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# Identification of a Subdomain in the Moloney Murine Leukemia Virus Envelope Protein Involved in Receptor Binding

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**We have mutated amino acids within the receptor-binding domain of Moloney murine leukemia virus envelope in order to identify residues involved in receptor binding. Analysis of mutations in the region of amino acids 81 to 88 indicates that this region is important for specific envelope-receptor interactions. None of the aspartate 84 (D-84) mutants studied bind measurably, although they are efficiently incorporated into particles. D-84 mutants have titers that correspond to the severity of the substitution. This observation suggests that D-84 may provide a direct receptor contact. Mutations in the other charged amino acids in this domain (R-83, E-86, and E-87) yield titers similar to those of wild-type envelope, but the affinity of the mutant envelope in the binding assay is decreased by nonconservative substitutions in parallel to the severity of the change. These other amino acids may either provide secondary receptor contacts or assist in maintaining a structure in the domain that favors efficient binding. We also studied other regions of high hydrophilicity. Our initial characterization indicates that amino acids 106 to 111 and 170 to 188 do not play a major role in receptor binding. Measurements of relative binding affinity and titer indicate that most mutations in the region of amino acids 120 to 131 did not significantly affect receptor binding. However, SU encoded by mutants H123V, R124L, and C131A as well as C81A could not be detected in particles and therefore did not bind measurably. Therefore, the region encompassed by amino acids 81 to 88 appears to be directly involved in receptor binding.**

Infection of cells by enveloped viruses depends upon viral proteins that bind the virus to the target cell and promote fusion of the viral and cellular membranes. In retroviruses, specifically murine leukemia viruses (MuLVs), these functions are performed by glycoproteins encoded by the retroviral envelope gene (*env*) (27). A precursor is processed to yield a subunit that traverses the viral membrane (TM) and a subunit maintained on the viral surface (SU) by associating with TM (13).

MuLVs can be grouped into five subtypes—ecotropic, amphotropic, polytropic, xenotropic, and 10A1—based on their host range (23). The choice of viral hosts is mediated by the different cell surface receptors utilized by viruses of different subtypes for entry. The sequences required for receptor choice have been localized to the 5′ end of the retroviral *env* gene by combining DNA fragments from cloned viral genomes to form chimeric viruses (3, 22, 26). Within this receptor-binding domain, variation in the sequence of SU is largely confined to three segments: two variable segments, called VRA and VRB, that interrupt conserved sequences at the amino terminus of SU, and a hypervariable, proline-rich domain that follows the sequence of the amino-terminal domain (3). The host range determined by chimeric proteins produced by combining sequences from amphotropic, polytropic, and xenotropic envelope proteins is determined by VRA (3, 22). However, the results obtained suggest that the interaction between VRA and the other two domains is also important for envelope function.

The ecotropic MuLVs have been studied more extensively than other retroviral subtypes, and numerous ecotropic MuLV genomes have been isolated. In spite of this, little is known about the envelope protein sequences that are involved in binding to the ecotropic retrovirus receptor (ecoR). The sequences necessary for specific interaction with ecoR must be contained within the first 237 amino-terminal amino acids, since chimeric envelope proteins incorporating these sequences direct efficient ecoR-mediated entry (19). In addition, soluble proteins encoding 229 or more amino acids comprising the complete amino-terminal domain of the ecotropic envelope can bind to the ecoR and block binding and entry by ecotropic virus particles (2, 11, 29). A chimeric protein encoding only the amino-terminal 88 amino acids of the Moloney MuLV (MoMuLV) envelope can still mediate entry via the ecoR, albeit with reduced efficiency, suggesting that at least some specific interactions are mediated by these first 88 amino acids (19). Numerous insertion and site-directed mutations have been made in the binding domain of the Friend MuLV and MoMuLV envelopes (4, 5, 8, 10, 15, 25). While many of these mutations prevent the propagation of the virus, none have been shown to interfere with receptor binding. The exceptions to this are mutations that remove a site for N-linked glycosylation (N-166 in MoMuLV and N-169 in Friend MuLV) that can alter the relative efficiency with which the virus infects target cells from different species (4, 8). While these mutants infect NIH 3T3 cells normally, their ability to infect some cell types is diminished. It is likely that their altered tropism is due to altered receptor recognition.

Significant progress has been made in understanding the role that sequences in the ecotropic receptor play in virus binding. By using chimeric receptor proteins, the sequences necessary for ecotropic virus receptor binding have been localized (1, 28). Fragments of mouse ecoR were substituted into the human receptor homolog, which is incapable of binding ecotropic MuLV, yielding a functional protein. The specific amino acids responsible for this difference were identified by changing residues in the human protein to the corresponding

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residues in the mouse ecoR sequence. Another study utilized the observation that Friend MuLV infects *Mus dunni* cells efficiently while MoMuLV does not. A point mutation in the *M. dunni* ecoR that changes the sequence to more closely resemble the NIH 3T3 ecoR allows the modified receptor to mediate entry of MoMuLV (6).

