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In Vitro Binding of Purified Murine Ecotropic Retrovirus Envelope Surface Protein to Its Receptor, MCAT-1

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An amino-terminal portion of the Friend murine leukemia virus (MLV) envelope surface protein {SU, residues 1 to 236 [SU:(1-236)]} and its receptor, MCAT-1, were each purified from insect cells after expression by using recombinant baculoviruses. Friend SU:(1-236) bound specifically to *Xenopus* oocytes that expressed MCAT-1 with an affinity (K_d, 55 nM) similar to that of viral SU binding to permissive cells. Direct binding of Friend SU:(1-236) to purified MCAT-1 was observed in detergent and after reconstitution into liposomes. Analysis of binding demonstrated that MCAT-1 and Friend SU:(1-236) interact with a stoichiometry of near 1:1. These findings demonstrate that the amino-terminal domain from the SU of ecotropic murine retroviruses contains an MCAT-1 binding domain.

Type C retroviruses in mammals and birds can be classified into groups by host range and interference (22). For type C murine leukemia viruses (MLVs), this classification is determined largely by differences in the surface protein (SU) of the virus envelope (5). Infection by viruses within each group requires binding to the same or closely related receptors expressed on the membranes of permissive cells. Receptors for several MLVs have been identified, and their expression has been correlated with SU binding (2, 24). Expression of the murine cationic amino acid transporter (MCAT-1) is sufficient to establish susceptibility to ecotropic MLV infection when expressed in nonpermissive cells (2). Moreover, binding of ecotropic SU to the membrane of permissive cells requires a short stretch of residues in the extracellular loop connecting the third and fourth membrane-spanning domains of MCAT-1 (1). In the simplest model, direct binding of SU on the virus surface to a domain in MCAT-1 containing these residues can induce the conformational changes in the associated transmembrane protein (TM) that are required to mediate fusion of the virus to the host cell membrane. Alternatively, additional, unidentified membrane proteins may function in concert with MCAT-1 in binding to SU and/or inducing the subsequent conformational changes that result in fusion. Recent findings suggest the latter model best characterizes the function of chemokine receptors in human immunodeficiency virus infection (26).

Previously, studies of chimeric proteins, constructed by exchanging portions of SU from different MLVs, localized receptor specificity to the amino-terminal domain (5, 15, 19). Indeed, expression of a truncated protein containing this portion of the ecotropic Friend 57 SU was sufficient to establish superinfection interference, consistent with binding and down-regulation of receptor expression (4). In the studies reported here, we have expressed and purified the amino-terminal domain of Friend MLV SU and MCAT-1 in insect cells by using baculovirus vectors and then studied their interaction in vitro after purification.

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MATERIALS AND METHODS

Construction of expression plasmids encoding MCAT-1 and Friend SU. The vector pSP64T (16) was modified by (i) insertion of the *Bam*HI-to-*Xba*I portion of the polylinker from pCDNA3 (Invitrogen) at the *Bgl*II/*Sal*I site, (ii) reinsertion of the deleted *Bgl*II-to-*Sma*I fragment of pSP64T at the *Bam*HI/*Pvu*II site and addition of a second polylinker containing *Nsi*I, *Bgl*II, *Apa*I, and *Pst*I sites at the *Pst*I site, and (iii) insertion of a 140-bp fragment encoding (in tandem) a factor Xa protease site (amino acids IEGR), three copies of the hemagglutinin (HA) peptide (YPYDVPYA [10]) recognized by monoclonal antibody 12CA5 (Boehringer Mannheim), and a six-histidine motif at the *Xho*I/*Pst*I site in the polylinker (Flu_3-his_6 tag). The new vector was designated pRD67:Flu_3-his_6.

A clone encoding envelope SU of Friend MLV (reference 12; GenBank accession no. J02192) in plasmid pUC13 (a gift from A. Pinter) was modified by addition of a 5′ *Eco*RI site at bp −40 relative to the initiator ATG and a 3′ *Xho*I site at bp 810 that permitted insertion into pRD67:Flu_3-his_6 with an in frame fusion at the 3′ end to the sequences encoding the protease cleavage site and Flu_3-his_6 tag.

A cDNA encoding the ecotropic retrovirus receptor (MCAT-1) with the C-terminal protease cleavage site and Flu_3-his_6 tag (MCAT-1t) was constructed by using PCR to place an *Eco*RI site at bp −50 relative to the initiator ATG and to replace the stop codon of MCAT-1 (residue 622) with an in-frame *Xho*I recognition site and inserted into the polylinker of pRD67:Flu_3-his_6. To avert the possibility of PCR-induced mutations, the *Bam*HI-to-*Sph*I fragment of this plasmid was excised and replaced by the equivalent fragment from the original MCAT-1 cDNA clone. The absence of *Taq* polymerase-induced changes in the remaining portion (<10% of the coding region) was confirmed by nucleotide sequencing.

Expression of recombinant proteins in oocytes and mammalian cells. Capped mRNAs encoding MCAT-1 or MCAT-1t were transcribed and injected into *Xenopus laevis* oocytes, and after 3 days, transport of L-[^{14}C]arginine and exogenous binding of ^{125}I-SU proteins were measured as described elsewhere (11). In some experiments, detergent extracts of oocytes were prepared and solubilized proteins separated on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels, transferred to nitrocellulose filters, and immunoblotted with monoclonal antibody 12CA5 or a polyclonal antibody raised against a C-terminal peptide of MCAT-1 (11).

For expression in mammalian cells, the cDNA encoding MCAT-1t was inserted into the expression vector pCDNA3 (Invitrogen), using *Eco*RI and *Apa*I sites. Receptor function was confirmed by using ecotropic β-galactosidase-encoding virus after transient expression in nonpermissive human 293 cells (2).

Protein expression in insect cells. Recombinant insect viruses encoding the recombinant envelope and receptor proteins were made by cloning the Friend SU or MCAT-1t insert from the pRD67:Flu_3-his_6 into the baculovirus vector pVL1393 (Pharmingen) adjacent to the *polH* promoter element vector backbone, using the 5′ *Eco*RI and 3′ *Bgl*II sites. Each of the resulting plasmids was co-transfected with linear baculovirus virus DNA (Pharmingen) into *Spodoptera frugiperda* Sf9 insect cells, and recombinant viruses encoding the correct proteins were recovered and amplified in Hi5 cells (Invitrogen) propagated in Excel-405 medium (JRH Biosciences). For protein purification, Hi5 cells were grown in suspension cultures (400 ml/2-liter baffled flask) rotated at 100 rpm at 27°C. Cells in log growth phase were infected at a multiplicity of infection of 10.

