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Role of Basic Residues in the Subgroup-Determining Region of the Subgroup A Avian Sarcoma and Leukosis Virus Envelope in Receptor Binding and Infection

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Receptor specificity in avian sarcoma and leukosis viruses (ASLV) maps to the central region of the envelope surface protein, SU. Two hypervariable regions, hr1 and hr2, within this region of SU are the principal determinants of receptor specificity. The cellular receptor for subgroup A ASLV, Tva, utilizes a 40-residue, acidic, cysteine-rich sequence for viral binding and entry. This domain in Tva is closely related to the ligand-binding domain of the low-density lipoprotein receptor (LDLR). Ligands bind to LDLR via the interaction of clustered basic residues in the ligand with the acidic cysteine-rich domains of the receptor. Analysis of the ASLV envelope sequences revealed a cluster of basic residues within hr2 that is unique to the subgroup A viruses, suggesting a possible role for these residues in receptor recognition. Therefore, the effects of altering these basic residues on subgroup A envelope expression, receptor binding, and infectivity were examined. Most of the mutant proteins were transported to the cell surface and processed normally. Receptor binding was diminished approximately 50% by alanine substitution at amino acid R213 or K227, whereas substitution by alanine at R210, R223, or R224 had no effect. However, when coupled with mutations at R213 or K227, changes at R223,R224 reduced envelope binding by 90%. Mutation of all five basic residues abrogated receptor binding. The effect of the hr2 mutations on ASLV envelope-mediated infection did not parallel the effect on receptor binding. Residues 210, 213, 223, and 224 were important for efficient infection, while mutations at residue 227 had little effect on infectivity. These results demonstrate that the basic residues in the ASLV envelope have roles in both receptor recognition and post-receptor binding events during viral entry.

Enveloped viruses utilize glycoproteins on their surfaces to specifically recognize receptors on the host surface and to catalyze fusion of the viral and host membranes. Entry into host cells by most retroviruses, including the avian sarcoma and leukosis viruses (ASLV), does not depend on low pH (12, 16, 17, 24). Therefore, it has been suggested that specific receptor binding triggers the presumed conformational changes in envelope protein necessary for virus-host membrane fusion (22). ASLV are classified into five major subgroups (A through E) based on receptor specificity and infection interference. The envelope protein of ASLV consists of two subunits, SU (gp85) and TM (gp37), that are produced by proteolytic cleavage of a precursor (Pr95). Within the central region of the SU glycoprotein is the subgroup-determining region (sdr), containing sequences that vary among the ASLV subgroups and that determine the receptor specificity of the virus. Sequence heterogeneity in sdr is confined to three short stretches (6 to 12 amino acids in length) of variable residues called v1, v2, and v3 and two larger regions (30 to 50 amino acids in length) called hr1 and hr2 (3, 4, 7, 8). hr1 and hr2 appear to be the main determinants of receptor utilization; therefore, these regions are presumably involved in the recognition of the different ASLV receptors.

Genetic and biochemical studies have demonstrated that Tva is the cellular receptor for subgroup A ASLV [ASLV(A)] (2, 5, 10, 27). Sequence analysis of *tva* has revealed that this receptor is a small membrane-associated protein, and that it contains a single, 40-residue, cysteine-rich motif first identified in the low-density lipoprotein receptor (LDLR). This cysteine-rich motif is sufficient for ASLV receptor function, since it efficiently mediated ASLV(A) infection when appended to a heterologous membrane-spanning domain (20, 28). Furthermore, mutations within this region affect the ASLV(A) receptor function of Tva (28). The ASLV(A) envelope protein has been demonstrated to interact directly with Tva (5, 10). Together, these results demonstrate that this 40-residue region of Tva is necessary and sufficient for ASLV(A) receptor function and suggest the direct interaction of ASLV envelope with the cysteine-rich motif in Tva.

LDLR is a cell surface glycoprotein that regulates plasma cholesterol levels by mediating the uptake of low-density lipoprotein, the major cholesterol transport protein in human plasma (13). More than half of the 767-residue extracellular domain of LDLR is composed of cysteine-rich repeated sequences, each roughly 40 amino acids in length and containing 6 cysteines. The first seven imperfect repeats form the ligand-binding domain of LDLR (9, 21). The carboxyl terminus of each of the ligand-binding repeats contains a cluster of negatively charged amino acids postulated to interact with lipoprotein. Motifs homologous to the ligand-binding repeat of LDLR, including the one in Tva, have been found in a number of proteins with a diverse array of putative functions. It appears likely that this cysteine-rich LDLR motif constitutes one-half of an extracellular protein-protein interaction motif. The LDLR ligand-binding motif consensus contains 6 invariant cysteine residues and 11 other amino acids that are highly conserved. Overall, the highest degree of conservation in this motif is at the carboxyl-terminal end, where acidic residues are

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clustered. Mutation of these acidic residues in LDLR significantly impairs ligand binding (9, 21).

The receptor binding domains from a number of proteins that interact with LDLR family members have been characterized (15, 18, 19, 26). A common feature is the importance of basic residues for receptor binding. The structure of one of the LDLR ligands, apolipoprotein E, has been determined and reveals that the basic residues required for LDLR binding are clustered in a surface patch (26). This patch is proposed to bind directly to the acidic residues in the ligand-binding domain of the LDLR. Thus, ligand binding by LDLR appears to involve ionic interactions between acidic amino acids in the receptor repeats and clustered basic residues in apolipoprotein.

Comparison of the amino acid sequences of the subgroup-determining region of the five major ASLV subgroups revealed that within the hr2 region, the Schmidt-Ruppin A (SR-A) envelope contains seven basic residues and an overall charge of +6, while all the other subgroup envelopes are either neutral or only slightly charged in this region. Five of the basic amino acids within the hr2 region are conserved in all subgroup A viruses and are unique to this subgroup. Given the importance of basic residues for the LDLR-ligand interaction, we hypothesized that these unique basic amino acids might be important for EnvA to bind its receptor, Tva. To address this question, all seven basic amino acids of the hr2 region in SR-A envelope protein (EnvA) were changed, either individually or in combination, and the mutant envelopes were evaluated for protein expression, Tva binding, and viral infectivity. Our results indicate that several of the basic residues in EnvA hr2 are important for receptor binding. Furthermore, a number of the hr2 mutations affected EnvA-mediated infection but not receptor binding.