Knowledge of the molecular contacts between the ecotropic MuLV envelope and its receptor would substantially facilitate the understanding of the process of virus entry. In this study, we have sought to elucidate the protein segments and amino acids that are involved in receptor binding. The receptor-binding domain of ecotropic MuLV contains three hydrophilic domains that are highly constrained by disulfide bonds (16). We have mutated the residues of these domains and measured the ability of replication-incompetent retroviral pseudotypes to bind to ecoR on NIH 3T3 cells by flow cytometry. We have identified sequences necessary for receptor binding and propose an essential role for sequences within one of these constrained loops, amino acids 81 to 88.

## MATERIALS AND METHODS

**Plasmids and mutagenesis.** Cee+ was made by excising a fragment containing the cytomegalovirus promoter, the MoMuLV envelope gene, and the simian virus 40 early-gene polyadenylation signal from plasmid CEE (19) with *Hin*dIII and *Not*I, filling in the *Not*I site with Klenow fragment, and recloning the fragment into Bluescript II SK+ (Stratagene, La Jolla, Calif.) that had been digested with *Sma*I and *Hin*dIII. Cee+ single-stranded DNA corresponding to the noncoding strand was produced by superinfecting cultures of JM109 transformed with Cee+ with R408 helper phage and purifying the single-stranded DNA as described before (24).

Mutagenic oligonucleotides were designed to produce the desired codon by single or double point mutations. Cee+ single-stranded DNA (2.5 μg) was mixed with 2 pmol of the mutageneic oligonucleotide, and mutagenesis was performed as described before (20). The presence of the mutated nucleotide in the resulting clones was confirmed by PCR sequencing (AmpliTaq Cycle Sequencing; Perkin Elmer, Foster City, Calif.). DNA prepared from the clones was digested with restriction enzymes to exclude plasmids that were rearranged or contained deletions.

**Transfection, virus collection, and titer.** Cee+ derivatives encoding mutant envelope proteins were transformed into *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.), and DNA was prepared from overnight cultures with a Plasmid Maxi Kit (Qiagen, Chatsworth, Calif.). The plasmid DNA was repurified by chromatography on ChromaSpin +TE 400 columns (5 Prime-3 Prime, Boulder, Colo.), precipitated with ethanol, and resuspended in sterile $H_2O$.

GPNZ cells (19) are NIH 3T3-derived preproducer cells expressing *gag* and *pol* of MoMuLV and the retroviral vector LBgSN (18). GPNZ cells ($6 \times 10^5$) were plated on a 100-mm tissue culture dish in D10 medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM glutamine). Sixteen hours later, the medium was removed and replaced with fresh D10. After an additional 4 h, cells were transfected with 40 μg of plasmid per plate by using calcium phosphate (9). After 16 h, the precipitate was removed and replaced with D10 for 8 h, at which time the D10 was replaced with Ultradoma (Bio Whittaker, Walkersville, Md.). After 48 h at 37°C, the supernatant was collected from the two plates, pooled, and concentrated 8- to 10-fold with a CentriPrep 100 ultrafilter (Amicon, Beverly, Mass.). This concentrated supernatant was used for Western immunoblotting and for the binding and titer assays.

The concentrated supernatants were diluted in D10, and titers were determined from β-galactosidase expression as previously described (19), with the following modifications: 72 h after virus addition, the cells were stained for β-galactosidase expression; monolayers were fixed in 0.5% gluteraldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.06 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$), washed three times in PBS, and stained overnight at 37°C in PBS containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; U.S. Biochemical, Cleveland, Ohio) per ml.

**Protein quantitation by Western analysis.** Aliquots of concentrated supernatants (see above) were diluted to 10 ml with Ultradoma and centrifuged at 4°C for 2 h at 30,000 rpm in an SW41 rotor. Viral pellets were resuspended in 30 μl of 2× sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.01% bromphenol blue, 1.4 M 2-mercaptoethanol), boiled for 5 min, and electrophoresed in 8 to 16% polyacrylamide gels (Novex, San Diego, Calif.). Gels were transferred to supported nitrocellulose (BA-S85; Schleicher and Schuell, Keene, N.H.) and blocked overnight at 4°C with 5% bovine serum albumin (BSA; fraction V; U.S. Biochemical, Cleveland, Ohio) in 1× TBS (50 mM Tris-HCl [pH 7.5], 150 mM NaCl). Blots were incubated at room temperature for 2 h with a 1:50 dilution of goat anti-gp70 (lot 79S656; Quality Biotech, Camden, N.J.) and a 1:500 dilution of goat anti-p30 (lot 78S221; Quality Biotech) in 1× TBSTB (1× TBS with 0.25% Tween 20 and 2% BSA). This was followed by a 1-h incubation at room temperature with rabbit anti-goat immunoglobulin G (IgG; Organon Teknika-Cappel, Durham, N.C.) diluted 1:500 in 1× TBSTB and a 1-h incubation at room temperature with 5 μCi of $^{125}$I-protein G (Amersham, Arlington Heights, Ill.) in 1× TBSTB. The radioactivity associated with each band was quantitated with a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, Calif.) and the ImageQuant software after 12 to 20 h of exposure.

