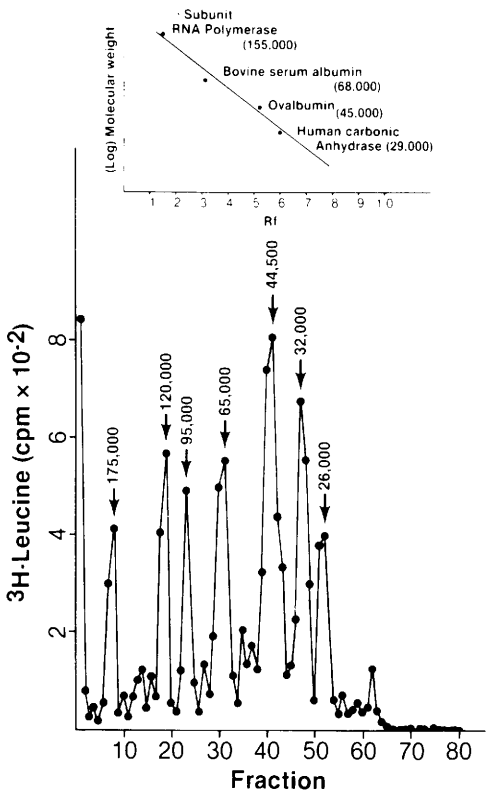

FIG. 2. SDS-Polyacrylamide gel electrophoresis of reduced [^{3}H]leucine polypeptides solubilized from anti-gp71 precipitates of MMB-cross-linked oligomers. Aliquots were removed from MMB-cross-linking reaction mixtures and precipitated with thymocyte-adsorbed gp71 antiserum as described in Table 1. Washed pellets were reduced in a solution containing 3% β-mercaptoethanol 2 *M* NaCl and 0.01 *M* Tris-Cl, pH 7.8 (constant mixing for 24 hr at 4°) recentrifuged to remove insoluble precipitates and dialyzed against SPB buffer prior to SDS-PAGE analysis. The molecular weights were estimated from semilogarithmic plots using the log of molecular weight versus migration ratio of the protein standards: β subunit of *Escherichia coli* RNA polymerase, 155,000 M_r, bovine serum albumin 68,000 M_r, ovalbumin 45,000 M_r, and human carbonic anhydrase 29,000 M_r.

molecule reacts with the free amino groups of gp71 and cell surface polypeptides and the added thiol group function introduced by the MMB molecule is mildly oxidized to form disulfide-linked oligomers (12). From the reported length of the MMB molecule, (12) 15 Å, the inference is made that the thymocyte surface polypeptides and bound gp71 observed are spatially orientated with this distance after the initial binding reaction in order to participate in the cross-linking reaction. Initial cross-linking reactions were attempted on washed viable thymocytes (no NP-40 stripping) removed from ^{125}I-labeled gp71 binding reaction mixtures after gp71 saturation was achieved. Unfortunately, there is no specificity in the cross-linking reaction in that the imidate function of the reagent reacts with the amino group of all neighboring cell surface proteins which are within effective cross-linking distance. In our system, this resulted in extremely large insoluble oligomers which were too complex for further analysis; the utilization, however, of NP40-solubilized cell surface components in these studies although not the method of choice does provide a technically analyzable experimental system. The noncleavable bifunctional cross-linking reagent dimethyl suberimidate was also used to generate gp71·cell surface polypeptide oligomers, however the linked complexes formed were mostly insoluble and the advantage of dissociating the components of the oligmer and subsequent identification was also lost (irreversible linkage). Advantage is taken of the retained antigenicity of the gp71 molecule after the cross-linking reaction to selectively absorb out those linked complexes containing gp71 from the heterogeneous pool of thymocyte surface proteins not within distance for effective cross-linking and therefore presumably not involved in the binding of gp71. This brings up a consideration of cell membrane fluidity and cautions against absolute interpretations of the data. It is well established that membrane components diffuse freely with diffusion rates much higher relative to similar molecules in solution (14), and membrane proteins have been estimated to traverse 200 to 1400 nm/sec (15). Some of the thymocyte polypeptides observed in the cross-linked complex do not necessarily have to be a constant neighbor of the gp71 binding site but can also become companion molecules via migration (post facto) after the initial binding. Conversely, other molecules may leave the vicinity or become buried in the cell membrane after the binding reaction and become unavailable to participate in the reaction.

