JOURNAL OF VIROLOGY, Feb. 1986, p. 413–421
0022-538X/86/020413-09$02.00/0

Vol. 57, No. 2

Monoclonal Antibody to the Amino-Terminal L Sequence of Murine Leukemia Virus Glycosylated *gag* Polyproteins Demonstrates Their Unusual Orientation in the Cell Membrane

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Received 26 December 1984/Accepted 1 July 1985

To analyze cell surface murine leukemia virus *gag* protein expression, we have prepared monoclonal antibodies against the spontaneous AKR T lymphoma KKT-2. One of these antibodies, 43-13, detects an AKR-specific viral p12 determinant. A second monoclonal antibody, 43-17, detects a novel murine leukemia virus-related antigen found on glycosylated *gag* polyproteins (gp95^{gag}, gp85^{gag}, and gp55^{gag}) on the surface of cells infected with and producing ecotropic endogenous viruses, but does not detect antigens within these virions. The 43-17 antibody immunoprecipitates the precursor of the cell surface *gag* protein whether in its glycosylated or unglycosylated state, but does not detect the cytoplasmic precursor of the virion *gag* proteins (Pr65^{gag}). Based on these findings, we have localized the 43-17 determinant to the unique amino-terminal part of the glycosylated *gag* polyprotein (the L domain). We have determined that gp95^{gag} contains L-p15-p12-p30-p10 determinants, whereas gp85^{gag} lacks the carboxyterminal p10 determinant, and gp55^{gag} lacks both p30 and p10 carboxy terminal determinants. Analysis of cell surface *gag* expression with the 43-17 antibody leads us to propose that the L domain plays a crucial role in (i) the insertion and orientation of murine leukemia virus *gag* polyproteins in the cell membrane and (ii) the relative abundance of expression of AKR leukemia virus versus Moloney murine leukemia virus glycosylated *gag* polyproteins in infected cells.

The murine leukemia virus (MuLV) *gag* genes are initially transcribed from a full-length (35S) RNA species (25, 65). One of the translation products of this message is a 65,000-molecular-weight polyprotein (Pr65^{gag}) (36, 53, 64) which contains the virion *gag* structural proteins in the order NH_2-p15-p12-p30-p10-COOH (3, 44). This polyprotein is subsequently phosphorylated within p12 (50) and cleaved sequentially to release the individual *gag* peptides for packaging into the virion core (1, 53, 64).

The discovery of a glycosylated form of the *gag* polyprotein at the surface of AKR lymphomas (31, 63) led to the recognition of a second pathway of *gag* gene processing. In Moloney MuLV-infected cells treated with tunicamycin, a 75,000-molecular-weight polyprotein (Pr75^{gag}) is detected which has been implicated as the precursor of glycosylated *gag* polyproteins (9, 15, 50). Tryptic peptide mapping has shown that Pr65^{gag} and Pr75^{gag} share all *gag* virion structural proteins, but that Pr75^{gag} contains a unique amino-terminal portion of 5,000 to 7,000 molecular weight termed "L" because it contains the leader (10, 48, 49) or left-hand sequence. However, the precise role of the L domain in *gag* protein processing and cell surface expression is not known.

To investigate further the mechanisms of cell surface *gag* expression, we have prepared monoclonal antibodies to cell surface *gag* antigens of spontaneous AKR lymphomas by using xenogeneic immunizations. In this paper we report the isolation and characterization of a rat monoclonal antibody, 43-17, which detects an antigenic determinant localized to or dependent upon the unique amino-terminal L domain of the glycosylated *gag* polyprotein (10, 49). Using the 43-17 antibody, we present evidence that the L domain plays a crucial role in the orientation and expression of AKR virus *gag* polyproteins in the cell membrane.

MATERIALS AND METHODS

Mice. AKR/J mice were originally obtained from the Jackson Laboratory and were bred and raised in our own animal facilities.

Antibodies. 43-13 and 43-17 are rat immunoglobulin G-2b hybridomas prepared by immunization of Fischer rats with the spontaneous AKR T lymphoma KKT-2. The preparation of these two antibodies has been described elsewhere (34). Monospecific rabbit anti-p30 serum was prepared by repeated immunization with Moloney virus p30 antigen purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Goat antisera against Rauscher MuLV p30, p15, p12, p10, and gp70 were obtained from J. Gruber, National Cancer Institute. Rat anti-C58NTD serum was prepared by repeated immunization of Wistar/Furth rats with syngeneic rat C58NTD T lymphoma cells. This serum has broad reactivity with MuLV proteins (17).

Cells. Summaries of the cell lines used in this study are presented in Tables 1 and 3. All in vitro tumor cell lines are maintained in culture in our laboratory in RPMI-1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum. The BW5147 lymphoma was obtained from R. Hyman of the Salk Institute, San Diego, Calif. The BL/VL_3, BL/AQR, and BL/RL-12-NP cell lines were generously provided by M. Lieberman, Stanford University. The Abelson lymphomas L1-2 and RAW112 were obtained from R. Coffman, Stanford University. The Moloney MuLV-induced lymphoma MBL-2 was provided by W. Green, Frederick Hutchinson Cancer Research Center, Seattle, Wash. The three spontaneous AKR T lymphomas, TK1, TK5, and TK38 (obtained from E.

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Butcher, Stanford University), are maintained by serial in vivo passage in irradiated (400 rads) AKR/J mice.

Viruses. The characteristics of the cloned viruses in this study are summarized in Table 2. Ecotropic viruses are maintained in the III6A feral mouse fibroblast cell line (obtained from T. Pincus, Vanderbilt University, Nashville, Tenn.); dualtropic and xenotropic viruses are maintained in the CCL64 mink lung fibroblast cell line (from J. Hartley, National Institutes of Health). Fibroblast cell lines are grown in culture in our laboratory in minimal essential medium (GIBCO) supplemented with 10% fetal calf serum. The cloned AKR L-1, WN1802N, WN1802B, and AKR-6 viruses were obtained from T. Pincus. BL/Ka(N), BL/Ka(B), BL/Ka(X), and RadLV/VL_3 viruses were provided by M. Lieberman. Cloned MCF 247 virus was obtained from J. Hartley.