MATERIALS AND METHODS

Cells, tissue culture, and reagents. 293T cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and 300 μg of Geneticin (GIBCO BRL) per ml. Mouse monoclonal antibody 1D3, recognizing the amino terminus of herpes simplex virus gD glycoprotein, was a gift from Gary Cohen and Roz Eisenberg (University of Pennsylvania). Purified anti-ASLV(A) envelope cytoplasmic tail antibody was previously described (10). Goat anti-murine leukemia virus (MLV) AKR capsid p30 antibody was purchased from Quality Biotech Inc. (Camden, N.J.).

Plasmids and mutagenesis. A 209-bp DNA fragment from the gD glycoprotein gene of herpes simplex virus type 1 was amplified by PCR with primers OS44 (5′-AATGGTACCAAGCTTCAGCGCGAACG-3′) and OS43 (5′-CAAAGGT TCCCTGGCTGGTCTTTGCCGCGAAAGC-3′). This fragment encodes the leader peptide and the N-terminal 21 amino acids of the mature form of gD. This 21-amino-acid region is specifically recognized by a mouse monoclonal antibody, 1D3. The amplified gD segment was fused at the 7th codon of the processed SR-A envelope gene by a two-step PCR procedure. This DNA fragment was then cloned into the envelope gene in expression vectors pCB6 and pcDNA3 by using *Kpn*I and *Xba*I.

To facilitate exchange of mutant hr2 regions into gDEnvA, silent mutations were introduced to generate unique restriction sites that flank the hr2-encoding region. *Bst*EII and *Bsp*EI sites were engineered into gDEnvA by PCR mutagenesis with primers OS81 (5′-CGGTGCATATGGTTACCGATTTTGGAACATG -3′) and OS84 (5′-CCGCAACATCCGGAGGCATTACCCA-3′). This construct is called the gDEnvA cassette (see Fig. 2A) and was used for the hr2 mutagenesis.

The EnvA hr2 mutants were constructed by using the gDEnvA cassette. Mutations were generated by PCR site-directed mutagenesis as previously described (20). After *Bst*EII and *Bsp*EI digestion, the mutated hr2-coding fragments were cloned into the gDEnvA cassette digested with *Bst*EII and *Bsp*EI. All mutants were confirmed by DNA sequence analysis.

Expression and characterization of the hr2 mutant proteins. Envelope proteins were expressed by using a transient-transfection protocol. Transfection of 293T cells with DNA from "wild-type" (wt) gDEnvA and hr2 mutants used a standard $CaPO_4$ procedure and a chloroquine shock as previously described (20). For protein analysis, the transfected cells were induced with 10 mM butyrate overnight, and cell monolayers were lysed with Triton lysis buffer (20) 48 h posttransfection. Cell surface protein biotinylation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were carried out as previously described (20).

ELISA binding assay of the hr2 mutant proteins to Tva. An enzyme-linked immunosorbent assay (ELISA) of the binding of the envelope hr2 mutant proteins to Tva was carried out as described elsewhere (1). Briefly, 96-well plates were coated with monoclonal antibody 1D3 and then used to capture gDEnvA or hr2 mutant proteins from cell lysates. The volume of lysate used was titrated so as to be sufficient to saturate the antibody on the plate with the gD-tagged envelope. After envelope binding, the plates were washed, and then 5.6 ng of biotin-labeled soluble Tva (sTva) was added per well and allowed to bind. All incubations were carried out at 4°C for 1 h each except for the antibody coating, which was performed overnight. After washing, the bound sTva was detected by using streptavidin-horseradish peroxidase and 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) (Pierce). Plates were read after incubation for 30 min at room temperature with a Molecular Dynamics ELISA reader at 405 nm.

Analysis of EnvA function. To produce envelope protein from a vector compatible with the MLV pseudotype system, the *Xba*I/*Bam*HI fragments, containing the hr2-coding region from wt *envA* and hr2 mutants, were subcloned into an *envA* vector (pCB6) digested with *Xba*I and *Bam*HI. These constructs were designated sM1, sM2, etc., corresponding to the original constructs in pcDNA-3 (M1, M2, etc.).

A modification of a transient MLV packaging system (23) was used to assay the function of the EnvA mutants. In brief, plasmid DNA encoding either wt EnvA or the hr2 mutants was mixed with plasmids encoding MLV Gag-Pol (pHIT60), and with a packagable MLV genome carrying a β-galactosidase marker gene (pHIT111). Typically, 15 μg each of pHIT60 and pHIT111 and 25 μg of EnvA DNA were used. In some cases, the amount of envelope-encoding plasmid was increased to adjust for low expression of the mutant envelope. DNAs were introduced into 293T cells by $CaPO_4$ transfection as described above. Forty-eight hours posttransfection, media containing the virus were collected, and cells and debris were pelleted for 10 min at 1,500 × *g*. The supernatants were stored at −80°C as viral stocks. Cell lysates were harvested with Triton lysis buffer and stored at −20°C until MLV capsid p30 and EnvA expression was analyzed.

To analyze virion incorporation, 3 ml of viral stock was layered onto a 7-ml cushion of 20% sucrose in phosphate-buffered saline and centrifuged at 25,000 rpm for 1 h and 15 min in an SW41 rotor. Pelleted virions were lysed with Triton lysis buffer and subjected to SDS-PAGE. Western blot analysis was used to detect MLV p30 with a polyclonal anti-Gag antibody. EnvA was detected with a polyclonal antipeptide antibody made to the cytoplasmic tail of subgroup A envelope.

The titer of the pseudotype virus was determined by seeding NIH 3T3 cells stably expressing wt Tva in 6-well plates at 1.5×10^5 cells/well the day prior to infection. Serial 10-fold dilutions of the viral stocks, in 2 ml of medium, were used to infect the cells. Forty-eight hours after infection, cells were fixed with 2% paraformaldehyde and stained for β-galactosidase activity. β-Galactosidase-positive cells were enumerated by microscopic examination of the plates. Titers of virus were expressed as numbers of positive cells per milliliter of viral stock.