A standard curve was generated by pelleting twofold serial dilutions of supernatant that was collected in Ultradoma from cultures of the producer line GP+E86/LNCX (29) and concentrated with a Minitan filtration unit (Millipore, Bedford, Mass.) with polysulfone plates with a 300,000-molecular-weight cutoff PTMK. These dilutions were processed for Western analysis, the radioactivity in each band was plotted against the dilution factor, and a standard curve was generated by linear regression. Aliquots of the concentrated transfected cell supernatants were processed in parallel with the standard curve, and the amount of gp70 in each aliquot was quantitated by assigning relative protein concentrations from the standard curve. Western analysis of the dilutions also allowed the sensitivity of the assay to be assessed.

**Quantitation of cell binding by viral particles.** Virus-cell binding was measured by a modification of the assay of Kadan et al. (14). NIH 3T3 cells were suspended with trypsin. A total of $2\times 10^5$ cells were mixed with 1 ml of concentrated supernatant and incubated at 4°C for 3 h with gentle mixing. The cells were washed twice with 10% normal goat serum in 1× PBS (10% goat serum). The cells were suspended in 250 μl of undiluted supernatant from hybridoma 83A25 (7) and incubated for 1 h at 4°C. Following two washes, the cells were incubated for 30 min at 4°C in a 1:75 dilution of fluorescein isothiocyanate-labeled goat anti-rat IgG (Kirkegaard and Perry, Gaithersburg, Md.) diluted in 10% goat serum. Following two final washes, the cells were resuspended in 4% paraformaldehyde in 1× PBS, kept covered on ice, and subjected to analysis by flow cytometry (FACS Star Plus; Becton Dickenson, San Jose, Calif.). The machine was calibrated by using latex spheres of known fluorescence intensities (Rainbow Calibration Particles; Spherotech, Libertyville, Ill.), and a standard curve was generated by plotting the fluorescence intensities of the beads against the mean fluorescence value obtained from the FACS. Binding by individual samples was assessed by using this standard curve to determine the fluorescence intensity that corresponded to the mean of the fluorescent signal for that sample. The fluorescence of individual samples was normalized to the amount of gp70 quantitated by Western blotting in that sample, and this ratio was adjusted to take into account differences in the volume of sample that was used for the binding assay and for the gp70 Western blot. Binding was always done in parallel with serial dilutions of high-titer supernatant from GP+E86/LNCX. This procedure allowed us to confirm that the fluorescent signal varied linearly with the amount of virus added and to assess the assay's sensitivity.

**Immunoprecipitation of labeled proteins.** At 72 h following the addition of DNA to transfected cells, the D10 was removed, and the monolayers were washed with cell-labeling medium (DMEM without methionine and cysteine and with 10% dialyzed fetal bovine serum). The cells were incubated in this medium for 2 h at 37°C, and then the medium was replaced with cell-labeling medium containing 50 μCi each of [$^{35}$S]methionine and [$^{35}$S]cysteine (Amersham) and incubated at 37°C for the desired time. For immunoprecipitation from the supernatant, medium was pipetted from the monolayers and centrifuged briefly at 200× *g*, and then the supernatant was diluted with an equal volume of lysis buffer II (10 mM sodium phosphate [pH 7.4], 150 mM NaCl, 0.2% SDS, 1% sodium deoxycholate) and placed on ice. Cell layers were washed twice with ice-cold PBS, scraped into 1 ml of lysis buffer I (10 mM sodium phosphate [pH 7.4], 150 mM NaCl, 1% Triton X-100), and transferred to a 1.5-ml tube. Each sample was vortexed for 1 min and spun for 10 min at 4°C in a microcentrifuge at 16,000 × *g*. The supernatant was transferred to a new tube and diluted with an equal volume of lysis buffer II. Both cell pellet and supernatant samples were vortexed for 1 min and kept on ice for 20 min. A total of 1.5 volumes of immunoprecipitation buffer (1:1 mix of lysis buffers I and II) containing a 1:100 dilution of goat anti-gp70 antiserum (lot 79S656; Quality Biotech) was added to each sample, and the samples were incubated overnight at 4°C. Protein G-Sepharose (Sigma, St. Louis, Mo.) (50 μl) equilibrated in immunoprecipitation buffer was added to each sample, and the samples were incubated for 2 h at 4°C. The Sepharose was collected by spinning at 200 × *g*, and the pellets were washed twice each in wash buffer I (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), wash buffer II (100 mM Tris-HCl [pH 7.5], 500 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40), and wash buffer III (10 mM Tris-HCl [pH 7.5]) sequentially. Pellets were resuspended in 30 μl of 2× SDS gel loading buffer and boiled for 5 min, and the matrix was pelleted by centrifugation for 1 min at 200 × *g*. The supernatant was loaded on an 8% polyacrylamide gel. The gel was fixed in 5% acetic acid–5% isopropanol and washed with water, and the signal was enhanced by soaking the gel in Autofluor (National Diagnostics, Atlanta, Ga.). The resulting dried gels were exposed to film (BioMax MR; Eastman Kodak, Rochester, N.Y.) and quantitated with a PhosphorImager (Molecular Dynamics) following overnight exposure.