Virions were purified from the supernatant fluids of virus-producing fibroblasts and lymphoma cells by Sepharose 4B chromatography (35) into phosphate-buffered saline (PBS), pH 7.4. Virus preparations were diluted to a final concentration of 0.1 unit of absorbance at 260 nm per ml in PBS before use.

FACS analysis. Cell suspensions of normal thymus or tissue culture cell lines were prepared in cell-suspending medium plus 5% newborn calf serum. Subconfluent fibroblast cell lines were harvested by incubation in Ca- and Mg-free PBS with 10 mM EDTA for 10 min at 37°C, followed by gentle pipetting to remove nonadherent cells. After one wash in cell-suspending medium–5% newborn calf serum, cells were adjusted to a concentration of 10^8 cells per ml. A 0.01-ml sample of cells was added to a conical tube together with 0.04 ml of hybridoma antibody supernatant or antiserum. After incubation for 15 min on ice, the cells were washed once over a calf serum underlayer. The pellet was suspended in 0.01 ml of fluorescein isothiocyanate-labeled second-stage antibody. For rat antibodies, the second stage used was a column-purified rabbit antiserum specific for rat immunoglobulin. Sheep anti-rabbit immunoglobulin serum was obtained from the Institute Pasteur, Paris, France; rabbit anti-goat immunoglobulin serum was purchased from Meloy, Inc., Springfield, Va. After incubation with the second-stage antibody for 15 min on ice, the cells were washed once over calf serum and suspended in PBS plus 1 mM sodium azide for fluorescence-activated cell sorter (FACS) analysis. All first- and second-stage antibodies were pretitered to give maximum staining intensity at the appropriate dilution.

Radioimmunoassay. A cell-binding radioimmunoassay was performed in 96-well flexible microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). Cell suspensions were prepared in cell-suspending medium–5% newborn calf serum and adjusted to 2.5×10^7 cells per ml. Then 2.5×10^5 cells were added to each well with 0.04 ml of hybridoma supernatant. After incubation for 4 h at 4°C, the wells were washed three times with PBS plus 5% calf serum. A second-stage ^{125}I-labeled rabbit anti-rat immunoglobulin (25,000 to 50,000 cpm in 0.025 ml) was added to each well and incubated for an additional hour at 4°C. After three washes, the wells were cut with a hot wire device and transferred directly to tubes for counting.

Antibody binding to virions was determined by solid-phase immunoassay in 96-well flexible microtiter plates. Wells were coated for 16 h at 4°C with 0.05 ml of a solution (0.1 unit of absorbance at 260 nm per ml) of Sepharose-4B purified virions in PBS–1 mM sodium azide. In some cases, virions were disrupted by freeze-thawing or detergent lysis before incubation on the plates. After three washes with PBS–5% calf serum, 0.05 ml of hybridoma supernatant was added to each well. After incubation for 2 h at 4°C the wells were again washed with PBS–5% calf serum. ^{125}I-labeled rabbit anti-rat immunoglobulin serum (25,000 to 50,000 cpm per 0.025 ml) was added to each well and incubated for 1 h at 4°C. After three washes in PBS–5% calf serum, the wells were cut with a hot wire device and transferred to tubes for counting.

Metabolic labeling with [^{3}H]leucine and tunicamycin treatment. KKT-2 cells were harvested from tissue culture and washed two times in PBS. The cells were suspended at a concentration of 10^7 cells per ml in leucine-free minimal essential medium supplemented with 5% fetal calf serum dialyzed against saline. Cells were labeled with 200 μCi of [^{3}H]leucine per ml for 1 h at 37°C in a 5% CO_2 incubator. Cells to be treated with tunicamycin were preincubated with 2 μg of tunicamycin per ml for 1 h before labeling with [^{3}H]leucine.

Cell surface iodination and endo F treatment. Lactoperoxidase-catalyzed iodination was performed on washed KKT-2 cells at a concentration of 2×10^7 cells per ml in PBS (69). One millicurie of ^{125}I was used to label 10^7 cells, and the reaction was stopped by the addition of sodium azide. After labeling, the cells were washed three times in cell-suspending medium–5% newborn calf serum. Viability was always greater than 95% as determined by trypan blue exclusion. In experiments involving endo-β-*N*-acetylglucosaminidase F (endo F), cells were treated by the method of Elder and Alexander (12) after surface iodination.

Immunoprecipitation. Labeled cells were lysed into phosphate lysis buffer plus 1 mM sodium azide (68) at a concentration of 2×10^6 cells per ml, and clarified by centrifugation at 27,000 rpm for 1 h. One milliliter of lysate was added to either 20 μl of monoclonal antibody supernatant concentrated 15-fold by 50% ammonium sulfate precipitation (in PBS) or with 5 μl of neat antiserum. After overnight incubation at 4°C, 60 μl of a 1-mg/ml solution of affinity-purified rabbit anti-rat immunoglobulin antibodies in PBS was added to those samples containing rat monoclonal antibodies. After an additional 2-h incubation, immune complexes were precipitated with 60 μl of a 10% suspension of *Staphylococcus aureus*. The *S. aureus* A pellets were washed four times with phosphate lysis buffer–1 mM sodium azide, and immunoprecipitates were eluted into SDS sample buffer containing 2-mercaptoethanol by boiling for 3 min.