Purification of truncated envelope proteins. Three days after baculovirus infection, culture supernatants were collected, filtered (0.2-μm-pore-size filter),

and incubated for 1 h at room temperature with Ni^{2+}-charged chelating resin (20 ml of resin/liter of medium; Pharmacia). The resin was collected with a sintered glass funnel, packed into a column, and washed with 10 column volumes of 50 mM imidazole–500 mM NaCl–10 mM sodium phosphate (pH 6.4). The bound protein was eluted with 500 mM imidazole–500 mM NaCl–10 mM sodium phosphate (pH 6.4). Protein was dialyzed overnight against 500 mM NaCl–20 mM HEPES (pH 7.4) and then dialyzed for 4 h against 50 mM NaCl in the same buffer. A precipitate which contained the recombinant SU protein formed. This was pelleted, redissolved in 300 mM imidazole (pH 8.0) to 1 mg/ml, and digested with 1% (wt/wt) factor Xa protease (New England Biolabs) for 48 h at room temperature. After digestion, the sample was diluted with 10 volumes of 50 mM NaCl–20 mM morpholineethanesulfonic acid (MES; pH 6.0) and applied to a Mono S ion-exchange column (Pharmacia). Elution of recombinant protein was performed by using an NaCl gradient (50 to 500 mM in 20 mM MES [pH 6.0]). Digested recombinant Friend SU, residues 1 to 236 [Fr SU:(1-236)], eluted as a sharp peak at 200 mM NaCl. On SDS-polyacrylamide gels, this protein was detected by silver staining (Quicksilver kit; U.S. Biochemical) or Coomassie brilliant blue staining as a single, broad band with an estimated molecular mass of 35 kDa.

Envelope proteins were ^{125}I labeled by using iodo-beads as instructed by the manufacturer (Pierce). Typically 1 mCi of carrier-free $Na^{125}I$ (NEN-DuPont) was used per 0.1 mg of protein.

Purification of MCAT-1t. The following protocol is based on a 1-liter culture; typically 4-liter batches were prepared. Two days after infection with baculovirus encoding MCAT-1t, Hi5 cells were harvested by centrifugation at 1,000 × *g*. Pelleted cells were swollen by resuspension in 100 ml of hypotonic buffer (15 mM KCl, 5 mM sodium citrate [pH 6.3]) containing 2× protease inhibitor cocktail (0.1 mg each of Pefabloc-SC [Boehringer Mannheim], *N*-*p*-tosyl-L-lysine chloromethyl ketone [TLCK], and *N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK] per ml) and disrupted using a probe sonicator (Branson) at power setting 3 in 10-s bursts for 1 min. After addition of 100 ml of 500 mM sucrose–50 mM sodium phosphate-citric acid buffer (pH 6.4), the lysate was clarified by centrifugation at 1,000 × *g* for 30 min at 4°C. Crude membranes were prepared from the supernatant by centrifugation (100,000 × *g* for 30 min at 4°C [23]). The membranes were rinsed with 250 mM sucrose in 50 mM sodium phosphate-citric acid buffer (pH 6.4), snap frozen in liquid nitrogen, and stored at −80°C.

Crude membranes were thawed on ice and dissolved in 35 ml of 10 mM sodium deoxycholate (Sigma)–250 mM NaCl–10% glycerol–20 mM sodium phosphate buffer (pH 8.0) containing reversible protease inhibitors (leupeptin [0.5 μg/ml], aprotinin [2.0 μg/ml], and pepstatin A [0.5 μg/ml]). The pellet was suspended and sheared through an 18-gauge needle. After centrifugation at 2,000 × *g* for 5 min, the supernatant was increased to 800 ml and incubated with 10 ml of Ni^{2+}-charged chelating resin (Pharmacia) by stirring. After 30 min, the resin was collected with a sintered glass funnel, washed with 3 volumes of sodium deoxycholate buffer, and packed into a 1.6- by 20-cm column (Pharmacia). The resin was washed sequentially with 5 column volumes each of (i) 250 mM NaCl–20 mM sodium phosphate buffer (pH 8.0), (ii) 500 mM NaCl–20 mM sodium phosphate buffer (pH 8.0), (iii) 500 mM NaCl–140 mM NaH_2PO_4–30 mM citric acid–50 mM imidazole (pH 6.4) (stringent wash), and (iv) 250 mM NaCl–35 mM NaH_2PO_4–7.5 mM citric acid (pH 6.4). MCAT-1t eluted as a single peak with 250 mM NaCl–80 mM NaH_2PO_4–60 mM citric acid (pH 4.0). Buffer for washes and elution contained 8 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Sigma) and 10% glycerol and were chilled on ice before use. Buffer was exchanged by applying the peak of eluted protein to a PD-10 column (Pharmacia) preequilibrated with 10 mM CHAPS in 150 mM NaCl–10% glycerol–20 mM HEPES buffer (pH 7.4). MCAT-1t eluted in the void volume. In some experiments, the remaining protein was applied to a monoclonal antibody 12CA5 column, and washed with 250 mM NaCl–10% glycerol–8 mM CHAPS–35 mM NaH_2PO_4–7.5 mM citric acid (pH 6.4), and bound protein was eluted with 150 mM NaCl–10% glycerol–8 mM CHAPS–17 mM NaH_2PO_4–9.8 mM citric acid (pH 3.0). The column was made by mixing saturating amounts of biotin-coupled monoclonal antibody 12CA5 (Boehringer Mannheim) with streptavidin beads (Pierce). The pH of the eluate was neutralized by buffer exchange as described above.

Detection of proteins during purification. The absorbance of column eluates was monitored continuously at 280 nm. To monitor the elution of recombinant proteins, 0.5-μl samples of each fraction were spotted onto nitrocellulose filters and immunoblotted with monoclonal antibody 12CA5. In some experiments, eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (13) and visualized by silver staining (Quick-Silver kit; U.S. Biochemical) or transferred to nitrocellulose and immunoblotted with monoclonal antibody 12CA5 (1:1,000) followed by a goat anti-mouse horseradish peroxidase (HRP) conjugate (Pierce). Biotin-coupled protein was detected on immunoblots by using streptavidin-HRP conjugate (Pierce), used at a dilution of 1:2,000. Antibody-HRP or streptavidin-HRP bound proteins were identified by using the Amersham ECL substrate. Protein concentration was measured by using either protein-gold (Integrated Separation Systems) or bicinchoninic acid (Pierce).