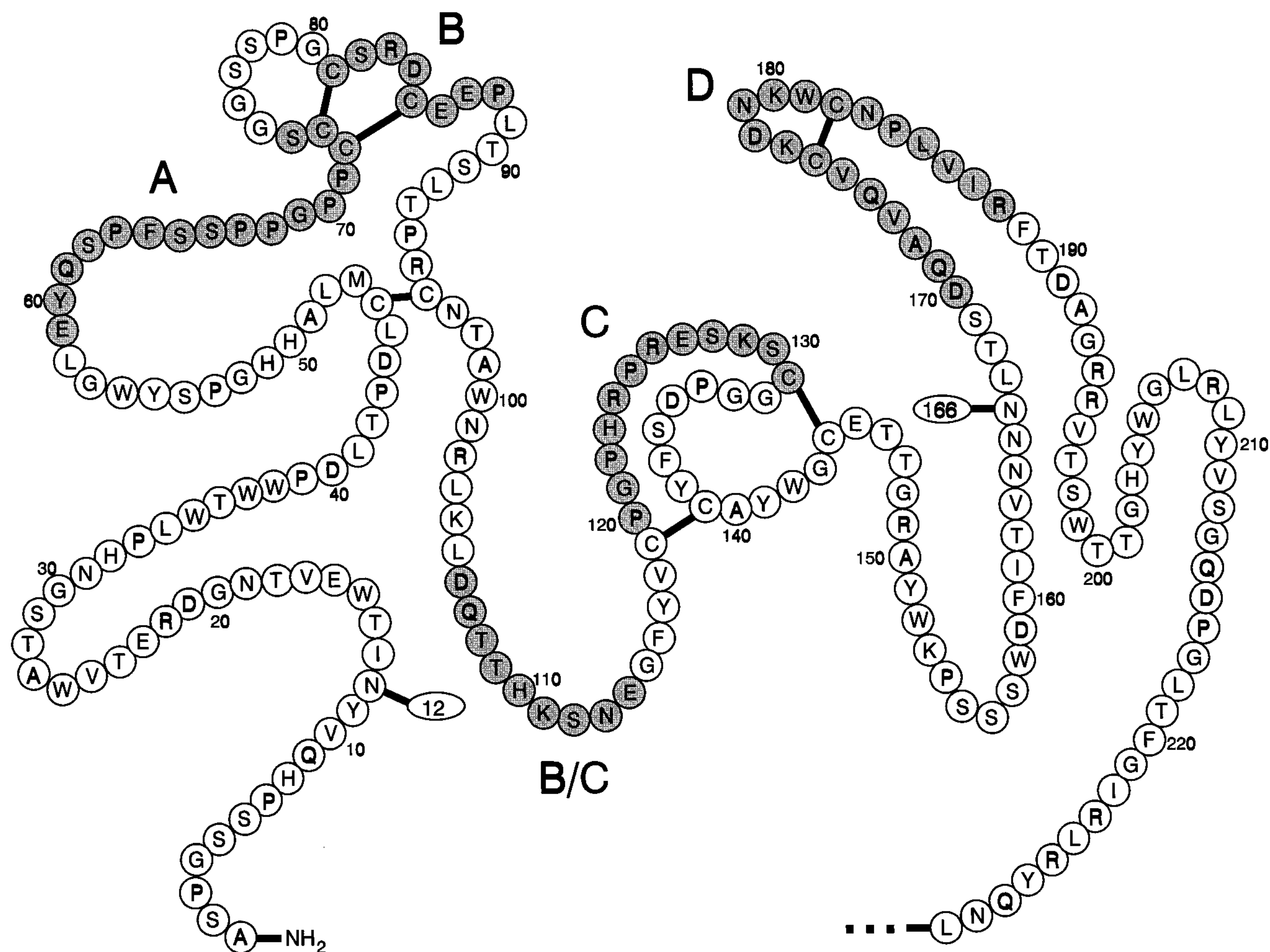


FIG. 1. Receptor-binding domain of MoMuLV envelope protein. The amino-terminal 229 amino acids of the MoMuLV SU protein are depicted to illustrate the disulfide-bonding pattern of this domain (based on Linder et al. [16]). Numbering starts with the $NH_2$ terminus of the mature SU protein. The two sites of N-linked glycosylation (12 and 166) are also shown. The segments of the protein domain that were selected for mutagenesis are shaded. Four domains within constrained disulfide-linked loops were designated A, B, C, and D. A fifth hydrophilic domain is designated B/C.