SDS-PAGE. Labeled samples (25 to 50 μl) were run on SDS-PAGE gradient slab gels (20 to 5%) for 4 h at 150 V. Gels were fixed in methanol-acetic acid-water (50:10:40) for 1 h and then rinsed in distilled water for 1 h. Gels were treated with 1 M sodium salicylate for an additional hour, dried, and then exposed to Kodak XAR-5 film for 1 to 3 days by using an intensifying screen.

RESULTS

Production of monoclonal antibodies specific for cell surface *gag* polyproteins. A Fischer rat was immunized against the AKR spontaneous T-lymphoma cell line KKT-2, and its spleen was fused with the NS-1 myeloma cell line. To identify antibodies reactive with virus-encoded or induced nonvirion antigens, 100 hybridoma supernatants were screened initially against KKT-2 cells and purified KKT-2 virions. Fifty clones were found to react with both cells and virions; these were excluded from this study. To eliminate antibodies reactive with normal cellular antigens, the remaining 50 hybrids were screened against a panel of normal

TABLE 1. Distribution of 43-13, 43-17, and p30 antigens on tumor cell lines[a]

Cell line	Cell type	Strain of origin	Mode of induction	Median fluorescence[b]		
				43-13	43-17	p30
KKT-2	T lymphoma	AKR	Spontaneous	478	436	260
TK1	T lymphoma	AKR	Spontaneous	916	812	400
TK5	T lymphoma	AKR	Spontaneous	344	344	152
TK38	T lymphoma	AKR	Spontaneous	736	876	336
BW5147	T lymphoma	AKR	Spontaneous	4	3	3
BL/VL_3	T lymphoma	C57BL/Ka	Radiation leukemia virus	0	28	16
BL/AQR	T lymphoma	C57BL/Ka	Radiation leukemia virus	0	68	20
S49	T lymphoma	BALB/c	Mineral oil	0	22	52
YAC-1	T lymphoma	A/Sn	Moloney MuLV	1	11	181
MFT-2	T lymphoma	(C57BL/6 × BALB/c)F_1	Moloney MuLV	3	38	167
CL_2FT_2	T lymphoma	(C57BL/6 × BALB/c)F_1	Moloney MuLV	4	320	898
L691	T lymphoma	C57BL	Radiation	0	1	1
BL/RL-12NP	T lymphoma	C57BL/Ka	Radiation	2	3	7
EL-4	T lymphoma	C57BL/6	Chemical carcinogen	4	300	172
LSTRA	Fc receptor-positive lymphoma	BALB/c	Moloney MuLV	0	200	780
RAW112	Pre-B lymphoma	BALB/c	A-MuLV (Moloney MuLV)	0	84	130
NS-1	Plasmacytoma	BALB/c	Mineral oil	8	1,148	688
P815	Mastocytoma	DBA/2	Chemical carcinogen	4	371	164

[a] Cells were stained with the indicated antibody followed by a fluorescein isothiocyanate-labeled second-stage antibody. Analysis was performed by FACS.
[b] Median fluorescence above background staining standardized to a fluorescence gain of 8. Background staining was determined independently for each cell line using as a first-stage control either a nonbinding monoclonal antibody of irrelevant specificity (for 43-13 and 43-17) or an affinity-purified rabbit anti-rat immunoglobulin serum (for anti-p30).

lymphoid cells. Only two of these hybrids, designated 43-13 and 43-17, were negative on all lymphoid cells (data not shown). These two hybridoma lines were cloned at limiting dilution and grown to larger volume for further characterization of their monoclonal antibodies.

With the FACS, the two hybridomas were screened against a panel of tumors representing a variety of different cell types and modes of induction (Table 1). The 43-13 antibody is specific for AKR lymphomas; no binding was observed for any other tumor regardless of origin. In contrast, the 43-17 hybridoma detects an antigen found on a variety of tumors in addition to those of AKR origin. The highest level of 43-17-detected antigen is on the myeloma NS-1. Only three cell lines tested were totally negative for the 43-17 antigen. Two of these are radiation-induced lymphomas which do not produce detectable MuLV virions: L691 and BL/RL-12-NP (33). Surprisingly, the spontaneous AKR lymphoma BW5147 was also negative for both the 43-13 and 43-17 antigens. However, this cell line has been maintained for years in culture and no longer produces detectable levels of MuLV (33).

To investigate further the relationship between virus-encoded antigens and the expression of 43-13 and 43-17 antigens, the same tumor lines were stained with a heterologous rabbit anti-p30 serum. The occurrence of the 43-13 and 43-17 antigens was found to correlate closely with that of p30 expression on the cell surface (Table 1). All cell lines which are 43-17 positive are also positive for p30, although there are quantitative differences in the expression of the two antigens. These findings are supported by immunoprecipitation with the 43-13 and 43-17 antibodies of molecules of approximately 95,000, 85,000, and 55,000 molecular weight from ^{125}I-surface-labeled KKT-2 cells (Fig. 1). The two higher-molecular-weight proteins are precipitated also by the anti-p30 serum and thus correspond to the glycosylated *gag* polyproteins ($gp95^{gag}$ and $gp85^{gag}$) previously identified on spontaneous AKR lymphomas (30, 55, 63). The additional 55,000-molecular-weight protein precipitated by the 43-13 and 43-17 antibodies is not found in the anti-p30 immunoprecipitation and is the subject of a separate communication (Kooistra et al., manuscript in preparation).

Both 43-13 and 43-17 antibodies were initially negative when screened for binding to intact KKT-2 virions. However, since most of the virion *gag*-related proteins are located in the internal core of the virus particle (39), we subjected KKT-2 virions to disruption before performing the binding assay (Table 2). In parallel with its distribution on tumor cells and virus-infected cells, the 43-13 antigen is found only on AKR ecotropic and dualtropic viruses. In contrast to these findings with the 43-13 antibody, we have been unable to detect the 43-17 antigen on any disrupted virion preparation (Table 2), regardless of the amount of antibody or virus used in the assay (data not shown). Thus we conclude that the 43-17 antigen is specific for the cell surface form of the glycosylated *gag* polyprotein.