Reconstitution of purified proteins into phospholipid vesicles. A lipid mixture in chloroform [90% (wt/wt) phosphatidylcholine (bovine heart), 10% (wt/wt) crude bovine brain polar lipid extract (Avanti Polar Lipids, Birmingham, Ala.), 0.1% (wt/wt) *N*-(lissamine rhodamine B sulfonyl) diacyl phosphatidylethanolamine] was dispensed into glass tubes under argon gas and lyophilized for 5 h. The dry film was rehydrated under argon with 45 μl of rehydration buffer (120 mM NaCl, 20 mM KCl, 20 mM HEPES [pH 7.4]) per 2.5 mg of lipid with vigorous mixing. Twelve microliters of 0.5 M CHAPS–2.5 mg of lipid was added to this suspension, vortex mixed, and warmed to 37°C until it became transparent. Typically, 4 μg of MCAT-1t in 1 ml of rehydration buffer with 8 mM CHAPS was added to the lipid-detergent mixture (1 mg of lipid) and dialyzed for 48 h at room temperature against 500 ml of rehydration buffer containing 10 ml of a 50% (wt/vol) solution of Biobeads SM-2 (Bio-Rad). A 50,000-molecular-weight-cutoff dialysis membrane (Spectra/Por; Spectrum Medical Industries) was used. After dialysis, lipid content of samples was determined from the absorbance at 550 nm of the rhodamine-labeled lipid and also by lipid-derived phosphate determination (18).

Detergent binding assay. One hundred micrograms of purified Fr SU:(1-236) or amphotropic 4070 SU:(1-205) was reacted with 60 nmol of sulfo-NHS-Biotin (Pierce) for 30 min at room temperature in 100 mM sodium phosphate buffer (pH 8.0). Unincorporated biotin was removed by using a NAP-10 column (Pharmacia). Fifty microliters of Ultralink streptavidin beads (Pierce) was then primed by incubation (15 min) with the biotin-coupled protein. Before use, the beads were washed three times in 150 mM NaCl in 20 mM HEPES buffer (pH 7.4). Samples containing MCAT-1t dissolved in 10 mM CHAPS–10% glycerol–150 mM NaCl–1 mM $CaCl_2$–0.1 mM $MgCl_2$–20 mM Tris-HCl (pH 7.4) (assay buffer, unless specified otherwise) were incubated with the SU-primed or unprimed beads. After 1 h at room temperature, the beads were washed three times in assay buffer, and bound protein was solubilized by resuspension in 2% SDS–8 M urea–100 mM Tris-HCl (pH 6.8). Samples were then spotted onto nitrocellulose or separated by SDS-PAGE and analyzed by immunoblotting. The same assay was also used to analyze MCAT-1t-reconstituted liposomes except that the assay buffer was modified by omitting detergent.

Liposome flotation assay. Biotin-coupled Fr SU:(1-236) (0.1 μg) was mixed with 5 μg of liposomes containing MCAT-1t or no protein in 300 μl of assay buffer and incubated at room temperature with gentle mixing. After 1 h, the reaction mixture was mixed with an equal volume of 20% (wt/vol) dextran (average molecular weight, 74,000; Sigma) in assay buffer and dispensed into the bottom of an SW55 ultracentrifuge tube (Beckman); 1.5 ml of 7.5 and 5% dextran solutions and 0.3 ml of assay buffer were sequentially layered above the reaction mixture. After centrifugation at 159,000 × *g* for 1 h at 15°C, 0.4-ml fractions were collected. Lipid content was determined by assaying for rhodamine-tagged phosphatidylcholine by absorbance at 550 nm.

RESULTS

Purification of the MCAT-1 Friend SU binding domain. It has been established that expression of MCAT-1 is required for ecotropic MLV binding (2) and for infection (20). Also, expression of ecotropic MLV SU blocked MCAT-1-dependent binding of exogenous SU and decreased L-arginine transport to less than 40% of the control value, consistent with binding-induced downregulation of MCAT-1 (11). Battini et al. reported that expression of the amino-terminal domain of Friend MLV SU is sufficient to establish superinfection interference, suggesting this portion of SU contains the MCAT-1 binding domain (4). This hypothesis was supported by our studies in frog oocytes, which showed that expression of the ecotropic Friend 57 SU protein truncated just before the proline-rich region (residue 236) decreased MCAT-1-dependent L-arginine transport and exogenous Moloney MLV SU binding to the same extent as expression of native ecotropic gp85 envelope protein (Fig. 1).

To further characterize the interaction between Fr SU:(1-236) and MCAT-1, binding was studied after purification. To facilitate purification, Friend SU (residues −34 to 236) was modified at the carboxyl-terminal end by addition of a protease cleavage site (factor Xa; IEGR), the influenza virus HA epitope tag, and a six-histidine motif and then expressed in insect cells by using a recombinant baculovirus. The recombinant protein was recovered from the medium of virus-infected cells by Ni^{2+} chelation (Fig. 2A, lanes 1 to 3). After elution in buffer containing 0.5 M imidazole (lane 3), the carboxyl-terminal tag was removed by factor Xa cleavage (lane 4), and the Friend SU product was purified to homogeneity by anion-exchange chromatography (lanes 5 and 6). Sequencing revealed that the amino-terminal residues of the purified SU protein (lane 5) are AAPGSSPHQV, confirming correct cleav-

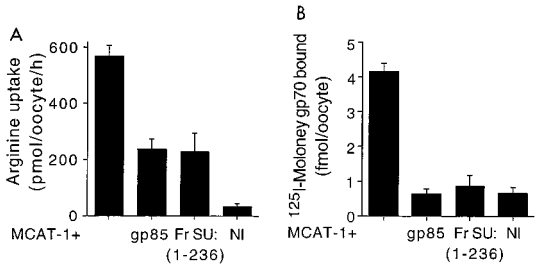

FIG. 1. Arginine uptake (A) and exogenous binding of ^{125}I-Moloney MLV SU (B) were measured in oocytes injected with MCAT-1 mRNA (10 ng) alone or with either mRNA encoding Fr SU:(1-236) (50 ng) or Moloney MLV gp85 (50 ng).

age (14) of the 34-residue signal peptide. The mobility of Fr SU:(1-236) on gel filtration chromatography (Superdex 75; Pharmacia) fell between those of the marker proteins lysozyme (12.5 kDa) and aprotinin (6.5 kDa), suggesting that Fr SU:(1-236) is a monomer (Fig. 2B).