## RESULTS

**Mutagenesis of MoMuLV envelope.** The amino-terminal domain of the ecotropic MuLV envelope protein involved in receptor binding has been shown to be a compact, disulfide-linked structure (16) that is diagrammatically represented in Fig. 1. The amino acid sequences contained in these disulfide-bonded loops are among the most variable and most hydrophilic in the protein (as determined by the method of Hopp and Woods [12]) and therefore would be predicted to be on the surface of the protein. Four hydrophilic loop segments were chosen for study: regions A, B, C, and D encompass amino acids 59 to 74, 81 to 88, 120 to 131, and 170 to 188, respectively. An additional hydrophilic sequence located between the B and C regions, called B/C, was also studied. These protein segments are shaded in Fig. 1.

Single-amino-acid changes were generated by oligonucleotide site-directed mutagenesis. The initial set of mutants was made by changing hydrophilic side chains to hydrophobic side chains with similar volume and vice versa. Mutations involved the minimum number of nucleotide changes necessary to yield the desired amino acid. At some positions, specifically in the B and C regions, we made additional mutations that either better preserved the hydrophilic nature of the positions being mutated or tested specific models that were developed in the course of the work about the function of the residues.

**Screening of envelope mutations.** Initial characterization of the envelope mutations was done by testing supernatants from transiently transfected GPNZ cells for the ability to transduce NIH 3T3 cells. These data are summarized in Table 1. Only a few mutations produced a substantial decrease in the titer of the replication-incompetent pseudotyped particles. Of 51 mutations tested, 7 produced no titer and 3 produced a minimal titer. Of these mutations, four changed cysteine residues, and three others were clustered in the histidine and arginine residues at the beginning of region C. Two mutations altered the aspartate residue at position 84.

A subset of these and other mutants were tested for their ability to bind to NIH 3T3 cells and to be incorporated into vector particles. Of the mutations that displayed defects in transduction, only C176A, D84V, and D84K clearly incorporated envelope into particles, as measured by Western analysis (Table 1). The data also indicated that pseudotypes of several mutants were more defective in binding, relative to wild-type envelope pseudotypes, than they were in transduction (e.g.,

TABLE 1. Functional analysis of mutations in the MoMuLV envelope[a]

| Protein[b] | Transduction[c] | Binding[d] | gp70[e] |
|---|---|---|---|
| Wild type | +++ | +++ | + |
| Y60L | ++ | | |
| S65A | ++ | | |
| S66A | ++ | | |
| P67S | − | | |
| C72A | − | | |
| S74A | +++ | | |
| C81A | + | − | − |
| S82A | +++ | +++ | + |
| R83L | +++ | ++ | |
| R83E | ++ | ++ | |
| D84V | + | | + |
| D84K | − | − | + |
| C85A | ++ | + | + |
| E86V | +++ | ++ | + |
| E86K | +++ | + | |
| E87V | +++ | +++ | + |
| E87K | +++ | + | |
| P88A | +++ | +++ | |
| D106V | +++ | +++ | |
| Q107L | +++ | +++ | |
| T108V | +++ | ++ | |
| T109V | +++ | | |
| K111L | +++ | +++ | |
| P120T | | +++ | |
| P122V | +++ | +++ | + |
| P122S | +++ | + | |
| H123V | − | − | − |
| H123Y | − | − | |
| R124L | − | − | − |
| R124Y | | + | |
| P125V | +++ | +++ | + |
| P125S | | +++ | |
| R126L | +++ | ++ | + |
| R126Y | | +++ | |
| E127V | +++ | +++ | + |
| E127S | | +++ | |
| S128A | +++ | +++ | + |
| S128D | | +++ | |
| K129L | ++ | +++ | + |
| S130A | +++ | +++ | + |
| C131A | − | + | − |
| D170V | +++ | ++ | + |
| Q171L | +++ | ++ | + |
| V173T | +++ | ++ | + |
| Q174L | +++ | ++ | + |
| V175T | +++ | +++ | + |
| C176A | + | | + |
| K177L | ++ | | |
| K180L | +++ | +++ | |
| K180E | +++ | +++ | |
| R188L | +++ | | + |

[a] Mutants are grouped according to the segments identified in the text: amino acids 59 to 74 (A), 81 to 88 (B), 106 to 114 (B/C), 120 to 131 (C), and 170 to 188 (D). An empty cell indicates that an assay was not performed on a particular mutant.
[b] Mutants are identified by the amino acid in the wild-type protein followed by the residue number and the amino acid found in the mutant protein.
[c] +++, 30 to 100% of wild-type titer; ++, 1 to 30% of wild-type titer; +, detectable but <1%; −, no detectable titer.
[d] +++, 30 to 100% of wild-type binding; ++, 5 to 30% of wild-type binding; +, detectable but <5%; −, no detectable binding.
[e] Presence (+) or absence (−) of gp70 in particles, determined by Western analysis.

C85A and E86K [Table 1]). This observation suggested that the binding assay might be able to discriminate finer changes in envelope-receptor interactions than could be discerned by measuring titer.