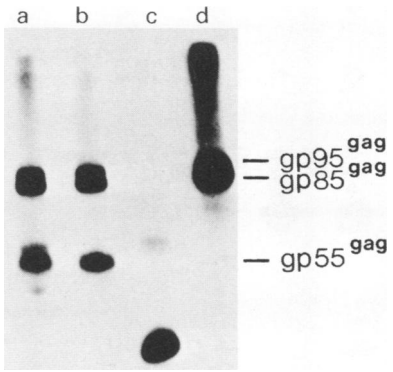

FIG. 1. Immunoprecipitation of ^{125}I surface-labeled proteins from KKT-2 cells by monoclonal antibodies. KKT-2 cells were surface labeled by lactoperoxidase-catalyzed iodination with 1 mCi of ^{125}I per 2×10^7 cells in PBS. Cell lysates were prepared and immunoprecipitated with (a) 43-13, (b) 43-17, (c) anti-Thy-1, or (d) anti-p30. Immunoprecipitated proteins were separated on an SDS-PAGE gradient slab gel (20 to 5%) and detected by autoradiography.

TABLE 2. Distribution of the 43-13 and 43-17 antigens on disrupted virions[a]

Expt	Virus of cell[b]	cpm bound/well[c]		
		43-13	43-17	Control
1	Virions			
	KKT-2 SL	870	113	74
	KKT-4 SL	486	111	101
	MCF 247	411	65	71
	RadLV/VL_3	63	69	53
	Moloney clone 2	62	60	66
2	Cells			
	KKT-2	2,102	1,401	78
	L691	98	136	124

[a] Virus and cell binding were determined in a two-stage radioimmunoassay with the unlabeled monoclonal antibody as the first stage and a ^{125}-I-labeled rabbit anti-rat immunoglobulin serum as the second stage. A solution of 0.1 mg of virus per ml in PBS freeze-thawed three times was used to coat the wells for the virus binding assay. For the cell binding assay, 2.5×10^5 lymphoma cells were used per well. The amount of first- and second-stage antibodies used per well was the same in both cases.

[b] Virus was purified by Sepharose-4B chromatography (23) from supernatant fluids of the following virus producing cell lines: KKT-2 SL producing KKT-2 spontaneous AKR lymphoma; KKT-4 SL producing KKT-4 spontaneous AKR lymphoma; MCF 247 producing CCL64 mink fibroblasts; RadLV/VL_3 producing BL/VL_3 radiation leukemia virus-induced lymphoma; Moloney MuLV producing III6A mouse fibroblasts. L691 is a nonproducer radiation-induced T lymphoma.

[c] The binding assays were performed in triplicate. Control values were determined for each virus and cell with medium alone used instead of first-stage antibody.

Molecular characterization of the 43-13 and 43-17 antigenic determinants. When immunoprecipitation was performed on extracts of [^{3}H]leucine labeled KKT-2 cells (Fig. 2), the 43-13 antibody was found to precipitate both the precursor of the virion *gag* structural proteins (Pr65^{gag}) and the 180,000-molecular-weight *gag-pol* precursor (Pr180$^{gag\text{-}pol}$). The 43-13 antibody also immunoprecipitates p12-related peptides from metabolically labeled AKR virus-infected cells and their supernatants (data not shown).

In contrast, the 43-17 antibody does not immunoprecipitate either Pr65^{gag} or Pr180$^{gag\text{-}pol}$, although it precipitates glycosylated *gag* polyproteins (gp95^{gag} and gp85^{gag}) from the same cell extract (Fig. 2). This suggests that the 43-17 determinant is specific for the glycosylated form of the *gag* polyproteins. Such an antigenic determinant could reside either in the unique N-terminal portion of the *gag* polyprotein (L) or in some posttranslational modification specific for the glycosylated *gag* proteins (e.g., the carbohydrate side chains). The Moloney MuLV glycosylated *gag* polyprotein gPr80^{gag} has been shown to contain three N-linked carbohydrate groups, one in the L or p15 domain and the other two in the p30 domain (47). To test whether N-linked carbohydrates contributed to 43-17 detected antigens, KKT-2 cells were labeled after pretreatment with tunicamycin (28, 61). In the presence of tunicamycin, the 43-17 antibody immunoprecipitates an 85,000- to 87,000-molecular-weight protein (Fig. 3) from KKT-2 cells, demonstrating that 43-17 binding is not dependent on N-linked glycosylation of the *gag* polyprotein. Additional evidence against the possibility that the 43-17 antibody detects a carbohydrate-related antigen is provided by experiments with endo F, which cleaves both high-mannose and complex glycoproteins (12). KKT-2 cells were surface iodinated by the lactoperoxidase method, subjected to endo F digestion or mock digestion, lysed, and then immunoprecipitated with various monoclonal antibodies and antiviral antisera (Fig. 4). In contrast to the three cell surface *gag* polyproteins found on untreated cells (gp95^{gag}, gp85^{gag}, and gp55^{gag}), endo F treatment produces three deglycosylated products with increased electrophoretic mobility consistent with polypeptides of approximately 85,000, 75,000, and 45,000 molecular weight. This experiment demonstrates that all three cell surface *gag* polyproteins possess endo F-sensitive N-linked carbohydrates, and that removal of these carbohydrate groups does not alter the antigenic determinant recognized by the 43-17 antibody. Based on these data, we propose that the 43-17 antibody detects a nonglycosylated determinant localized to or dependent upon the unique N-terminal L domain of the cell surface *gag* polyproteins.