RESULTS

Hypervariable region 2 of EnvA contains a cluster of basic residues unique to subgroup A viruses. Receptor specificity in ASLV maps to two variable domains, hr1 and hr2, in the subgroup-determining region of the SU glycoprotein. Since both ASLV(A) envelope and LDLR ligands recognize a highly conserved cysteine-rich motif in their cell surface receptors, we asked whether the subgroup-determining region of EnvA and the LDLR ligands have any common feature that might be involved in receptor recognition. In LDLR ligands, basic residues are essential for binding the LDLR-like motif (15, 18, 19, 26). Although there is no primary sequence homology between ASLV(A) envelope and any of the known ligands for LDLR family members, comparison of the sequences of the subgroup-determining region of EnvA and sequences for several LDLR ligands reveals similar concentrations of basic residues (Fig. 1). Furthermore, comparison of the sdr sequences from the five major subgroups (A through E) revealed that these basic residues in hr2 are unique to EnvA proteins (Fig. 1). Six of these basic residues, R205, R210, R213, R223, R224, and K227 (numbered according to the sequence of the mature SR-A envelope protein), are found in all subgroup A envelope sequences. In addition, an arginine residue at position 202 is unique to SR-A, while myeloblastosis-associated virus type 1 contains a lysine residue at 208 and Rous-associated virus type

Net charge	Virus	Subgroup	202	205	210	213	223/224	227
				*	*	*	**	*
+6	SR-A	(A)	QVGR...QYRCGNARSPRPGLPEIQCTRRGGK					
+6	RAV-1	(A)	---Q...--------R----H--T--------					
+6	MAV-1	(A)	---Q...-----K-------S--T--I-----					
0	RAV-2	(B)	-MR-N..WSI-QDVWGR..-P--NW--ST--T					
+2	PrC	(C)	-T.-N..T----DVGGT..-L---W.RGK--I					
-1	SR-D	(D)	-IR.N..YSI-EDVWG-..-L--SW-A-T--T					
-1	RAV-0	(E)	HRFDNFDI-T--DVQTV..KS--K--VGG--I					
Human apo E-3			LRVRLASHLRKLRKRLLR					

FIG. 1. Amino acid sequence alignments of hr2 of envelope proteins of ASLV subgroups. Alignment of the sequence of the hr2 region from representative subgroup A, B, C, D, and E ASLV envelope proteins. The basic residues in ASLV(A) envelope hr2 region are underlined. Dashes indicate identical residues, while dots indicate gaps. Asterisks denote conserved basic residues in the hr2 region of subgroup A viruses. The sequence of the receptor binding domain of human apolipoprotein E-3 is shown on the bottom, with the clustered basic residues required for binding underlined. RAV, Rous-associated virus; MAV, myeloblastosis-associated virus; PrC, Prague C.

1 has a unique arginine at position 211. Because of the high density of basic residues in hr2, the net charge of this region in the subgroup A envelope proteins is strongly positive (+6). In contrast, the hr2 regions of the other major ASLV subgroups contain few basic residues and are either neutral or negatively charged (Fig. 1). A similar analysis of the sequences in hr1 of ASLV(A) did not identify any basic residues which were unique to the subgroup A envelope protein (data not shown). Therefore, we focused our mutational analysis on the basic residues of the EnvA hr2 region to determine if these residues are involved in the envelope-receptor interaction.

Production of an epitope-tagged envelope protein. To facilitate studies on interactions between EnvA and Tva, an epitope tag was appended onto the amino terminus of EnvA. This region of Env was chosen for insertion of the epitope tag because we had previously appended sequences to the amino terminus of EnvA in a replication-competent Rous sarcoma virus vector with no effect on viral replication (27a). The epitope tag consists of the N-terminal 21 amino acids from mature gD envelope protein of herpes simplex virus and is recognized by the monoclonal antibody 1D3.

The gDEnvA construct was generated by PCR and cloned into an expression vector as described in Materials and Methods. The tagged envelope protein was produced in 293T cells by using a transient-transfection protocol. gDEnvA was efficiently expressed and processed (see Fig. 3A and B, lanes Wt). In addition, the surface localization of gDEnvA was verified by biotinylation of transfected cells with a membrane-impermeant reagent (Fig. 3, lanes Wt). Two assays were used to determine if the epitope-tagged envelope protein retained receptor binding ability. First, a precipitation assay that relies on the capacity of gDEnvA in cell lysates to be coprecipitated with a purified, soluble, histidine-tailed form of the receptor, sTva, was employed (1). After pelleting and washing, gDEnvA was readily detected by Western blotting with monoclonal antibody 1D3, whereas no binding to the Ni^{2+} agarose beads was detected when sTva was omitted (data not shown). The second assay used to examine gDEnvA binding is ELISA based and measures binding of biotinylated sTva to gDEnvA. In this assay, a monoclonal antibody that recognizes the gD epitope tag was used to capture gDEnvA from cell lysates onto a 96-well plate. Subsequently, biotin-labeled sTva was added and allowed to bind to gDEnvA proteins. After washing, bound sTva was detected by using streptavidin-horseradish peroxidase and a colorimetric assay. The assay is sensitive to both EnvA and Tva concentrations and can be saturated for either component. Using this assay, we have determined that the affinity binding constant for Tva-EnvA is approximately 1.5 nM (1). From these experiments, we conclude that epitope-tagged EnvA clearly retains receptor binding activity.

Generation and expression of hr2 mutations. To allow efficient mutagenesis of the EnvA hr2 region, two unique endonuclease restriction enzyme recognition sites (*Bst*EII and *Bsp*EI) that do not alter the EnvA protein sequence were introduced into the sequences flanking hr2, producing the construct called the gDEnvA cassette (Fig. 2A). As expected, the gDEnvA cassette produced a protein indistinguishable from wt gDEnvA upon transient expression in 293T cells (data not shown). The hr2 mutants described below were constructed by using this cassette vector and a PCR-based mutagenesis procedure (see Materials and Methods).

Initially, 10 mutants in the hr2 region of the gDEnvA cassette were created by changing residues R210, R213, R223, R224, and K227 to either alanine or isoleucine residues individually or in combination (Fig. 2B). The Mx mutant contains a PCR-introduced mutation of G226Q and a deletion of K227. To analyze envelope expression, the mutants were introduced into 293T cells by $CaPO_4$ transfection and cell lysates were harvested 48 h posttransfection. Expression of the envelope