Unlike the mutations described above, mutations in the B/C and D regions appear to have little or no effect (Table 1). While we cannot rule out a function for these regions in receptor binding, we conclude that other regions appear to play a larger role. Several mutations in the A region have been characterized, and some of these mutations were minimally defective in transduction. Two mutations (P67S and C72A) produced no titer. We chose to focus on the mutations in the B and C regions, since they appeared to produce the most interesting phenotypes in this initial screen.

**Quantitative envelope binding to 3T3 cells.** GPNZ cells were transiently transfected with plasmids expressing mutant envelope proteins or wild-type envelope, and the replication-incompetent pseudotyped particles were collected in serum-free medium and concentrated as described in Materials and Methods. Serial dilutions of these supernatants were added to NIH 3T3 cells, and the cells were later assayed for the appearance of β-galactosidase-expressing clones. The titers obtained with nearly all of the mutants were within 1 log unit of those obtained by transfecting wild-type envelope (Table 2). In contrast, pseudotypes of mutants D84K, H123V, R124L, and C131A did not measurably transduce NIH 3T3 cells. D84V- and C85A-transfected cells did produce active particles, but their titers were consistently 2 to 3 log units lower than wild-type envelope.

In order to quantitate differences in the binding affinities of mutant envelope pseudotypes, we employed a modification of the FACS binding assay (14). For differences in binding to reflect differences in the affinity of the envelope for its receptor and to account for differences in envelope expression or incorporation into particles, binding must be normalized to the amount of SU present on retroviral particles. The amount of SU that could be pelleted with retroviral particles was therefore quantitated. Bare particles represent the vast majority of material recovered by centrifugation of supernatants from transiently transfected GPNZ cells. Since the amounts of CA (p30) produced by transfected and mock-transfected cells are similar (Fig. 2), quantitation of CA serves as a control for the recovery of particles by centrifugation. Western blotting was used to quantitate the amount of SU incorporated into vector particles by comparing the amount of protein in a sample with serial dilutions of a standard ecotropic supernatant (see Materials and Methods) (Fig. 2). The amounts of envelope incorporated into particles for mutations at R-83 and D-84 and for wild-type envelope are similar. We were unable to detect SU in supernatants produced by cells transfected with C81A, H123V, R124L, or C131A (data not shown). The level of SU in these particles, if any is present, can be no more than 10% of that found in the wild type (i.e., Cee+-transfected cell) supernatants, since the limit of detection of these Western blots is 1 log unit less than the amount of protein found in supernatants produced by cells transfected with wild-type envelope-encoding plasmid.

Comparison of the binding affinities of envelope mutants

TABLE 2. Quantitative binding and transduction of particles pseudotyped with MoMuLV mutants

| Mutant | Relative titer[a] (% ± SEM) | Relative binding[b] (% ± SEM) | Mutant | Relative titer (% ± SEM) | Relative binding (% ± SEM) |
|---|---|---|---|---|---|
| C81A | 6.5 ± 3.5 | 0 | P122V | 85 ± 55 | 50* |
| S82A | 42 ± 1.5 | 20 ± 20 | H123V | 0 | 0 |
| R83K | 9.0 ± 5.0 | 60 ± 30 | R124L | 0 | 0 |
| R83L | 75 ± 5.0 | 20 ± 10 | P125V | 33 ± 7.0 | 50* |
| R83E | 30 ± 10 | <10 | R126L | 55 ± 5.0 | 60 ± 20 |
| D84E | 30 ± 19 | <10 | E127V | 57 ± 13 | 60 ± 20 |
| D84S | 50 ± 20 | <10 | S128A | 140 ± 0.0 | 90 ± 20 |
| D84V | 0.4 ± 0.3 | <10 | K129L | 13 ± 2.0 | 60 ± 20 |
| D84K | 0 | <10 | S130A | 220 ± 180 | 50 ± 0.0 |
| C85A | 0.3 ± 0.3 | <10 | C131A | 0 | 0 |
| E86V | 75 ± 5.0 | 100 ± 60 | | | |
| E86K | 35 ± 5.0 | <10 | Wild type | 100 | 100 |
| E87V | 95 ± 35 | 40 ± 20 | | | |
| E87K | 100 ± 17 | <10 | | | |
| P88A | 120 ± 100 | 30 ± 4.0 | | | |

[a] Titer is expressed as percentage of that obtained in parallel transfection with the wild-type envelope. Results are averages of two or more experiments. A titer of 0 indicates that transduction was never observed with pseudotypes of this mutant.