Our immunoprecipitation of an 85,000-molecular-weight

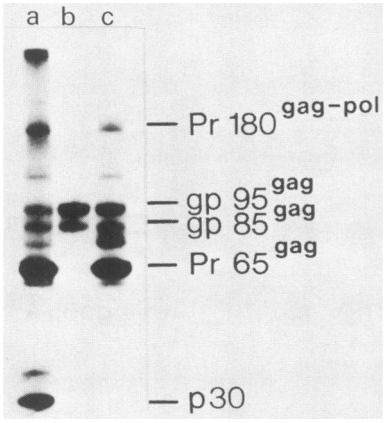

FIG. 2. Immunoprecipitation of [^{3}H]leucine-labeled proteins from KKT-2 cells by monoclonal antibodies. KKT-2 cells were labeled with 200 μCi of [^{3}H]leucine per ml for 1 h at 37°C in leucine-free minimal essential medium. Cell lysates were prepared and immunoprecipitated with (a) anti-p30, (b) 43-17, or (c) 43-13. Immunoprecipitated proteins were separated on an SDS-PAGE gradient slab gel (20 to 5%) and detected by fluorography.

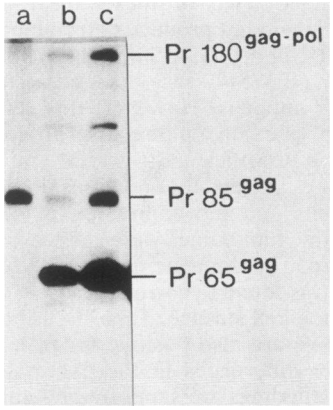

FIG. 3. Effect of tunicamycin pretreatment on immunoprecipitation of [^{3}H]leucine-labeled proteins from KKT-2 cells. KKT-2 cells were preincubated with 2 μg of tunicamycin per ml for 1 h at 37°C in leucine-free minimal essential medium. Then 200 μCi of [^{3}H]leucine per ml was added, and cells were incubated for an additional hour. Cell lysates were prepared and immunoprecipitated with (a) 43-17, (b) 43-13, or (c) anti-p10. Immunoprecipitated proteins were separated on an SDS-PAGE gradient gel (20 to 5%) and detected by fluorography.

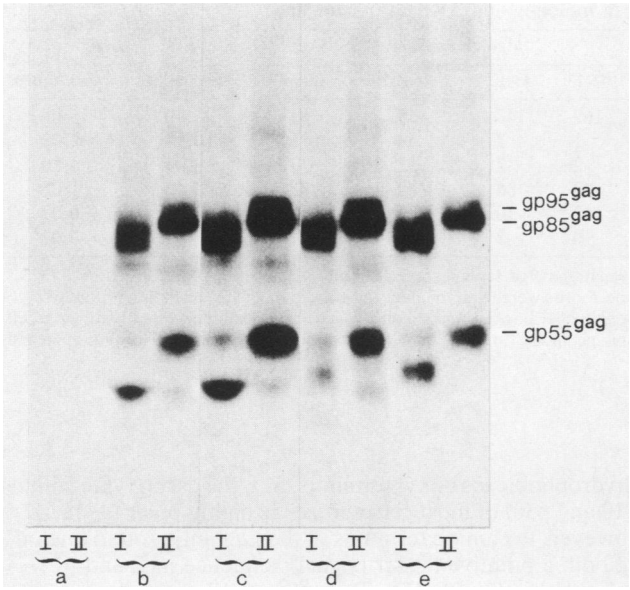

FIG. 4. Effect of endo F treatment on immunoprecipitation of [^{3}H]leucine-labeled proteins from KKT-2 cells. KKT-2 cells were surface labeled by lactoperoxidase-catalyzed iodination with 1 mCi of ^{125}I per 2×10^7 cells in PBS. Labeled cells were either mock digested (lanes II) or digested with endo F (lanes I) by the method of Elder and Alexander (12). Cell lysates were prepared and immunoprecipitated with (a) normal rat serum, (b) 43-17, (c) 43-13, (d) anti-p15, or (e) anti p12. Immunoprecipitated proteins were separated on an SDS-PAGE gradient gels (20 to 5%), and detected by fluorography.

protein from tunicamycin-treated KKT-2 cells with 43-17 and anti-p10 antibodies suggest that this protein is the precursor of the glycosylated *gag* polyproteins. In analogy to Pr75^{gag} in the Moloney MuLV system, we have termed this protein Pr85^{gag}. These findings are in contrast with those of Ledbetter (29), who reported that a 75,000-molecular-weight protein is the precursor of the glycosylated *gag* in AKR lymphoma cells. We have also detected a 75,000-molecular-weight protein in AKR tumors with 43-13 and anti-p10 antibodies, but not with 43-17 (Fig. 2). However, this molecule is unlikely to represent the glycosylated *gag* precursor, since it is found in cells not treated with tunicamycin. Since glycosylation occurs coordinately with protein synthesis in the rough endoplasmic reticulum (26, 46, 52), the unglycosylated precusor of the cell surface *gag* should be synthesized only when glycosylation is blocked. Thus, in cells infected with Moloney MuLV, the Moloney glycosylated *gag* precursor (Pr75^{gag}) is detected reproducibly and in a large amount only when cells are pretreated with tunicamycin (9, 50). Further characterization of the 85,000- and 75,000-molecular-weight proteins in AKR tumors and their role in *gag* gene processing is the subject of a separate communication (Kooistra et al., manuscript in preparation).