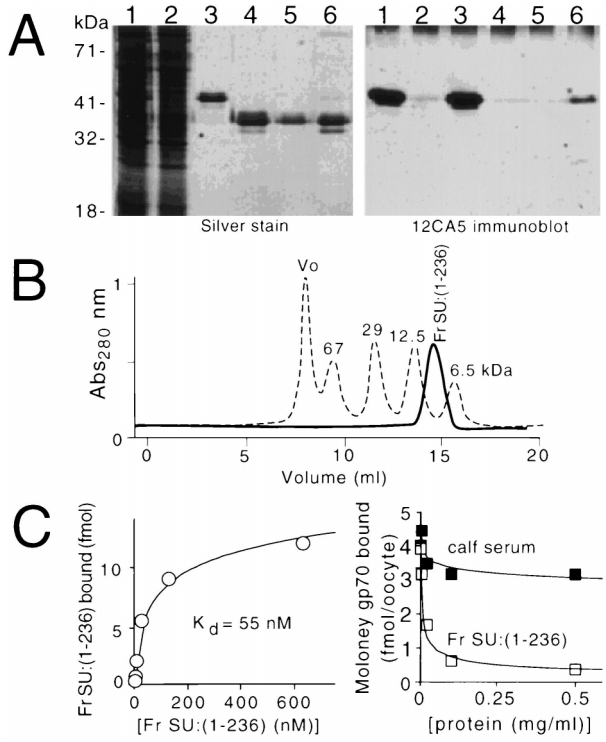

FIG. 2. Purification of the truncated envelope from recombinant baculovirus-infected insect cell culture supernatants. (A) Protein yield was monitored by SDS-PAGE, and proteins were detected by silver staining (left panel) or immunoblotting using monoclonal antibody 12CA5, which recognized influenza virus epitope-tagged protein (right panel). For silver staining, 0.5 μg of protein was loaded per lane; for immunoblotting, 1 ng was used. Lanes: 1, culture supernatant; 2, nickel column flowthrough; 3, elution from nickel column with imidazole followed by precipitation by dialysis and resolubilization in imidazole buffer (see Materials and Methods); 4, after cleavage with factor Xa protease; 5, major peak from ion-exchange chromatography; 6, fraction following major ion-exchange peak. (B) Size exclusion chromatography of Fr SU:(1-236), using Sephadex-75. The column was calibrated with proteins of the molecular masses indicated. The 280-nm absorbance of the eluate is indicated. (C) Binding of ^{125}I-labeled Fr SU:(1-236) to oocytes injected with mRNA encoding MCAT-1 was measured and yielded a K_d of 55 nM by nonlinear regression analysis (left panel). Binding of ^{125}I-labeled Moloney SU to oocytes injected with mRNA encoding MCAT-1 was measured in the presence of increasing amounts of either unlabeled Fr SU:(1-236) or calf serum (right panel).

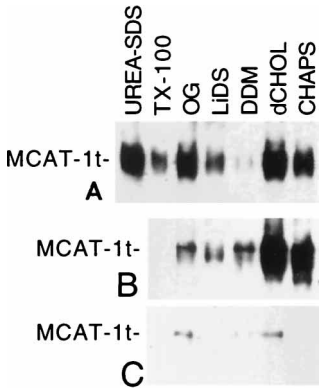

FIG. 3. Detergent extraction and binding of MCAT-1t to beads coupled to Fr SU:(1-236). (A) After MCAT-1t-containing insect cell membranes were extracted with either Triton X-100 (TX-100; 0.5 mM), octylglucoside (OG; 30 mM), lithium dodecyl sulfate (LiDS; 5 mM), dodecylmaltoside (DDM; 0.5 mM), sodium deoxycholate (dCHOL; 12 mM), or CHAPS (10 mM), the relative amount of soluble MCAT-1t was determined by immunoblotting. The above described extracts were incubated with streptavidin beads coupled to biotinylated Fr SU:(1-236) (B) or with beads alone (C). MCAT-1t bound to beads was eluted with 2% SDS–8M urea and detected by immunoblotting.

^{125}I-labeled Fr SU:(1-236) bound specifically to frog oocytes that expressed MCAT-1 (Fig. 2C, left panel). Binding to these membranes was saturable, and the affinity (K_d, 55 nM) was comparable to that of virus-purified Moloney MLV SU binding to permissive murine fibroblasts (7). In addition, Fr SU:(1-236) was able to compete with ^{125}I-labeled Moloney MLV SU gp70 purified from virions for binding to membranes of frog oocytes that express MCAT-1 (Fig. 2C, right panel). We conclude that Fr SU:(1-236) contains a functional receptor binding domain.

Function and purification of recombinant MCAT-1. To facilitate purification, MCAT-1 was modified by addition of the carboxyl-terminal tag used for purification of Fr SU:(1-236). Addition of the tag had no detectable effect on MCAT-1-dependent L-arginine transport or ^{125}I-SU binding in frog oocytes (data not shown). Also, expression of the tagged MCAT-1 (MCAT-1t) conferred susceptibility of human 293 cells to infection by the ecotropic MLV vector encoding β-galactosidase (BAG). The titer of the BAG virus infection on these cells is comparable to that on cells that express the unaltered receptor protein. Therefore, the presence of the carboxyl-terminal tag produced no detectable alteration in transport or receptor function.

MCAT-1t was expressed in insect cells by using a recombinant baculovirus. The efficiency of MCAT-1t extraction from the membrane fraction of these cells was monitored by immunoblotting using monoclonal antibody 12CA5, which recognizes the influenza virus HA epitope tag. Extraction of MCAT-1t in buffers containing either sodium deoxycholate (12 mM), CHAPS (10 mM), or octylglucoside (30 mM) was comparable to that with 2% SDS–8 M urea and exceeded that with lithium dodecyl sulfate (5 mM), Triton X-100 (0.5 mM), and dodecylmaltoside (0.5 mM) (Fig. 3A). The function of detergent-extracted MCAT-1t was monitored by measuring binding to biotin-coupled Fr SU:(1-236) bound to streptavidin beads. Preliminary experiments demonstrated that the streptavidin-biotin linkage was stable in detergent, but Fr SU:(1-236) was completely released from beads by addition of 8 M urea to 2% SDS (data not shown). After 1 h of incubation in detergent extracts containing MCAT-1t, the Fr SU:(1-236)-charged beads were washed and MCAT-1t and Fr SU:(1-236) was eluted with 2% SDS–8 M urea. Substantial binding of soluble