In addition to the molecular weight estimations, a partial identification of those thymocyte polypeptides cross-linked to gp71 was achieved using immunoprecipitation analysis before and after MMB cross-linking. The use of antisera directed against products of the major histocompatibility gene complex (H-2^d and Ia antigens) demonstrated the cross-linked dependent precipitation of ^{125}I-labeled gp71. Those thymocyte polypeptides observed in reduced gp71 immune precipitates migrating in the molecular weight region of 45,000 M_r correspond to the reported molecular weights for H-2 antigens (16). The suggestion that products of the major histocompatibility complex are involved in the interaction of viral structural components with the cell surface is not new. Fluorescent antibody experiments have demonstrated that capping of H-2 antigens resulted in the cocapping of antigens reactive with antiserum directed against envelope glycoproteins of R-MuLV (17). Viral modification of H-2 antigens specify virus infected syngeneic cells as targets of cytotoxic T-lymphocytes (18) and a hypothesis has been advanced suggesting that the H-2 molecules function as an adapter capable of interacting with viral antigens on the target cell surface (17). We have recently demonstrated the affinity of H-2 antigens for the envelope glycoprotein of R-MuLV (19) and a gene within the H-2 complex has been mapped which influences the formation of molecular complexes of the H-2^b gene product with Friend virus (FV) molecules in the plasma membrane of infected cells (20). As a consequence, inclusion of H-2^b molecules into FV, cocapping of FV, and H-2^b antigens, and target cell generation have been described. The data presented in this study also suggests that H-2 is expressed on the cell surface or moves after the initial binding event to a position near the binding site for gp71, and adds biochemical evidence supporting the physical association of gene products of the histocompatibility locus and viral antigens. Although evidence for the association of H-2 antigens and gp71 on the cell surface is considerably strong, we cannot by experimental design rule out the possibility of the reassociation of H-2 antigens and gp71 during the solubilization procedure prior to linking.

The cross-linked dependent precipitation of ^{125}I-labeled gp71·thymocyte surface polypeptides with antisera to products of the "gag" gene is surprising. Both p30 and p15 antisera precipitate approximately 17.5 and 6%, respectively, of the input gp71 only if it has been cross-linked with MMB. The expression of gag gene products in particular p30 gag precursor on the cell surface of infected (21) and normal mouse cells during embryogenesis (22) has been reported. The major polypeptide found in reduced (p30) immune precipitates of cross-linked gp71·thymocyte surface proteins migrates on SDS–PAGE in the range of 65,000–85,000 M_r (data not shown). A 65,000 M_r polypeptide is observed in the gel profile of Fig. 2 (fractions 28–32). This corresponds in the range of the molecular weight reported for the gag precursor 65,000–85,000 M_r (23) and therefore suggest possibly that viral precursor molecules are in the vicinity of the receptor for gp71.

In conclusion, the data we have presented supports previous observations that products of the major histocompatibility locus interact with viral antigens on the cell surface. Preliminary evidence is also presented which suggest that gag gene products are also localized in the vicinity of the binding site for gp71. These studies have also demonstrated that the use of *cleavable* bifunctional cross-linking reagents followed by the specific immunoprecipitation of the molecule of interest (gp71) is a valuable tool for studying the interaction of molecules on the cell surface.

Summary. The 71,000 M_r major envelope glycoprotein (gp71) of Rauscher murine leukemia virus has been shown to bind to susceptible cells *in vitro*. [^{3}H]Leucine surface polypeptides bound to ^{125}I-labeled gp71 were stripped from BALB/c thymocytes with nonionic detergent and chemically linked with the cleavable bifunctional reagent methyl 4-mercaptobutyrimidate. Soluble disulfide-linked ^{125}I-labeled gp71 bound thymocyte surface polypeptide oligomers were precipitated with adsorbed gp71 antisera and after reductive cleavage, molecular weights (SDS–PAGE) of approximately 23,000, 32,000, 45,000, 65,000, 90,000, and 170,000 M_r were seen. Experiments demonstrating the *cross-linked-dependent* immunoprecipitation of ^{125}I-

labeled gp71·thymocyte surface polypeptide oligomers with antiserum having reactivity to products of the major histocompatibility locus (H-2^d and Ia) and also for the gag gene products (p30 and p15) are described. The involvement of these thymocyte surface polypeptides in structuring the functional receptor for ecotropic oncornaviruses is discussed.

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