Virus specificity of the 43-13 and 43-17 antibodies. To investigate further the virus specificity of 43-13 and 43-17 expression, the nontransformed III6A feral mouse fibroblast line was infected with a series of cloned viruses (Table 3). Infection of III6A cells with either AKR ecotropic or dualtropic virus results in 43-13 and 43-17 antigen expression. All other endogenous ecotropic viruses induce 43-17 antigens, but not 43-13 antigens. Since exogenous ecotropic Moloney virus fails to induce the expression of either 43-13 or 43-17 antigens, it seemed possible that these cell surface molecules are dependent on infection by or expression of endogenous viruses. To test this hypothesis further, mink lung fibroblasts were infected with endogenous xenotropic and dualtropic viruses. Although the dualtropic AKR mink cell focus-forming virus MCF 247 induces 43-13 and 43-17 antigens in mink cells, the same cells infected with the endogenous C57B1 xenotropic virus or the endogenous AKR xenotropic virus were negative for both antigens. Since the MCF 247 virus is a recombinant between AKR dualtropic envelope and AKR ecotropic *gag* genes (13, 43, 45), this suggests that the expression of the 43-13 and 43-17 antigens is related to expression of either AKR-specific endogenous ecotropic *gag* gene products (43-13) or of endogenous ecotropic *gag* gene products in general (43-17).

TABLE 3. 43-13 and 43-17 binding to MuLV-induced cell surface antigens[a]

Tumor or cell line[b]	Infecting virus[c]			Median fluorescence[d]		
	Class	Designation	Strain of origin	43-13	43-17	Anti-C58NTD
III6A	N ecotropic	AKR L-1	AKR	118	107	137
III6A		Gross passage A	AKR	1	94	150
III6A		BL/Ka(N)	C57BL/Ka	0	54	91
III6A		WN1802N	BALB/c	0	33	46
III6A	B ecotropic	RadLV/VL$_3$	C57BL/Ka	1	23	92
III6A		BL/Ka(B)	C57BL/Ka	1	10	36
III6A		WN1802B	BALB/c	1	80	104
III6A	NB ecotropic	Moloney clone 2	Uncertain	0	4	100
CCL64	Dualtropic	MCF 247	AKR	45	41	61
CCL64	Xenotropic	BL/Ka(X)	C57BL/Ka	0	2	18
CCL64		AKR-6	AKR	1	2	22
III6A				0	0	13
KKT-2				66	42	94
BL/RL-12-NP				0	0	0

[a] cells were stained with the indicated antibody followed by a fluorescein isothiocyanate-labeled second-stage antibody. Analysis was performed by FACS.
[b] III6A is a feral mouse fibroblast cell line; CCL64 is a mink lunk fibroblast cell line; KKT-2 is a spontaneous AKR T-cell lymphoma; BL/RL-12-NP is a nonproducer radiation-induced lymphoma from a C57BL/Ka mouse.
[c] Ecotropic viruses were maintained in the III6A mouse cell line; xenotropic and dualtropic viruses were maintained in the CCL64 mink cell line.
[d] Median fluorescence above-background staining standardized to a fluorescence gain of 1. Background staining was determined independently for each cell line with medium alone as a first-stage control.

TABLE 4. Differential cell surface expression of moloney and AKR virus antigens[a]

Cell line	Cell type	Strain of origin	Mode of induction[b]	Median fluorescence[c]				Ratios	
				43-13	43-17	Anti-gp70	Anti-p30	p30/gp70[d]	43-17/gp70
KKT-2	T lymphoma	AKR	Spontaneous	167	147	45	81	1.80	3.00
MBL-2	T lymphoma	C57BL/6	Moloney MuLV	1	2	58	8	0.14	0.02
L691	T lymphoma	C57L	Radiation	2	2	22	1	0.05	0.10
L691/A	T lymphoma	C57L	Infected with AKR L-1	83	86	82	38	0.46	1.05
L691/M	T lymphoma	C57L	Infected with Moloney MuLV	2	10	132	12	0.09	0.07
L1-2	Pre-B lymphoma	C57L	A-MuLV (Moloney MuLV)	0	1	52	1	0.02	0.02

[a] Cells were stained with the indicated antibody followed by a fluorescein isothiocyanate-labeled second stage antibody. Analysis was performed by FACS.
[b] AKR endogenous ecotropic AKR L-1 virus and exogenous ecotropic Moloney clone 2 virus were used to infect the nonproducer L691 cell line productively.
[c] Median fluorescence above background staining was standardized to a fluorescence gain of 1. Background staining was determined independently for each cell line using as a first stage control either a nonbinding monoclonal antibody of irrelevant specificity (for 43-13 and 43-17) or normal goat serum (for anti-gp70 and anti-p30 sera).
[d] The ratio of anti-p30 fluorescence to anti-gp70 fluorescence on the same cell line.
[e] The ratio of 43-17 fluorescence to anti-gp70 fluorescence on the same cell line.

Our finding that cloned Moloney virus infection of III6A does not lead to cell surface 43-17 expression is in contrast with the high level of 43-17 and p30 antigens on some of our currently maintained in vitro Moloney tumors (Table 1). However, all of the Moloney tumors tested arose in mouse strains (BALB/c or a) in which spontaneous expression of endogenous *gag* gene products is common (40). Thus, it is possible that the p30 and 43-17 antigens detected on these Moloney tumors may reflect in vitro activation of endogenous ecotropic virus *gag* genes rather than cell surface expression of Moloney virus *gag*-related proteins. To test this hypothesis, we compared gp70 and p30 expression on cells of C57L or C57BL background infected with or transformed by several murine retroviruses, including Moloney virus (Table 4). These mouse strains were chosen because they do not spontaneously activate endogenous ecotropic virus (27) or *gag*-related proteins (40) in culture. When normalized for gp70 expression, it is evident that cells infected by Moloney virus have a 5- to 10-fold lower amount of cell surface *gag* gene products as compared with those infected by AKR-type viruses (Table 4).