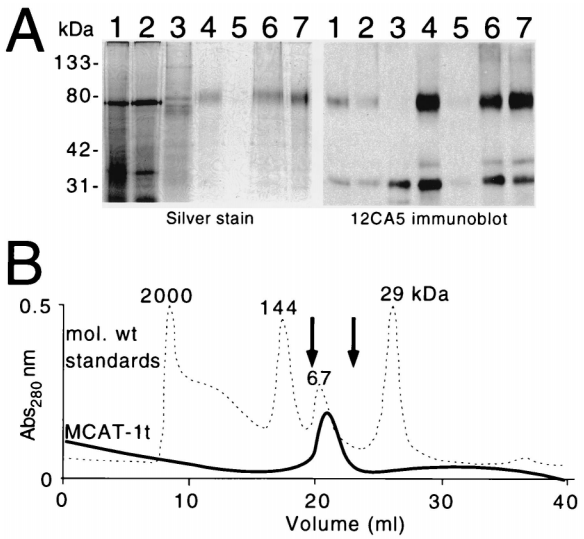

FIG. 4. (A) Purification of MCAT-1t by nickel column chromatography. After SDS-PAGE, purification was monitored by silver staining (left panel) or immunoblotting using monoclonal antibody 12CA5 (right panel). For silver staining, 0.5 μg of protein was loaded per lane; for immunoblotting, 1 ng was used. Lanes: 1, membrane preparation dissolved in sodium deoxycholate before application to chelating resin; 2, resin flowthrough; 3, eluate from wash in 50 mM, imidazole–30 mM citric acid; 4, eluate in CHAPS buffer (pH 4.0); 5, flowthrough after application to antibody 12CA5 column; 6, eluate from antibody column in CHAPS buffer (pH 3.0); 7, liposomes (5 μl) formed in the presence of 4 μg of MCAT-1t by dialysis in the presence of 0.9 mg of phosphatidylcholine PC and 0.1 mg of total brain lipid. (B) Size exclusion chromatography of MCAT-1t. MCAT-1t in CHAPS (8 mM) was applied to a Superose-6 column which had been calibrated with proteins of the indicated molecular masses. The 280-nm absorbances of eluted standards (broken line) and MCAT-1 (solid line) are indicated. Fractions between the arrows contained HA epitope-tagged protein as detected on immunoblots.

MCAT-1t to Fr SU:(1-236)-charged beads (Fig. 3B), but not uncharged beads (Fig. 3C), was observed in buffers containing sodium deoxycholate, CHAPS, or dodecylmaltoside, but binding was diminished in Triton X-100, octylglucoside, or lithium dodecyl sulfate. These experiments indicate that sodium deoxycholate and CHAPS are more effective than the other detergents in efficient extraction of functional MCAT-1t from insect cell membranes.

Purification of MCAT-1t was monitored by silver staining (Fig. 4A, left panel) and immunoblotting (right panel) after SDS-PAGE. A 450-mg membrane pellet from insect cells that express MCAT-1t was extracted in buffer containing sodium deoxycholate (lane 1). Ni^{2+}-chelating resin was incubated with this extract and then washed with 10 column volumes of detergent buffer containing imidazole (50 mM) and citric acid (30 mM) to remove loosely bound proteins (lanes 2 and 3). The final wash contained CHAPS (8 mM) in place of sodium deoxycholate, which formed a gel under the conditions (pH 4) required to elute MCAT-1t (lane 4). The yield of MCAT-1 from this protocol was 100 μg (>90% homogeneity), as assessed by silver staining (lane 4; summarized in Table 1). In some preparations, MCAT-1t was purified further by using antibody (12CA5) affinity chromatography. After application of MCAT-1t in CHAPS buffer at pH 7.5, Sephadex beads coupled to 12CA5 were washed extensively at pH 6 (lane 5), and MCAT-1t was eluted from the antibody at pH 3 (lane 6). Brief (less than 1-h exposure of MCAT-1t to pH 3 to 10 did not noticeably alter its binding properties. The 30- and 34-kDa proteins that copurified with MCAT-1 and were detected by antibody 12CA5 on the immunoblot (Fig. 4A, right panel) are

TABLE 1. Purification of MCAT-1t from crude insect cell membranes

Purification step	Total protein (μg)	Titer[a]	Fold enrichment (cumulative)
Whole cells	45,000	4,096	1
Membranes[b]	14,840	4,096	3
Not adsorbed to Ni resin[c]	13,040	1,600	15
Wash eluted	1,200	1,600	18
Acid-eluted protein	90	1,024	41
Imidazole eluted[d]	440	256	ND[e]
Not adsorbed to antibody resin[f]	5	64	ND
Eluted from antibody resin	35	512	ND[g]

[a] Determined by serially diluting samples 1:2-fold, applying the protein to nitrocellulose filters, and immunoblotting with 12CA5. The titer given is the reciprocal of the dilution factor and was normalized for the initial volume of the protein sample.
[b] Crude membranes were made from insect cells overexpressing MCAT-1t 48 h after infection with recombinant baculovirus.
[c] Membranes were dissolved in sodium deoxycholate buffer and applied to a nickel-charged chelating resin in batch.
[d] Material not eluted with imidazole following MCAT-1t elution.
[e] ND, not determined.
[f] Fifty micrograms of acid-eluted protein from the nickel resin was buffer exchanged and applied.
[g] No apparent increase in purity of protein acid eluted from the antibody column as determined by SDS-PAGE, but this step was included in some experiments to further ensure the homogeneity of the MCAT-1t protein.

probably proteolytic fragments of MCAT-1t. Proteolytic cleavage is likely to occur in insect cells, since these fragments were present in the initial membrane extracts, despite the presence of protease inhibitors. Since both retain the carboxyl-terminal tag, they copurified with MCAT-1t. These proteins migrated as a single broad peak between the protein standards bovine serum albumin (67 kDa) and cytochrome *c* (29 kDa) on a gel filtration column in the presence of CHAPS (Fig. 4B). No MCAT-1t was detected in the early column fractions containing proteins that would be multiples of the calculated molecular mass of MCAT-1t (67 kDa). Therefore, like Fr SU:(1-236), MCAT-1t was purified as a monomer.

Binding of purified MCAT-1t to truncated envelope. Purified MCAT-1t bound to Fr SU:(1-236)-charged streptavidin beads (Fig. 5A, lane 1, right panel) but not to beads charged with the equivalent amino-terminal region of amphotropic SU:(1-209) (lane 2, right panel) purified by using the same protocol (lane 2, left panel). The 30- and 34-kDa proteolytic fragments that copurified with MCAT-1t were not detected in the eluate obtained from Fr SU:(1-236)-charged beads, demonstrating that they lack binding activity. More than 90% of purified MCAT-1t could be adsorbed by excess Fr SU:(1-236)-charged beads (data not shown), demonstrating that less than 10% of purified MCAT-1t was irreversibly denatured during purification.