[b] Binding was determined by fluorescence and was normalized to the amount of SU in particles, expressed as a percentage of binding for wild-type envelope transfected in parallel. Results are averages of two or more experiments unless indicated by an asterisk, which are results of a single experiment. Mutants that do not measurably incorporate SU in particles are shown as having 0 binding.

revealed a role for the disulfide-linked loop structure of region B in receptor binding. None of the mutations at D-84 produced envelope proteins that bound measurably to NIH 3T3 cells in this assay (Table 2). Mutations that substitute for the other charged amino acids in this domain alter binding in a fashion that reflects the substitution made: greater differences in the chemical properties of the wild-type and mutant amino acids yield more extreme changes in activity. Thus, while R83K does not significantly affect binding, R83L decreases it and R83E eliminates it. Similarly, reversing the charges of the glutamate residues E-86 and E-87 by changing them to lysine reduces binding to below the limit of detection. Other mutations in this domain that incorporate wild-type levels of SU into particles (C85A, S82A, and P88A) also cause significant decreases in the binding of pseudotyped particles. By contrast, mutations in domain C yield proteins that either bind with wild-type affinity or produce small decreases in binding (Table 2).

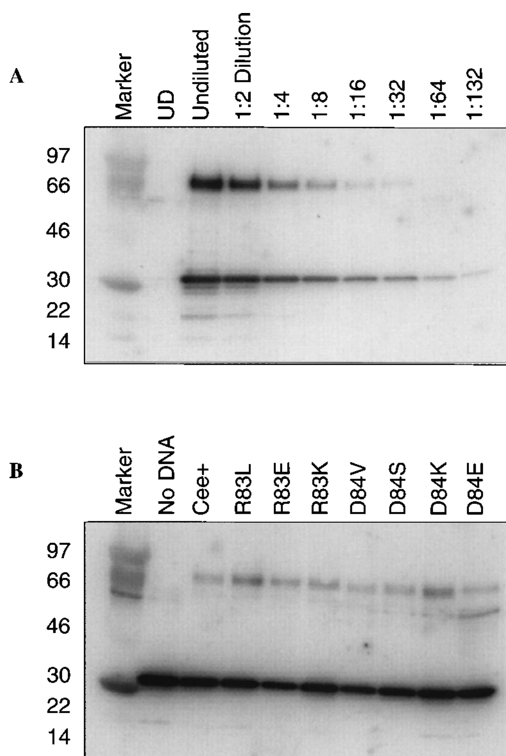


FIG. 2. Quantitation of SU and CA in retroviral particles. (A) Particles were pelleted from serial dilutions of ecotropic retroviral vector supernatant and quantitated by Western analysis with anti-SU and anti-CA antibodies to provide a standard curve. Ultradoma (UD) medium served as a control. (B) Quantitation of envelope in particles produced by GPNZ cells transfected with plasmids expressing wild-type (Cee+) or mutant envelope proteins. Sizes are shown at the left (in kilodaltons).

**Incorporation of envelope into vector particles.** Particles produced by cells transfected with the envelope mutants H123V, R124L, and C131A produce no titer. Along with the poorly transducing mutant C81A, these mutations form a group that produce proteins that are not measurably present in particles. To better understand the cause of this phenotype, metabolically labeled SU was immunoprecipitated. Figure 3 shows the result of immunoprecipitation of SU from supernatants of transiently transfected cells labeled overnight. While gp70 can be precipitated from medium from wild-type- and D84V-transfected cells, no SU could be detected in samples from the other mutants. Similarly, SU could not be immunoprecipitated from supernatants of C81A-transfected cells (data

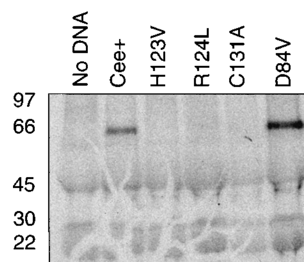


FIG. 3. Immunoprecipitation of SU from transfected-cell supernatants. GPNZ cells transiently transfected with plasmids expressing wild-type envelope or mutants were labeled overnight and immunoprecipitated with goat anti-SU antiserum. Sizes are shown at the left (in kilodaltons).

not shown). The envelope precursor Pr85 is present in immunoprecipitates of labeled cell lysates from cells transfected with C81A, R124L, and C131A but not from H123V-transfected cells (data not shown). This result suggests that the defect in C81A, R124L, and C131A is at the level of protein folding and/or protein transport.

## DISCUSSION

In order to identify amino acid residues that participate in the interaction of the ecotropic MuLV with its receptor, we have made mutations in the receptor-binding domain of the MoMuLV envelope protein. Mutations in a protein segment spanning amino acids 81 through 88 of the MoMuLV envelope (region B) interfere with the binding of pseudotyped retroviral vector particles to NIH 3T3 cells. Particularly informative are mutations in an aspartate residue at position 84: even conservative mutations (e.g., D84E) cause decreases in transduction, and all mutations at this position cause at least a 10-fold decrease in the cell-binding affinity of the particles. Other mutations in region B also inhibit virus-cell association, and the severity of these mutations correlate with how conservative the amino acid substitutions are. We conclude that D-84 is likely to provide a specific contact with ecoR, while other residues in this protein segment may either provide secondary contacts or help to maintain the three-dimensional structure necessary for optimal receptor binding.