DISCUSSION

43-17 antibody detects an antigenic determinant unique to glycosylated *gag* polyproteins. We have prepared monoclonal antibodies reactive with MuLV *gag* polyproteins in AKR lymphomas. One of these antibodies, 43-13, detects an AKR-specific p12-related determinant found both within the virion core and as part of cell surface glycosylated *gag* polyproteins. In contrast, the 43-17 antibody detects an antigen specific for glycosylated *gag* polyproteins of endogenous ecotropic viruses. The 43-17 antigen is not found within the virion or as part of the internal precursor of the viral core proteins ($Pr65^{gag}$), but is present on the precursor of the glycosylated *gag* polyproteins found in tunicamycin or endo F-treated cells. Since the MuLV glycosylated *gag* polyproteins have been shown to contain only three N-linked carbohydrate groups (47), the experiments with tunicamycin and endo F almost certainly rule out the possibility that the 43-17 antibody detects a carbohydrate determinant. Based on the presence of the 43-17 antigen on the precursor of the glycosylated *gag* but not on $Pr65^{gag}$, we propose that the 43-17 antigenic determinant is localized to or dependent on the unique amino terminus of the glycosylated *gag* polyprotein (L domain).

L domain may play a role in membrane insertion and cell surface expression of glycosylated *gag* polyproteins. Although most cell surface proteins are anchored in the membrane via a hydrophobic carboxyl terminus (32), the carboxyl terminus (P10 and p30) of most retroviruses is highly polar (4, 19, 42). However, the amino terminus of the *gag* polyprotein (including both a putative leader [signal] sequence in L and p15) is hydrophobic (2, 50, 57). Since the 43-17 antibody binds to the surface of intact cells via the unique amino terminus of the cell surface *gag* polyprotein (L), all or part of the L domain of the glycosylated *gag* should be retained after entry into the rough endoplasmic reticulum, in contrast to classical leader ("signal") peptides, which are cleaved during translocation (5, 8). Thus, it is possible that retention of hydrophobic L or p15 sequences or both after entry into the rough endoplasmic reticulum might allow for insertion of the glycosylated *gag* polyprotein into the membrane via the amino terminus. A similar mechanism has been proposed for insertion of the sucrase-isomaltase enzyme complex into the intestinal brush border membrane (6). The $Pr65^{gag}$ polyprotein has an added hydrophobic segment due to a posttranslational myristyl amino-terminal acylation of the first amino acid, glycine (20). We do not know if the L domain is similarly fatty acid acylated.

The strongest support for the hypothesis that cell surface *gag* polyproteins are anchored in the membrane via the amino terminus is provided by the identification of three cell surface forms of the glycosylated *gag* which differ at the carboxyl terminus: (i) $gp95^{gag}$, which contains L (43-17), p12 (43-13), p30, and p10 determinants (29, 31); (ii) $gp85^{gag}$, which contains L, p12, and p30, but not p10, determinants (62); and (iii) the newly identified $gp55^{gag}$, which contains L and p12, but not p30 or p10 determinants (Kooistra et al., manuscript in preparation). Naso et al. (37) have identified a similar molecule of 45,000 molecular weight in Rauscher MuLV-infected cells. These data suggest that the three AKR glycosylated *gag* polyproteins must be inserted into the membrane via the amino terminus, or else three different carboxyl-terminal anchoring sites would be required. If the glycosylated *gag* polyprotein were inserted into the membrane via the amino terminus, then sequential cleavage of $gp95^{gag}$ (or its precursor) from the carboxyl terminus inward would create the $gp85^{gag}$ and $gp55^{gag}$ molecules. One possible model for the structure and orientation of AKR virus glycosylated *gag* polyproteins in the membrane is shown in Fig. 5. Without more extensive analysis of the number and location(s) of transmembrane regions of these glycoproteins, the placement of the L domain in the cell membrane and the precise localization of the 43-17 determinant on it are still matters of conjecture.

Sequence changes within the amino terminus may explain

differential expression of cell surface *gag* polyproteins. Our conclusion that Moloney virus-induced tumors express only low levels of cell surface *gag* proteins is supported by the inability of ourselves (17, 23) and others (16, 18) to immunoprecipitate significant levels of cell surface *gag* polyproteins from cells productively infected with Friend-Moloney-Rauscher-type viruses. Edwards and Fan (11) detected *gag* proteins on the surface of Moloney virus-infected fibroblasts, but their data are consistent with our estimation of a 10-fold reduction in *gag* expression when compared with AKR virus-induced tumors.

Since we have shown that Moloney MuLV infection does not lead to high level expression of Moloney MuLV *gag* cell surface antigens (Table 4), the appearance of high levels of such antigens (Table 1) on Moloney MuLV-induced tumors previously typed to be low (e.g., LSTRA in our laboratory [17, 23]) could reflect the activation of endogenous ecotropic *gag* genes (40). Nowinski et al. (38) have identified such endogenous MuLV *gag* proteins on the surface of Friend-Moloney-Rauscher virus-infected cells. Similarly, Buetti and Diggelmann (7) have identified *gag* molecules on the surface of fibroblasts infected with Friend-Moloney-Rauscher-type viruses which are identical to AKR *gag* polyproteins by peptide mapping. Considering the known serological (58, 59) and amino acid (41) differences between the *gag* gene products of Friend-Moloney-Rauscher-type and AKR-type viruses, it is possible that endogenous AKR-like viral sequences have been activated in these BALB/c fibroblasts, analogous to the probable activation of such viruses in our in vitro Moloney tumors.