An indirect measure of the stoichiometry of SU binding to MCAT-1 was obtained using a batch of purified Fr SU:(1-236) that retained the influenza virus HA epitope tag. Beads charged with Fr SU:(1-236) were incubated with excess MCAT-1t in detergent; after washing, the relative amounts of eluted proteins were identified by densitometry of bands on an immunoblot (Fig. 5B). The ratios of eluted MCAT-1t to biotin-coupled Fr SU:(1-236) were 1.0 in CHAPS, 0.9 in sodium deoxycholate, 0.3 in dodecylmaltoside, and near zero in either octylglucoside or Triton X-100. Since the same epitope tag is present at the carboxyl terminus of both proteins, they are likely to be detected equally well by antibody 12CA5. Given that both Fr SU:(1-236) and MCAT-1t were purified as monomers, the binding ratio of near 1:1 in CHAPS and sodium

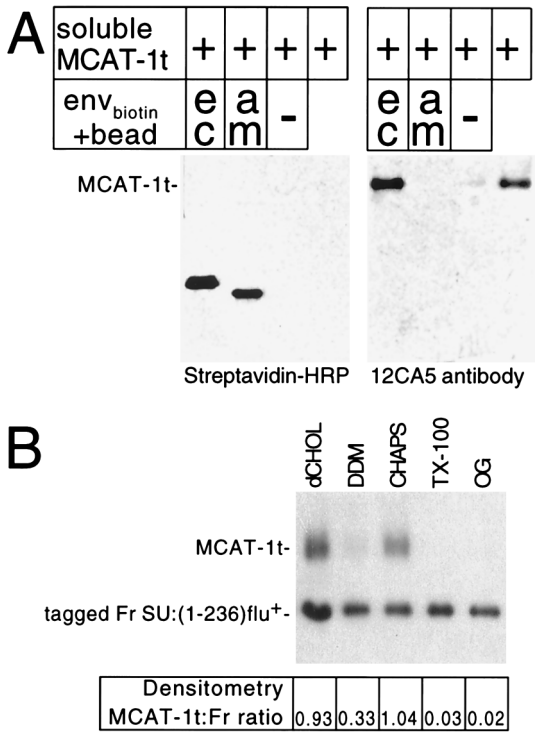

FIG. 5. Binding of purified MCAT-1t to Fr SU:(1-236). (A) The binding of purified MCAT-1t to streptavidin beads alone (−) or coupled to biotinylated ecotropic Fr SU:(1-236) (ec) or amphotropic SU:(1-209) (am) was measured. Protein was eluted from these beads with 2% SDS–8 M urea. Biotinylated Fr SU:(1-236) and amphotropic SU:(1-209) were detected on immunoblots by using streptavidin-HRP (left panel), and MCAT-1t was detected with antibody 12CA5 (right panel). (B) Stoichiometry of Fr SU:(1-236) and MCAT-1t interaction in buffers containing the detergent sodium deoxycholate (dCHOL; 12 mM), dodecymaltoside (DDM; 0.5 mM), CHAPS (10 mM), Triton X-100 (TX-100; 0.5 mM), or octylglucoside (OG; 30 mM) was measured by using streptavidin beads coupled to biotinylated Fr SU:(1-236) which retained the HA epitope tag. MCAT-1t and the tagged-Fr SU:(1-236) proteins were detected on immunoblots with antibody 12CA5 and analyzed by densitometry.

deoxycholate is consistent with a direct interaction between single molecules of each protein. An altered conformation of one or both of these proteins in the presence of octylglucoside or Triton X-100 is likely the cause of the reduced binding observed in these detergents.

Purified MCAT-1t was then reconstituted into artificial lipid bilayers (liposomes) through removal of CHAPS by dialysis in the presence of phosphatidylcholine (0.9 mg/ml) and total cow brain lipid (0.1 mg/ml). After dialysis, a uniform population of liposomes of 150-nm diameter were observed by fluorescence microscopy (0.1% rhodamine-tagged phosphatidylcholine was included during liposome formation) and by light scattering (Coulter N4 Plus). These liposomes were recovered by flotation on step gradients formed by using dextran (5%, 7.5%, and 10%, wt/vol) (Fig. 6A). Seventy percent of the lipid was recovered at the top of the gradient, as assessed in experiments that incorporated trace amounts of ^{3}H-labeled phosphatidylcholine in the lipid mixture. Incorporation of MCAT-1t into liposomes was verified by immunoblotting of fractions collected after centrifugation through dextran gradients (Fig. 6B). When MCAT-1t-containing liposomes were formed in excess lipid, more than 80% of MCAT-1t was recovered in the lipid-containing fraction at the top of the gradient (lane 8). Recovery of MCAT-1t from this fraction was saturable (plateau reached at 4 μg of protein/mg of lipid). When removal of CHAPS by dialysis was performed in the presence of excess MCAT-1t (more than 40 μg of MCAT-1t/mg of lipid), liposomes failed to form and MCAT-1t (perhaps in mixed micelles) was scattered throughout the middle of the gradient.

Liposomes formed in the presence of MCAT-1t were precipitated by Fr SU:(1-236)-charged streptavidin beads (data not shown), consistent with the presence of functional MCAT-1t. In addition, after incubation of 0.5 μg of Fr SU:(1-236) with 50 μl of MCAT-1t-containing liposomes (about 0.5 μg of MCAT-1t), 40% of Fr SU:(1-236) migrated to the top of the gradient (Fig. 6C, lane 8). Liposome-dependent migration of Fr SU:(1-236) to the top of the gradient correlated with incorporation of increasing amounts of MCAT-1t (range, 0.3 to 20 μg [Fig. 6D]). Addition of CHAPS to MCAT-1t-liposomes after incubation with Fr SU:(1-236) blocked migration of either protein to the top of the gradient (Fig. 6D, last set of bars), and in the absence of lipid, MCAT-1t precipitated and was pelleted. These findings support the conclusion that MCAT-1t is necessary and sufficient to bind Friend SU to membranes through interaction with residues 1 to 236.

DISCUSSION

The envelope protein of type C mammalian viruses can be divided into a variable amino-terminal portion, a proline-rich region similar to the immunoglobulin hinge region, and a conserved carboxyl-terminal domain (4). Chimeric SU proteins created by combining these regions from related mammalian type C retroviruses are functional, and receptor specificity is determined by the amino-terminal portion of SU (5, 17). In addition, Battini et al. (4) demonstrated that expression of the amino-terminal domain of Friend 57 SU is sufficient for superinfection interference, consistent with receptor binding. The studies reported here confirm direct binding between the amino-terminal domain of Friend SU to MCAT-1, using purified proteins.

The source of envelope protein in these studies was Friend 57 SU truncated (residues 1 to 236) just before the proline-rich region. After purification from insect cells, this protein bound to oocyte membranes that express MCAT-1 with an affinity comparable to that found for Moloney MLV SU gp70 binding to permissive cells (7). This finding suggests that Fr SU:(1-236) contains the entire MCAT-1 binding domain(s). Consistent with this conclusion, the equivalent portion of the amphotropic SU(1-209), purified by using the same protocol (Fig. 5), specifically binds to membranes that express its receptor, Pit2 (unpublished results).