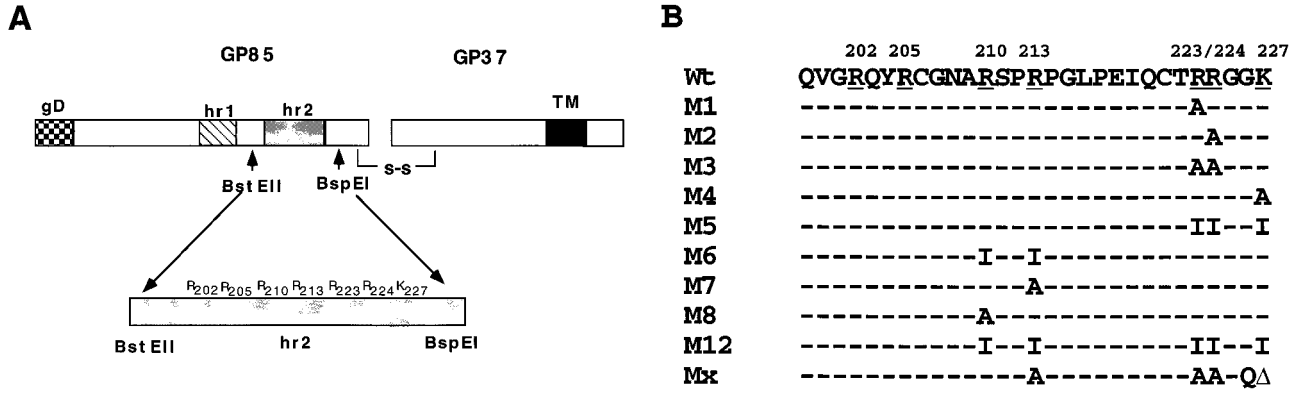

FIG. 2. (A) Diagram of the gDEnvA cassette, showing the locations of the gD epitope tag, hr1 and hr2, and the two restriction endonuclease sites (*Bst*EII and *Bsp*EI) introduced, flanking the hr2-coding region. The seven basic amino acids within the EnvA hr2 region are numbered according to the SR-A gp85 sequence. (B) Mutations in the EnvA hr2 region. The SR-A hr2 protein sequence is shown on the top with the basic residues underlined and numbered. Mutant names are on the left, and the amino acid substitutions are indicated below the residues replaced. Five residues unique to EnvA were changed to either alanine or isoleucine individually or in combination. Mutant Mx contains an alteration of G226 to Q and deletion of K227. Dashes indicate identical residues.

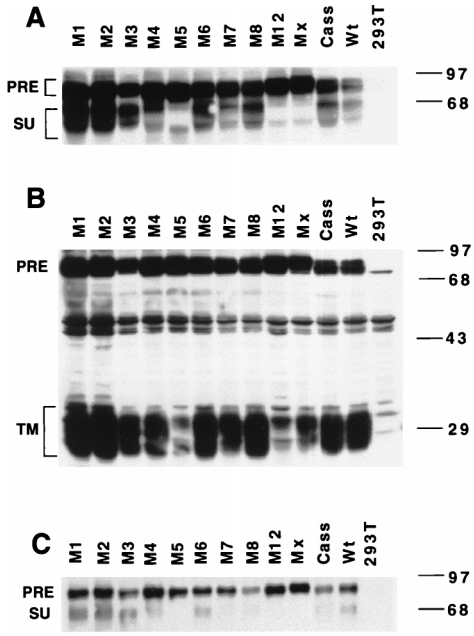

FIG. 3. Expression, processing, and surface labeling of EnvA hr2 mutant proteins. Bands representing the envelope precursor and surface proteins are labeled PRE and SU, respectively. Cass, gDEnvACassette; wt, gDEnvA; 293T, mock transfected. (A) Expression of EnvA hr2 mutant proteins in 293T cells. gDEnvA, gDEnvA cassette, and the hr2 mutants in pcDNA3 were transiently expressed in 293T cells. Cell lysates were harvested and analyzed by SDS-PAGE and Western blotting. The blot was probed with a mouse monoclonal antibody, 1D3, specific for the gD epitope. (B) Processing of EnvA hr2 mutant proteins. A blot identical to that shown in panel A was probed with a purified polyclonal anti-EnvA cytoplasmic tail antibody to assess the production of TM protein. (C) Analysis of surface expression by biotinylation. In a separate experiment, the hr2 mutant proteins were transiently expressed in 293T cells. Forty-eight hours posttransfection, surface proteins were labeled with biotin and then precipitated by streptavidin-agarose from cell lysates. After SDS-PAGE and blotting, the biotinylated surface proteins were detected with antibody 1D3.

proteins was assessed following SDS-PAGE by Western blotting with monoclonal antibody 1D3. The mutant proteins were efficiently expressed (Fig. 3A). In general, the pattern of expression appears similar for the mutants and wt gDEnvA, suggesting similar processing of the proteins. The upper two bands (labeled PRE in Fig. 3A) represent differentially modified forms of the EnvA precursor, while lower-molecular-weight forms (labeled SU) are the mature SU proteins. Mutants M5, M12, and Mx have greatly decreased levels of mature SU protein relative to the precursor, suggesting that these mutant proteins are inefficiently processed.

To further examine processing of the mutant envelope proteins, we utilized an antibody specific for the cytoplasmic tail of envelope (Fig. 3B). As expected, the envelope precursor was detected by this anti-EnvA cytoplasmic tail antibody. Also detected were several species of differentially modified transmembrane (TM) proteins. These proteins are detected only by anti-EnvA tail antibody, not by monoclonal antibody 1D3, which recognizes an N-terminal tag (compare Fig. 3A and B). In general, processing of the hr2 mutants is similar to that of wt EnvA. However, consistent with the results of analysis of SU shown in Fig. 3A, the amount of processing seen with mutants M5, M12, and Mx was decreased compared to that seen with wt EnvA.

Cell surface expression of the mutant envelope proteins was assessed in transiently transfected 293T cells by biotinylation with a membrane-impermeant compound. Biotin-labeled surface proteins were precipitated with streptavidin-agarose, analyzed by SDS-PAGE, and detected on Western blots by monoclonal antibody 1D3. It appears that all the hr2 mutant proteins were expressed on the cell surface (Fig. 3C). The lower levels of mature envelope protein seen in this experiment for the cassette and for the M4, M7, and M8 constructs were not reproducibly found (compare Fig. 3A and 8). In contrast, mutants M5, M12, and Mx were not processed efficiently (compare Fig. 3A, B, and C).

Effect of EnvA hr2 mutations on receptor binding. The ability of the mutant envelope proteins to bind receptor was investigated by using the quantitative ELISA-based assay described above. To analyze receptor binding properties of the hr2 mutant proteins, quantities of cell lysate containing hr2 mutant or wt gDEnvA protein sufficient to saturate the capture antibody were used. This ensured that similar levels of EnvA were present during the receptor binding reaction. Therefore, the binding data reflect the capacity of the captured Env to bind receptor. Furthermore, to ensure that receptor was not limiting in these binding experiments, the concentration of biotinylated sTva was approximately four times the K_d of the wt EnvA-Tva interaction. Finally, the experiments were repeated several times in triplicate with consistent results.