Since our data suggest that the amino-terminal portion of the *gag* polyprotein may play a critical role in membrane insertion, virus-encoded sequence differences between AKR and Moloney MuLVs in this region could determine the level of cell surface expression of glycosylated *gag* polyproteins in any of several ways, e.g., the following. (i) Amino acid changes from nonpolar to polar residues within the amino terminus of Moloney virus could prevent membrane association after entry into the rough endoplasmic reticulum. (ii) AKR virus glycosylated *gag* polyproteins may be less susceptible to posttranslational cleavage than Moloney virus polyproteins. (iii) AKR and Moloney virus 5′ transcription sites may differ, leading to differential production of mRNA coding for the glycosylated *gag* polyprotein between the two viruses. The Moloney MuLV DNA sequence (54) predicts an open reading frame of 333 nucleotides 5′ to the initiating AUG triplet of the p15 *gag* protein. Although there are three additional potential initiation sites in the leader region which could serve for translation of the glycosylated *gag* polyprotein, none of these is in the correct reading frame. This suggests that RNA processing may be necessary to create a spliced mRNA from which the Moloney virus glycosylated *gag* can be translated or that an unusual initiation site (e.g., GTG) may be used (51, 54). Saris et al. (47) have analyzed peptide maps of Moloney MuLV gp80^{gag} and Pr65^{gag} and conclude that 7 kilodaltons (kDa) of additional amino acids reside at the amino terminus of gp80^{gag}. They proposed that this unique sequence is a leader sequence which may be greater than 7 kDa in size, and which replaces part of the p15 sequence as well. In fact, they propose the amino-terminal unique sequence to be 10 to 20 kDa (47), a size approaching the additional L domain described here. We estimate the AKR L domain to be 18 to 20 kDa on the basis of the size differences of the glycosylated and nonglycosylated forms of surface pathway *gag* polyproteins and internal pathway *gag* polyproteins. Thus unglycosylated gp95^{gag} is ~85 kDa, ~20 kDa greater than Pr65^{gag}; unglycosylated gp85^{gag} is ~75 kDa, ~18 kDa greater than p15 plus p12 plus p30; and unglycosylated gp55^{gag} is ~45 kDa, ~18 kDa greater than p15 plus p12 (gp55^{gag} lacks both p10 and p30 determinants).

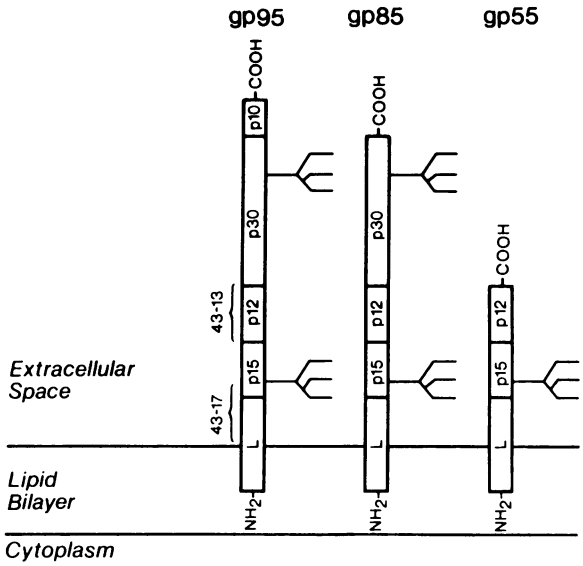

FIG. 5. Model for the structure and orientation of AKR virus glycosylated *gag* polyproteins in the plasma membrane. The viral glycosylated *gag* polyproteins are shown anchored in the plasma membrane via the amino-terminal L peptide. For the sake of simplicity, this model depicts the L peptide entirely within the lipid bilayer. However, other configurations are possible, e.g., the L peptide may be a transmembrane protein with its NH_2 terminus exposed to the cytoplasm, or a p15-L hydrophobic domain may actually insert into the membrane. More than one transmembrane crossing is possible; if so, the actual amino terminus could rest on the external face of the plasma membrane, with or without the 43-17 determinant. The relative size of the *gag* proteins and the location of the carbohydrate side chains are adapted from the data of Schultz et al. (48) for Rauscher MuLV.

We have examined the published DNA sequences 5′ of the p15 ATG translation start site for both Moloney MuLV (54) and AKR leukemia virus (14, 21). There is no continuous open reading frame which could encode an 18- to 20-kDa peptide, although it is possible to construct potential spliced messages which encode this size polypeptide using the 5′ long terminal repeat of AKR leukemia virus. If 43-17 in fact detects an 18- to 20-kDa amino-terminal peptide highly expressed in AKR leukemia virus-infected cells, it must be encoded by an unusual processing of 5′ sequences beginning in U3, since the published sequence has multiple stop codons in all three reading frames 5′ of the p15 ATG.

Role of cell surface *gag* antigen expression in the immune response to tumor viruses. Mice mount only a weak immune response to endogenous ecotropic *gag* gene products (24, 56), probably as a result of immunological tolerance to endogenous viral *gag* proteins expressed during embryogenesis (22, 60). Such immunological tolerance would not be expected to extend to exogenous viruses (such as Moloney MuLV) with antigenically distinct *gag* proteins. Our demonstration of differential expression of AKR and Moloney MuLV *gag* gene products suggests that selection for reduced cell surface *gag* expression may have occurred during repeated passage of the original Moloney virus stocks in postnatal mice.

The host response to virally transformed cells can be

viewed from another perspective. Anti-leukemia cell antibodies and cell-mediated immune responses to virion structural proteins must compete with intact, disrupted, and even accessory cell-processed virion antigens in the viremic host. However, the immune response to nonvirion antigens such as the 43-17 antigen should not be so affected. Thus, the immune response to nonvirion antigens may provide a more suitable model for immunodiagnosis and immunotherapy of tumor cells (66, 67).

ACKNOWLEDGMENTS

We thank Lily Hu for excellent technical assistance and Morris Dailey, Pam Fink, Howard Gershenfeld, and Jonathan Rosen for helpful criticism of the manuscript. We also thank John Elder for assistance with endo F experiments.

This work was supported by grant IM-56 from the American Cancer Society. E.A.P. is a Medical Scientist Trainee under Public Health Service grant GM-07365 from the National Institutes of Health.

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