Highly purified preparations of MCAT-1t have been obtained by sequential application of nickel chelation and antibody affinity chromatography in the presence of CHAPS and/or sodium deoxycholate. When purified in these detergents, more than 90% of MCAT-1t can bind to excess Fr SU:(1-236) coupled to beads. Given the high specific activity of the MCAT-1 preparation in these detergents, the existence of additional proteins that are required for SU binding and copurify with MCAT-1t is unlikely since these proteins were not identified by silver staining of purified MCAT-1t preparation after SDS-PAGE. This conclusion is consistent with the observation that MCAT-1 expression is sufficient to confer susceptibility to ecotropic MLV infection when expressed in cells from many nonpermissive species of mammals and birds (25).

Both Fr SU:(1-236) and MCAT-1 (in CHAPS) migrated as single peaks near their expected molecular weights on column chromatography and bound to each other with a stoichiometry of near 1:1. While it is possible that receptor-SU interaction could mediate oligomerization of either protein, the simplest

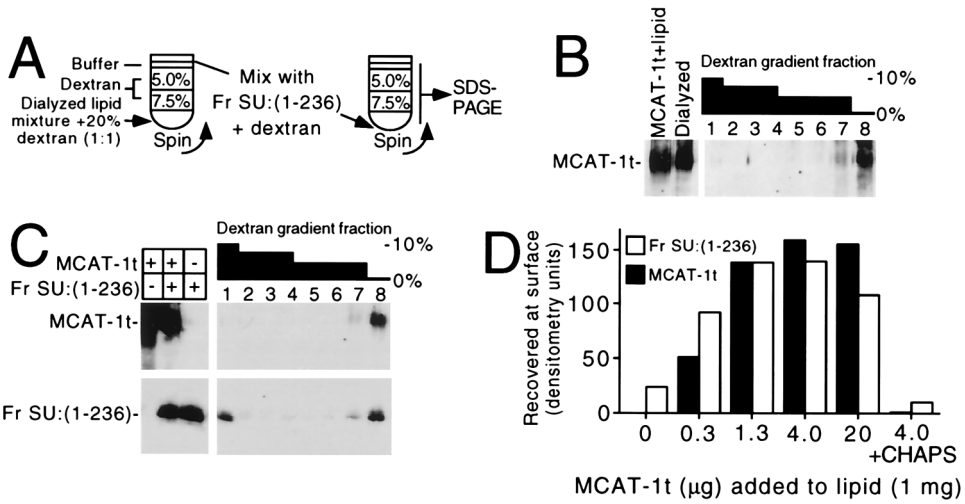

FIG. 6. Incorporation of MCAT-1t into liposomes and binding of MCAT-1t-containing liposomes to Fr SU:(1-236) in a detergent-free assay. (A) Schematic describing the assay. After dialysis to remove detergent from lipid–purified MCAT-1t mixtures, samples were applied to the base of a dextran step density gradient; after centrifugation, liposomes were recovered from the gradient surface, mixed with biotinylated Fr SU:(1-236), and reapplied to the base of a second dextran gradient. After centrifugation, fractions were collected and analyzed by SDS-PAGE. (B) Detergent was removed from detergent-solubilized lipid–MCAT-1t mixtures (first lane) by dialysis (second lane) and applied to the base of the dextran step gradient; after centrifugation, fractions 1 to 8 (from bottom to top) were collected. Liposomes were routinely found at the top of the gradient. (C) MCAT-1t-containing liposomes (first lane) were mixed with biotin-coupled Fr SU:(1-236) (second lane) and then reapplied to the base of the dextran gradient. The third lane is biotin-coupled Fr SU:(1-236) alone. Fractions 1 to 8 were collected; and MCAT-1t was detected by immunoblotting with antibody 12CA5 (upper panel), and biotinylated Fr SU:(1-236) was detected with streptavidin-HRP (lower panel). (D) The relative amount of MCAT-1t (solid bars) or biotinylated Fr SU:(1-236) (open bars) that was recovered at the surface of dextran step gradients was estimated by densitometry of bands after immunoblotting. Liposomes reconstituted with increasing amounts of MCAT-1t (0 to 20 μg) were used. CHAPS (10 mM) was added to one sample prior to centrifugation on the gradient. The amount of liposomes used in these assays was standardized by measuring the absorbance at 550 nm of mixtures that detected rhodamine-conjugated phosphatidylethanolamine which had been added (0.1%) to the lipid mixture prior to dialysis.

explanation of these findings is a direct interaction between a monomer of each protein. Indeed, amino acid substitutions in Fr SU:(1-236) that abrogate membrane binding and infection have been localized to a single region (variable region A) that is conserved in ecotropic MLV but not other subgroups of MLV (15). Analysis of the molecular structure of Fr SU:(1-236), recently obtained by X-ray crystallography (8), reveals that these residues are localized on one face of a loop at the top of the molecule, suggesting they define a discrete MCAT-1 binding domain. Determination of the molecular structure of Fr SU:(1-236) bound to the envelope binding domain from MCAT-1 will be required to confirm this conclusion.

At present, the Fr SU:(1-236) binding domain in MCAT-1 is not well defined. Residues within the extracellular loop connecting the third and fourth membrane-spanning domains are required for SU binding and infection, suggesting that they are part of the cognate SU binding site; however, to date, direct evidence in support of this conclusion has not been obtained. Specifically, competitive inhibition of SU binding by using peptides derived from this loop has not been observed (unpublished data). These peptides may fail to bind Fr SU:(1-236) because they cannot assume the conformation achieved in the context of the MCAT-1 loop. Indeed, conformation-dependent function of the SU binding domain of the receptor for the type C virus subgroup A avian leukosis virus has been reported (27). Alternatively, SU binding to MCAT-1 may require another, as yet unidentified domain whose conformation is critically influenced by residues in this loop. Indeed, detergent-dependent differences in MCAT-1t conformation may explain the reduced binding to Fr SU:(1-236) in octylglucoside or Triton X-100. Alternatively, those detergents that promote binding, such as sodium deoxycholate and CHAPS, may participate directly in SU binding by substituting for a specific lipid associated with MCAT-1 in the membrane. Indeed, enhancement of ecotropic MLV-induced syncytium formation in the presence of small amounts of amphotericin, a small hydrophobic molecule with detergent properties, has been reported (21). To explore this hypothesis, conditions have been established for study of MCAT-1 binding to SU in the absence of detergent after reconstitution in liposomes. In the experiments reported here, binding of Fr SU:(1-236) to MCAT-1 was observed after reconstitution in liposomes containing total brain lipids (10%), which may contribute specific lipids required for MLV infection of mammalian cells. Additional experiments comparing the binding properties of MCAT-1-containing liposomes of specific lipid composition can now be performed to test this hypothesis.