Analysis of receptor binding by the hr2 mutants is shown in Fig. 4 and summarized in Table 1. No binding was detected when capture antibody or envelope protein (Fig. 4, lane 293T) was omitted. In contrast, lysates from cells expressing the gDEnvA cassette or gDEnvA gave high, comparable signals. Receptor binding by envelope mutants M1 (R223A), M2 (R224A), M3 (R223A,R224A), and M8 (R210A) was consistently increased relative to gDEnvA, indicating that arginine 223, arginine 224, and arginine 210 are dispensable for Tva binding. Mutation of either R213 or K227 (M4, M6, and M7) decreased Tva binding to approximately 40 to 50% of the wt level. A triple mutant, R223I,R224I,K227I (M5), displayed

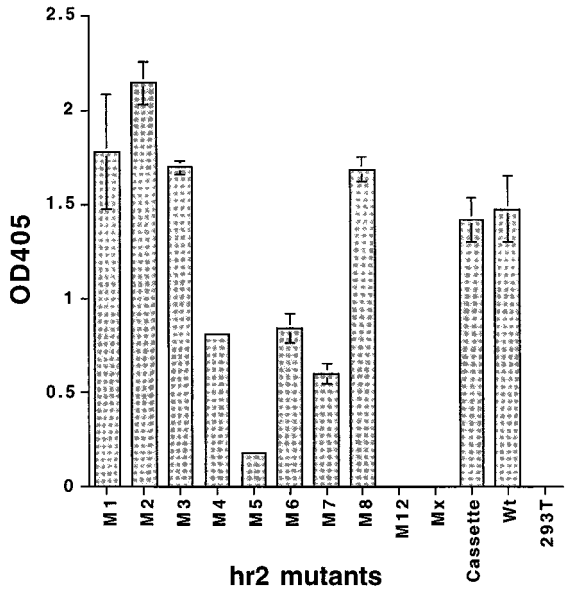

FIG. 4. Receptor binding activity of EnvA hr2 mutant proteins. Envelope protein in lysates from cells expressing gDEnvA or hr2 mutants was captured onto 96-well plates, and receptor binding was measured by an ELISA as described in Materials and Methods. OD405 (absorbance at 405 nm) represents the binding of soluble receptor protein; each value shown is the average from three wells. Bars indicate standard deviations. Where no bar is shown, the deviation is too small to be seen on this scale.

TABLE 1. Tva binding and infection properties of the EnvA hr2 mutants

Mutant	Mutation	Receptor binding (% of wt)[a]	IU/ml[b] (% of wt)
wt		100	3.6×10^4 (100)
M1	R223A	123	1.3×10^4 (36)
M2	R224A	148	8.3×10^3 (23)
M3	R223A,R224A	117	4.1×10^3 (11)
M4	K227A	56	1.5×10^4 (42)
M5	R223I,R224I,K227I	12	5.1×10^3 (14)
M6	R210I,R213I	59	8.6×10^2 (2)
M7	R213A	42	2.4×10^3 (7)
M8	R210A	117	1.0×10^4 (28)
M12	R210I,R213I,R223I,R224I,K227I	0	0 (0)
Mx	R213A,R223A,R224A,G226Q,ΔK227	0	0[c] (0)
M20	R213A,K227A	14	2.6×10^3 (7)
M21	R213A,R223A,R224A	11	1.7×10^3 (5)
M23	K227E	45	3.0×10^4 (83)
M24	K227S	64	1.5×10^4 (42)
M25	K227R	88	3.8×10^4 (105)
M26	R213E	64	8.5×10^2 (2)
M27	R213K	127	3.3×10^4 (92)
M28	R213S	91	2.0×10^3 (5)
M29	R202A,R205A	98	0[d] (0)

[a] Compiled from Fig. 4 and 7. Similar results were obtained in replicate experiments.
[b] β-Galactosidase infectious units (IU) per milliliter of viral stock.
[c] Poor EnvA incorporation into MLV virions.
[d] No detectable EnvA on MLV virions.

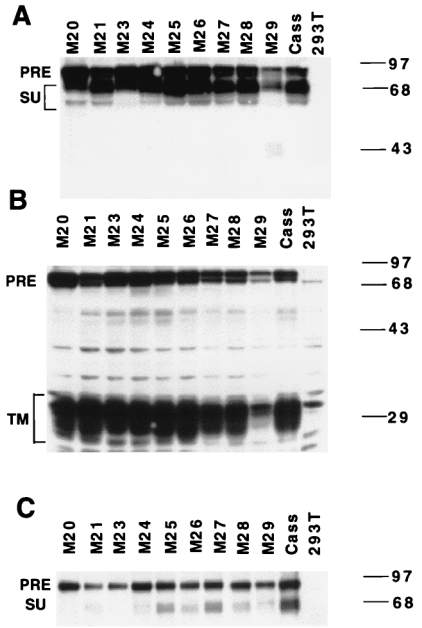

FIG. 6. Expression, processing, and surface labeling of EnvA hr2 mutant proteins diagrammed in Fig. 5. Procedures were as described in the legend to Fig. 3. PRE, Pr95; SU, surface, or gp85. (A) Expression of EnvA hr2 mutant proteins in 293T cells. (B) Processing of EnvA hr2 mutant proteins. (C) Surface expression of EnvA hr2 mutant proteins.

approximately 10% of the wt EnvA activity. Mutant envelopes containing alterations of four or five of the basic residues in hr2 (Mx and M12, respectively) did not have detectable receptor binding activity. These results appear to demonstrate that these basic residues in hr2, especially R213 and K227, play an important role in receptor recognition.

Role of EnvA residues R213 and K227 in receptor binding. To further examine the roles of R213 and K227 and also the potential involvement of residues 223 and 224 in Tva binding, additional mutations at these residues were generated (Fig. 5). The effect of combining R213 and K227 mutations was examined (M20). Also, since these two residues appear important for binding, the effect of conservative and nonconservative substitutions was examined with mutants K227E (M23), K227S (M24), K227R (M25), R213E (M26), R213K (M27), and R213S (M28). Basic residues at positions 202 and 205 are conserved between EnvA and EnvC and might therefore not be predicted

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                202  205      210 213           223/224 227
Wt        QVGRQYRCGNARSPRPGLPEIQCTRRGGK
M20       ----------A----------A
M21       ----------A---------AA---
M23       ----------------------------E
M24       ----------------------------S
M25       ----------------------------R
M26       ----------E----------
M27       ----------K----------
M28       ----------S----------
M29       ---A--A----------------------
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FIG. 5. Additional mutations within the hr2 region of SR-A EnvA. Shown are additional mutations generated to probe the effects of conservative and nonconservative substitutions at position 213 or 227; the effect of mutations at residues 223 and 224 on a residue 213 mutation was also investigated. In addition, residues 202 and 205 were changed to alanine.

to participate in specific receptor recognition. To address this question, a mutant (M29) that replaces both of these residues with alanine was constructed (R202A,R205A). Finally, the triple mutant R213A,R223A,R224A (M21) was constructed to test whether replacement of R223 and R224 has a synergistic effect with replacement of R213 on receptor binding, similar to the effect seen above with mutant M5 (R223I,R224I,K227I).