In this study, we have been able to discriminate between mutations in the ecotropic virus envelope that result in a range of defects from subtle to severe by using both transduction and a FACS-based binding assay. Many of the mutant envelopes produced pseudotyped particles that yield nearly wild-type titers. Comparison of titers produced by mutations at Asp-84 showed a range of titers, from nearly wild type for conservative substitutions to low or undetectable for divergent substitutions. It is likely that multiple interactions between amino acids on both envelope and receptor contribute to the overall binding affinity. Disruption of any individual interaction by our amino acid substitutions might produce changes in affinity that may be too small to be observed in the titer assay. Under the conditions used in the titer assay, a particle with less than wild-type affinity might still have sufficient opportunity to bind to a cell to permit transduction. Particles pseudotyped with mutant envelopes with less than wild-type affinity could therefore produce wild-type titers. In order to measure small changes in affinity, a significantly more sensitive assay was required. We selected conditions for use in the FACS-based assay that were stringent enough to detect differences in the relative amounts of virus bound to cells due to slight differences in affinity. The conditions used to bind particles to cells in this assay were sufficiently unfavorable that mutant envelope proteins with slightly reduced affinities could be distinguished from wild-type proteins. As a result, mutations that produced nearly wild-type titers could be distinguished by the FACS-based binding assay (e.g., E87V and E87K). We estimate that mutations that result in a decrease in affinity of more than 1 log unit relative to the wild type would not bind sufficiently to be detected. Therefore, several mutations that produced measurable titers bound too poorly to be quantitated in our assay. The binding assay did allow us to discern the effects of small disruptions of envelope-receptor interactions that would not otherwise be detected by the titer assay.

The amino-terminal domain of the ecotropic virus SU protein has been shown to be a compact, disulfide-linked structure (16, 17). The importance of the disulfide bridges in maintaining the structural integrity of the envelope protein is illustrated by mutations that disrupt them. All of the mutations that changed cysteine residues to alanine cause partial to complete loss of function, with some preventing the incorporation of the envelope into particles (C81A and C131A). In addition to these cysteine mutations, two other mutations, H123V and R124L, produce defective proteins that fail to be measurably incorporated into particles or produce detectable biological activity. It has been previously shown that a viral genome bearing a double mutation of R124G and R126A in Env cannot produce a productive infection but can block infection of cells stably expressing this mutant protein (25). This result suggests that while the mutant envelope can still bind to receptor, cells expressing it cannot form functional particles. Since residues 123 to 126 are invariant in all ecotropic virus envelope proteins (numbering is for MoMuLV), this observation suggests an essential role for these residues in maintaining the structure of SU. Other mutations in region C may produce small but measurable defects in binding by causing minor perturbations in the structure of the protein.

With the exception of mutations that severely inhibit envelope incorporation into particles, we have as yet been unable to demonstrate a role in receptor binding for any sequences carboxyl terminal to region B. With the exception of C176A, none of the mutations in the B/C or the D region produced clearly defective proteins. The significantly reduced biological activity of a chimeric envelope protein that fused the amino-terminal 88 amino acids of the ecotropic envelope protein to amphotropic virus sequences encoding the remainder of the head (19) may suggest a role for these sequences in interacting with ecoR. However, since the sequences of the ecotropic and amphotropic virus envelope proteins are quite divergent in this domain (3, 21), it is possible that the substituted amphotropic virus sequences do not permit ecotropic virus sequences in the amino terminus of SU to fold into the correct conformation to allow efficient binding of ecoR. While the findings reported herein suggest that amino acids 81 through 88 have a central role in binding to the ecotropic virus receptor, residues in other regions of the envelope cannot be excluded.

Mutagenesis of the ecotropic virus receptor protein has permitted the identification of amino acids that are required for efficient viral entry. From these studies, a tyrosine (Y-235), a glutamate (E-237), and perhaps a valine (V-233) residue need to be introduced into the human receptor sequence to convert this protein into a molecule that allows ecotropic retroviral entry (1, 28). A correlation between the critical amino acids in the receptor and the importance of D-84 in the ecotropic SU is not obvious. It is likely that envelope-receptor binding will be the result of multiple noncovalent interactions between the proteins. Some of the important molecular contacts between these proteins might involve amino acids that are conserved between the mouse and human receptors. Residues on both proteins that are sensitive to mutation also are likely to play a role in stabilizing protein conformations that promote efficient binding in addition to providing direct molecular contacts.

In summary, we have identified a segment of the ecotropic virus envelope, from amino acids 81 to 88, that participates in the binding of the ecotropic virus receptor and have identified D-84 as a likely candidate for a direct molecular contact. The data reported here also suggest that receptor binding may involve multiple envelope-receptor contacts and that sequences outside this segment may play a role in this interaction. The modified flow cytometry-based binding assay described here allows the sensitive discrimination of differences in binding affinity and should facilitate the elucidation of other molecular contacts. The identification of a constrained peptide sequence in the envelope protein that is essential for receptor

specificity suggests that these sequences could be modified to create proteins with novel binding specificities.

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