Although Fr SU:(1-236) was purified as a monomer, in virions, SU forms a trimer and is associated with TM (9). The structure of MCAT-1 in the membrane is unknown, but assembly into trimers would allow binding of one subunit to each SU in a trimer on the virion surface. Alternatively, interaction with a single MCAT-1 protein may be sufficient to induce the conformational change in the trimeric SU-TM required for infection. Further studies of this issue are required to create a clearer picture of the interaction of MCAT-1 with the virion surface. These details are likely to be important since interactions between several adjacent SU and receptor molecules are likely required to initiate membrane pore formation necessary for infection.

How MCAT-1 binding to SU leads to the conformational change in the TM protein required for infection remains unclear. Recently, residues near the amino terminus domain of Friend SU that are required for membrane fusion (but not SU binding) have been identified (3). This observation could be explained by an interaction between the amino-terminal domain of SU and TM, similar to the interaction between the amino termini of HA1 and HA2 along the threefold axis of the

influenza virus HA trimer (6). Alternatively, the interaction between the amino-terminal domain of SU and MCAT-1 identified here could trigger additional interactions between envelope and MCAT-1 and/or unidentified coreceptors. If the latter, these protein(s) must be highly conserved, since cells from all mammalian and avian species can be made susceptible to ecotropic MLV infection by expression of MCAT-1. The reconstitution of MCAT-1 into liposomes provides a reagent that may be suitable to determine if MCAT-1 is sufficient to induce ecotropic MLV envelope-dependent fusion.

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REFERENCES

1. **Albritton, L. M., J. W. Kim, L. Tseng, and J. M. Cunningham.** 1993. Envelope-binding domain in the cationic amino acid transporter determines the host range of ecotropic murine retroviruses. J. Virol. **67:**2091–2096.
2. **Albritton, L. M., L. Tseng, D. Scadden, and J. M. Cunningham.** 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell **57:**659–666.
3. **Bae, Y., S. M. Kingsman, and A. J. Kingsman.** 1997. Functional dissection of the Moloney murine leukemia virus envelope protein gp70. J. Virol. **71:** 2092–2099.
4. **Battini, J. L., O. Danos, and J. M. Heard.** 1995. Receptor-binding domain of murine leukemia virus envelope glycoproteins. J. Virol. **69:**713–719.
5. **Battini, J. L., J. M. Heard, and O. Danos.** 1992. Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic, and polytropic murine leukemia viruses. J. Virol. **66:**1468–1475.
6. **Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley.** 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature **371:**37–43.
7. **DeLarco, J., and G. J. Todaro.** 1976. Membrane receptors for murine leukemia viruses: characterization using the purified viral envelope glycoprotein, gp71. Cell **8:**365–371.
8. **Fass, D., R. A. Davey, C. A. Hamson, P. S. Kim, J. M. Cunningham, and J. M. Berger.** Structure of a retrovirus receptor-binding glycoprotein: murine leukemia virus SU domain at 2.0 Å resolution. Science, in press.
9. **Fass, D., S. C. Harrison, and P. S. Kim.** 1996. Retrovirus envelope domain at 1.7 angstrom resolution. Nat. Struct. Biol. **3:**465–469.
10. **Goldstein, D. J., and R. Schlagel.** 1990. The E5 oncoprotein of bovine papilloma virus binds to a 16 kd cellular protein. EMBO J. **9:**137–145.
11. **Kim, J. W., and J. M. Cunningham.** 1993. N-linked glycosylation of the receptor for murine ecotropic retroviruses is altered in virus-infected cells. J. Biol. Chem. **268:**16316–16320.
12. **Koch, W., G. Hunsmann, and R. Friedrich.** 1983. Nucleotide sequence of the envelope gene of Friend murine leukemia virus. J. Virol. **45:**1–9.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
14. **Linder, M., D. Linder, J. Hahnen, H. H. Schott, and S. Stirm.** 1992. Localization of the intrachain disulfide bonds of the envelope glycoprotein 71 from Friend murine leukemia virus. Eur. J. Biochem. **203:**65–73.
15. **MacKrell, A. J., N. W. Soong, C. M. Curtis, and W. F. Anderson.** 1996. Identification of a subdomain in the Moloney murine leukemia virus envelope protein involved in receptor binding. J. Virol. **70:**1768–1774.
16. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
17. **Morgan, R. A., O. Nussbaum, D. D. Muenchau, L. Shu, L. Couture, and W. F. Anderson.** 1993. Analysis of the functional and host range-determining regions of the murine ecotropic and amphotropic retrovirus envelope proteins. J. Virol. **67:**4712–4721.
18. **Morrison, W. K.** 1964. A fast simple and reliable method for the microdetermination of phosphorus in biological materials. Anal. Biochem. **7:**218.
19. **Ott, D., and A. Rein.** 1992. Basis for receptor specificity of nonecotropic murine leukemia virus surface glycoprotein gp70 SU. J. Virol. **66:**4632–4638.
20. **Perkins, C. P., V. Mar, J. R. Shutter, J. del Castillo, D. M. Danilenko, E. S. Medlock, I. L. Ponting, M. Graham, K. L. Stark, Y. Zuo, J. M. Cunningham, and R. A. Bosselman.** 1997. Anemia and perinatal death result from loss of the murine ecotropic retrovirus receptor mCAT-1. Genes Dev. **11:**914–925.
21. **Pinter, A., T. E. Chen, A. Lowy, N. G. Cortez, and S. Silagi.** 1986. Ecotropic murine leukemia virus-induced fusion of murine cells. J. Virol. **57:**1048–1054.
22. **Rein, A., and A. Schultz.** 1984. Different recombinant murine leukemia viruses use different cell surface receptors. Virology **136:**144–152.
23. **Thomas, T. C., and M. G. McNamee.** 1990. Purification of membrane proteins. Methods Enzymol. **182:**503.
24. **van Zeijl, M., S. V. Johann, E. Closs, J. M. Cunningham, R. Eddy, T. B. Shows, and B. O'Hara.** 1994. A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. Proc. Natl. Acad. Sci. USA **91:**1168–1172.
25. **Wang, H., R. Paul, R. E. Burgeson, D. R. Keene, and D. Kabat.** 1991. Plasma membrane receptors for ecotropic murine retroviruses require a limiting accessory factor. J. Virol. **65:**6468–6477.
26. **Wu, L., N. P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A. A. Cardoso, E. Desjardin, W. Newman, C. Gerard, and J. Sodroski.** 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. Nature **384:**179–181.
27. **Zingler, K., C. Belanger, R. Peters, E. Agard, and J. A. Young.** 1995. Identification and characterization of the viral interaction determinant of the subgroup A avian leukosis virus receptor. J. Virol. **69:**4261–4266.