Expression and processing of the mutant proteins was assessed in 293T cells as described above. As was seen previously, in general these additional mutants were well expressed (Fig. 6A), processed efficiently (Fig. 6B), and transported to the cell surface (Fig. 6C). An exception is the mutant R202A,R205A (M29). Consistently lower levels of expression and processing were observed for this mutant. In addition, a potential proteolytic fragment of approximately 40 kDa was detected in the M29 lysate (Fig. 6A, lane M29), suggesting that the M29 protein is unstable.

Analysis of Tva binding by these mutant proteins confirms the importance of R213 and K227 in receptor binding (Fig. 7). Mutation of both R213 and K227 to alanine (M20) resulted in low levels of receptor binding (14% of wt envelope). Replacement of K227 with an acidic residue (K227E; M23) had more effect on Tva binding than replacement with a polar amino acid (K227S; M24). Conservation of the basic character at this position in mutant M25 (K227R) only marginally diminished Tva binding. Similar results were obtained with the R213 mutants. An acidic substitution, R213E (M26), had more effect on Tva binding than a less severe substitution, R213S (M28). A conservative R213K (M27) change caused a slight increase in Tva binding. Together, these results strongly indicate that residues 213 and 227 are important for the binding of EnvA to its receptor. Furthermore, the data for mutants M25 and M27 suggest that maintaining positively charged characteristics at these two positions preserves efficient receptor binding. A mutant (M21) containing substitutions at positions 213, 223, and

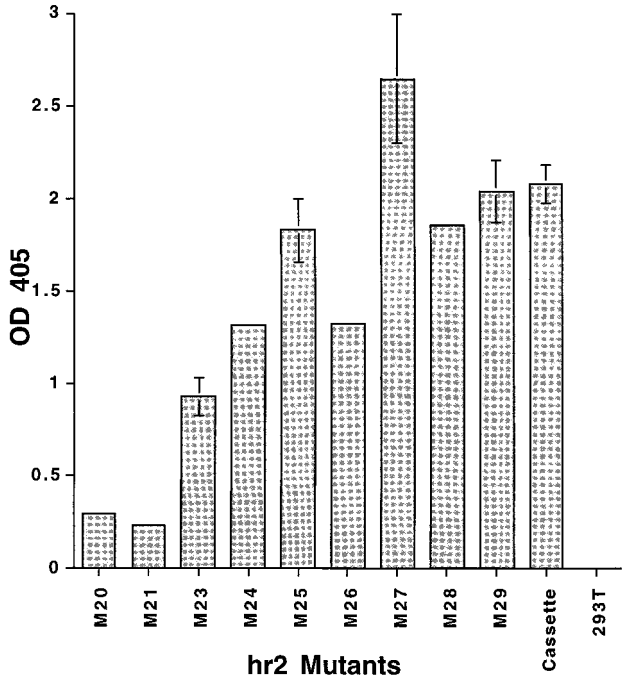

FIG. 7. Receptor binding activity of additional EnvA hr2 mutants. Binding of Tva was analyzed as described in the legend to Fig. 4 for the hr2 mutants diagrammed in Fig. 5.

224 reduced EnvA binding to Tva to 11% of the wt EnvA level, suggesting a synergistic effect similar to that observed with mutant M5 (see Fig. 4). The R202A,R205A mutation appeared to affect protein stability; however, this alteration had no significant effect on Tva binding, suggesting that these two residues probably are not involved in Tva binding but are important for envelope folding or stability.

Functional analysis of the envelope mutants. ASLV envelope protein is efficiently incorporated into MLV particles and can mediate entry of these pseudotypes into cells expressing Tva (14). Entry by the MLV(ASLV) pseudotyped virions requires the cellular receptor for ASLV(A); however, subsequent events depend solely upon MLV functions. Therefore, the effect of the hr2 mutants on EnvA-mediated infection was examined by incorporation of the ASLV envelope proteins into MLV virions using a modification of a transient virus production system described for MLV (23).

Plasmids encoding MLV Gag-Pol and ASLV Env, and a packagable MLV genome carrying a β-galactosidase marker, were cotransfected into human 293T cells. In some cases, the amounts of the envelope-encoding plasmids were adjusted to equalize the incorporation of Env into the virions. Virions were harvested 48 h after transfection. ASLV envelope incorporation into the virions was assessed by pelleting supernatants from transfected cells through a 20% sucrose cushion, resuspending the pelleted virions in lysis buffer, and analyzing them by SDS-PAGE and Western blotting with antibodies specific for EnvA and MLV Gag. wt ASLV EnvA and the majority of the hr2 mutant proteins were efficiently incorporated into the viral particles (Fig. 8A). In contrast, incorporation of M12 protein appeared less efficient, while Mx protein was poorly incorporated. M29 protein was not incorporated onto virions. The pelleted virions contained similar levels of MLV Gag (Fig. 8B). Thus, infectivity of these viruses reflects the capacity of the ASLV envelope to mediate entry and is not determined by differences in particle number or the level of envelope in the virions.

To assess the function of the ASLV envelope mutants, a series of 10-fold dilutions of the media from the transfected cells was used to infect an NIH 3T3 cell line stably expressing Tva. Forty-eight hours postinfection, the cells were stained for β-galactosidase activity and blue-stained cells were enumerated. The infection results are summarized in Table 1. The R202A,R205A mutation (M29) abolished incorporation of envelope into virions and, as expected, this mutant was unable to mediate infection. As might be predicted, mutants incapable of binding Tva were not infectious (M12 and Mx). A conservative mutation at residue 227 (K227R; M25) had no effect on infectivity, while other mutations at this position (K227A, K227S, and K227E) reduced infection by 20 to 60% compared to wt EnvA. In contrast, the single mutations affecting position 213, R213A (M7), R213S (M28), and R213E (M26), all had a significant impact on envelope function. The titers of viruses carrying these envelopes were reduced by 93 to 98% compared to wt. The only exception was the conservative R213K mutation (M27), which displayed essentially wt infectivity. Indeed, all mutant envelopes that contained a nonconservative substitution at residue 213 were severely impaired in function. Somewhat surprisingly, mutations that altered residues 223 or 224, R223A (M1), R224A (M2), R223A,R224 (M3), and R223I, R224I,K227I (M5), also significantly reduced infection. The M1, M2, and M3 mutants retained at least wt Tva binding but displayed only 11 to 36% of wt infectivity. Similarly, mutation of R210 (M8) slightly enhanced receptor binding but reduced infection by roughly 70%. Together, these results demonstrate that residues R210, R213, R223, and R224 are required for EnvA to function efficiently during infection.

DISCUSSION

Entry of enveloped viruses requires fusion of the viral and host membranes mediated by glycoproteins on the viral surface. For viruses that enter cells in a pH-independent manner, receptor binding to the envelope protein is not only required for specific host recognition but is also believed to be critical for triggering a series of conformational changes in the envelope that lead to membrane fusion (22). In this regard, binding of purified ASLV(A) receptor induces specific conformational changes in partially purified EnvA (11). Because receptor

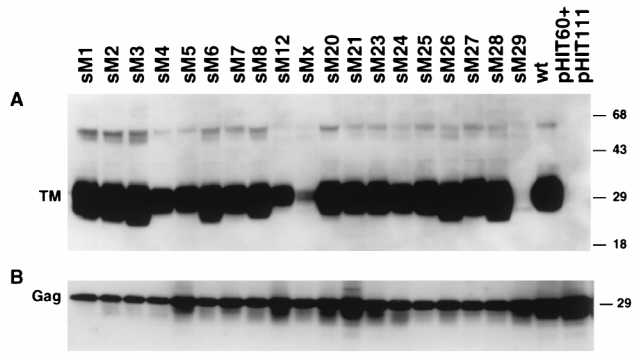

FIG. 8. Analysis of EnvA incorporation in MLV virions. Transient transfection of 293T cells with vectors expressing MLV Gag-Pol, an MLV packaging genome, and vectors expressing EnvA or the hr2 mutants was used to produce MLV pseudotyped with EnvA. To analyze proteins in virions, medium was collected off the transfected cells and the virions were pelleted through 20% sucrose. Pellets were resuspended in Triton lysis buffer and analyzed by SDS-PAGE and Western blotting. Blots were probed with either anti-EnvA cytoplasmic tail antibody (TM) (A) or anti-MLV capsid p30 antibody (Gag) (B).

binding is critical for initiating the process of viral entry, we attempted to identify residues of ASLV(A) envelope important for receptor interaction. Mutations were introduced into the subgroup-determining region of EnvA, and their effects on receptor binding and infectivity were analyzed separately. Our results indicate that two basic residues in the hr2 region of the ASLV(A) SU protein (R213 and K227) are important determinants of receptor binding. In addition, arginine residues at positions 223 and 224 appear to contribute to binding, since mutations at these positions potentiate the binding defect of the R213 and K227 mutants. Finally, four residues (R210, R213, R223, and R224) are critical for efficient ASLV envelope-mediated infection.

The ASLV(A) receptor, Tva, contains a small, cysteine-rich domain that determines envelope binding and confers susceptibility to infection (20, 28). Furthermore, two acidic residues near the carboxyl terminus of this domain in Tva are critical determinants of receptor function (28). In the LDLR, a very closely related motif is repeated seven times and constitutes the ligand-binding domain (9, 21). There is considerable evidence that a critical component of ligand binding by LDLR involves ionic interactions between acidic residues in the receptor and clustered basic residues in the ligand (9, 21, 26). Because of the importance of the LDLR motif of Tva for virus receptor function, we investigated whether basic residues in EnvA might perform a role analogous to that of the clustered residues in the LDLR ligands. Here we show that basic residues concentrated in the hr2 region of the envelope protein are indeed crucial for EnvA to bind receptor. These new data, coupled with the previous Tva results, support a model in which the EnvA-Tva interaction is mechanistically similar to the binding of LDLR to its ligands, having as a key determinant the ionic interaction of acidic residues in the receptor and basic residues on the virus.

The subgroup-determining region of ASLV Env has been proposed to be the site of receptor recognition based on the ability of this region to define the specific host range of the virus (3, 4, 7, 8). Indeed, a naturally occurring recombinant ASLV contains the subgroup E hr2 sequences in the background of a subgroup B envelope. Because this virus is able to infect cells normally resistant to subgroup B viruses, a direct role for hr2 in receptor recognition has been proposed (8). The mutations we have introduced into EnvA hr2, especially those in residues 213 and 227, demonstrate that, as predicted, hr2 contains at least part of the receptor binding domain of the ASLV envelope. Mutation of these residues significantly impairs receptor binding, as measured by an ELISA, and also diminishes envelope-mediated infectivity. We favor the hypothesis that these two residues directly contact the receptor, and our results are consistent with this; however, substantiation for this assumption awaits further experiments. It is unlikely that the mutations in hr2 had their effects because they caused major perturbations in the envelope protein structure, since most of the mutants were proteolytically processed and transported to the cell surface, processes that select strongly for correctly folded glycoproteins (6).

Mutation of two additional basic residues in hr2, R223 and R224, either alone or in combination (M1, M2, and M3) did not affect receptor binding negatively; however, substitutions at these residues did accentuate the effect of alterations at either R213 or K227. For example, the R213A mutant bound receptor at 42% the level of wt EnvA, while the triple mutant R213A,R223A,R224A had only 11% binding activity. Mutants M12 and Mx, in which all four of these residues (R213, R223, R224, and K227) were altered, did not detectably bind Tva, whereas an envelope with mutations at residues 213 and 227 retained roughly 15% receptor binding activity. Together, these results suggest that residues 223 and 224 contribute to receptor binding but are not absolutely required. Perhaps these amino acids serve to stabilize an interaction that is initiated by residues 213 and 227 or to maintain a three-dimensional structure of the hr2 region required for productive receptor binding. In this regard it is interesting that in apolipoprotein E, mutation of individual basic residues in the receptor binding domain diminishes, but does not completely abolish, LDLR binding, suggesting that multiple basic residues may cooperate in the binding function. Indeed, for all LDLR ligands where the requirement of the basic residues for receptor binding has been studied, it appears that multiple residues are involved (18, 19, 26).

The apparent importance of ionic interactions for EnvA-Tva binding explains the effect of Polybrene on infection by subgroup A viruses, which was first described over 25 years ago. The infectivity of many retroviruses, including MLVs, human immunodeficiency virus, and all ASLV subgroups except A, is significantly enhanced (up to 80-fold) by addition of the polycation Polybrene during adsorption of the virus. This enhancement is probably due to neutralization of negative surface charges on the virus and cell, which would normally repel and inhibit viral adsorption. In contrast, infection by ASLV(A) viruses is inhibited by Polybrene (25). Our results would suggest that the positive charges on Polybrene compete with the basic residues in EnvA for binding to Tva, thereby inhibiting infection.

Mutations at R213 and K227 diminished Tva binding similarly; however, substitutions at these positions had dramatically different effects on infectivity. Nonconservative alterations at K227 produced moderate defects in infectivity that roughly paralleled the observed decreases in receptor binding. In contrast, envelopes with nonconservative substitutions of R213 were significantly impaired in envelope function, regardless of the effect of the mutation on binding. All alterations at this residue, except for a conservative lysine substitution, produce titers of virus less than 10% those of wt. Similarly, mutations at residues 223 and 224 had no effect on receptor binding, but these substitutions dramatically impaired envelope function, causing roughly a 40 to 90% decrease in infectivity. A mutant with a substitution at residue 210 behaved similarly to the 223 and 224 mutants. These results suggest that residues 210, 213, 223, and 224 may be required at a stage of viral infection after receptor binding. Alternatively, these residues might promote a structure that is required for a productive interaction of envelope with receptor. We have previously demonstrated that Tva binding can trigger conformational changes in ASLV(A) envelope that appear relevant to the viral entry process (11). Perhaps these residues are essential for receptor binding to induce conformational changes in EnvA that are required for viral infection.

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REFERENCES

1. **Balliet, J. W., J. Berson, C. D'Cruz, J. M. Gilbert, J. M. White, and P. Bates.** Unpublished data.
2. **Bates, P., A. T. J. Young, and H. E. Varmus.** 1993. A receptor for subgroup-A Rous sarcoma virus is related to the low-density-lipoprotein receptor. Cell **74:**1043–1051.
3. **Bova, C. A., J. P. Manfredi, and R. Swanstrom.** 1986. env genes of avian retroviruses: nucleotide sequence and molecular recombinants define host range determinants. Virology **152:**343–354.
4. **Bova, C. A., J. C. Olsen, and R. Swanstrom.** 1988. The avian retrovirus *env* gene family: molecular analysis of host range and antigenic variants. J. Virol. **62:**75–83.
5. **Connolly, L., K. Zingler, and J. A. T. Young.** 1994. A soluble form of a receptor for subgroup A avian leukosis and sarcoma viruses (ALSV-A) blocks infection and binds directly to ALSV-A. J. Virol. **68:**2760–2764.
6. **Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius.** 1993. Folding and assembly of viral membrane proteins. Virology **193:**545–562.
7. **Dorner, A. J., and J. M. Coffin.** 1986. Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. Cell **45:**365–374.
8. **Dorner, A. J., J. P. Stoye, and J. M. Coffin.** 1985. Molecular basis of host range variation in avian retroviruses. J. Virol. **53:**32–39.
9. **Esser, V., L. E. Limbird, M. S. Brown, J. L. Goldstein, and D. W. Russell.** 1988. Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. J. Biol. Chem. **263:**13282–13290.
10. **Gilbert, J. M., P. Bates, H. E. Varmus, and J. M. White.** 1994. The receptor for the subgroup-A avian leukosis-sarcoma viruses binds to subgroup-A but not to subgroup-C envelope glycoprotein. J. Virol. **68:**5623–5628.
11. **Gilbert, J. M., L. D. Hernandez, J. W. Balliet, P. Bates, and J. M. White.** 1995. Receptor-induced conformational changes in the subgroup A avian leukosis and sarcoma virus envelope glycoprotein. J. Virol. **69:**7410–7415.
12. **Gilbert, J. M., D. Mason, and J. M. White.** 1990. Fusion of Rous sarcoma virus with host cells does not require exposure to low pH. J. Virol. **64:**5106–5113.
13. **Goldstein, J. L., M. S. Brown, R. G. Anderson, D. W. Russell, and W. J. Schneider.** 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. **1:**1–39.
14. **Landau, N. R., and D. R. Littman.** 1992. Packaging system for rapid production of murine leukemia virus vectors with variable tropism. J. Virol. **66:**5110–5113.
15. **Law, A., and J. Scott.** 1990. A cross-species comparison of the apolipoprotein B domain that binds to the LDL receptor. J. Lipid Res. **31:**1109–1120.
16. **McClure, M. O., M. Marsh, and R. A. Weiss.** 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7:**513–518.
17. **McClure, M. O., M. A. Sommerfelt, M. Marsh, and R. A. Weiss.** 1990. The pH independence of mammalian retrovirus infection. J. Gen. Virol. **71:**767–773.
18. **Nielsen, K. L., T. L. Holtet, M. Etzerodt, S. K. Moestrup, J. Gliemann, L. Sottrup-Jensen, and H. C. Thogersen.** 1996. Identification of residues in alpha-macroglobulins important for binding to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein. J. Biol. Chem. **271:**12909–12912.
19. **Orlando, R. A., and M. G. Farquhar.** 1994. Functional domains of the receptor-associated protein (RAP). Proc. Natl. Acad. Sci. USA **91:**3161–3165.
20. **Rong, L., and P. Bates.** 1995. Analysis of the subgroup A avian sarcoma and leukosis virus receptor: the 40-residue, cysteine-rich, low-density lipoprotein receptor motif of Tva is sufficient to mediate viral entry. J. Virol. **69:**4847–4853.
21. **Russell, D. W., M. S. Brown, and J. L. Goldstein.** 1989. Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. J. Biol. Chem. **264:**21682–21688.
22. **Sattentau, Q. J., J. P. Moore, F. Vignaux, F. Traincard, and P. Poignard.** 1993. Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. J. Virol. **67:**7383–7393.
23. **Soneoka, Y., P. M. Cannon, E. E. Ramsdale, J. C. Griffiths, G. Romano, S. M. Kingsman, and A. J. Kingsman.** 1995. A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res. **23:**628–633.
24. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.
25. **Toyoshima, K., and P. K. Vogt.** 1969. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. Virology **38:**414–426.
26. **Wilson, C., M. R. Wardell, K. H. Weisgraber, R. W. Mahley, and D. A. Agard.** 1991. Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. Science **252:**1817–1822.
27. **Young, J. A., P. Bates, and H. E. Varmus.** 1993. Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. J. Virol. **67:**1811–1816.

27a. **Young, J. A. T., P. Bates, and H. Varmus.** Unpublished data.

28. **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.