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Functional Dissection of the Moloney Murine Leukemia Virus Envelope Protein gp70

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The envelope protein of Moloney murine leukemia virus (Mo-MLV) is a complex glycoprotein that mediates receptor binding and entry via fusion with cell membranes. By using a series of substitution mutations and truncations in the Mo-MLV external envelope surface protein gp70, we have identified regions important for these processes. Firstly, truncations of gp70 revealed that the minimal continuous receptor-binding region is amino acids 9 to 230, in broad agreement with other studies. Secondly, within this region there are two key basic amino acids, Arg-83 and Arg-95, that are essential for receptor binding and may interact with a negatively charged residue(s) or with the pi electrons of the aromatic ring on a hydrophobic residue(s) in the basic amino acid transporter protein that is the Mo-MLV ecotropic receptor. Finally, we showed that outside the minimal receptor-binding region at amino acids 2 to 8, there is a region that is essential for postbinding fusion events.

Murine leukemia viruses (MLVs) have been classified on the basis of their host range, which is determined, in large part, by the *env*-encoded envelope proteins and their cognate receptors (19). The primary translation product of the MLV *env* gene is a precursor protein that is cleaved to produce an external envelope surface (SU) glycoprotein, gp70, and a transmembrane (TM) protein, p15E, by a cellular protease during its transport to the cell surface (17, 40, 41). In the virion, or at the time of viral budding, p15E is processed further by the viral protease which clips a short peptide (R peptide) from the carboxy terminus (13). The gp70 protein interacts with its cognate receptor via an undefined mechanism. The p15E protein is an integral TM protein which associates with gp70, possibly through both noncovalent interactions and disulfide bonds. It serves to anchor the envelope complex to the viral membrane and is also a vital component of the viral entry process as it mediates membrane fusion (26, 27).

Five different MLV subgroups have been defined as ecotropic, amphotropic, polytropic, xenotropic, and 10A1 (4, 5, 28). Comparative studies of these subgroups suggest that receptor recognition is determined by the amino-terminal half of the SU protein. Consistent with this is the observation of Heard and Danos (15) that an envelope fragment containing the N-terminal two-thirds of Friend MLV (Fr-MLV) gp70 could bind to the ecotropic receptor in an interference assay. It has also been reported that the receptor-binding determinants of the ecotropic and amphotropic envelopes are all located in the amino-terminal domain of the envelope protein, whereas the xenotropic and polytropic envelopes utilize additional sequences located in the proline-rich region (PRR) (5). Furthermore, Yu et al. (43) have reported that the first 229 amino acids of Moloney MLV (Mo-MLV) gp70 bind to the ecotropic receptor, and Mackrell et al. (23) have reported that amino acids 81 to 88 of Mo-MLV gp70 are necessary for receptor binding.

Analysis of disulfide bridges in Fr-MLV and mink cell focus-forming MLV SU proteins has indicated that variable region A (VRA) can form two hydrophilic loops (22) and the cysteine residues involved in the formation of these potential loop structures are conserved in type C envelope proteins. This suggests that the disulfide-constrained loops are important for the interaction of gp70 with its receptor on host cells. Among the subgroups of MLV, there are two hypervariable regions, called VRA and VRB, and a recognizable PRR in the SU proteins. Exchange of VRA and/or VRB between the envelope proteins of different MLV subgroups showed that VRA plays a dominant role in receptor choice (4).

In this study, we have defined further the minimal continuous binding region and identified key residues within that region. A surprising observation is that the amino terminus of gp70 is required for postbinding fusion events. This could have a significant impact on strategies for targeting of retroviral vectors to specific cell types.

MATERIALS AND METHODS

Gene constructs and mutagenesis. Fragments of the envelope protein and mutant envelopes were amplified from pHIT123, a construct expressing the full-length MLV envelope protein precursor (36). Soluble forms of the envelope fragments were produced by inserting a stop codon (TAG) at the end of the carboxy-terminal primers. The PCR products were inserted into pBluescript KS^+ (Stratagene) or pGEM-T (Promega) cloning vectors and sequenced. The inserts were then cloned into the pGWIHG (8) expression vector, which contains the cytomegalovirus promoter and the simian virus 40 origin of replication. The pGWIHG vector was used for expression of the gene constructs described in this report.

Oligonucleotide-directed mutagenesis was performed by using the Altered Sites (Promega) mutagenesis system as instructed by the manufacturer. Briefly, single-stranded DNA containing Mo-MLV gp70 in pALTER-1 was isolated with R408 helper phage, and 0.1 μg of single-stranded DNA was used in the mutagenesis reaction. All of the mutants were confirmed by dideoxy sequencing with the Sequenase system (United States Biochemical Corp., Cleveland, Ohio).

Cell lines, transfection, production of viruses, and virus titration. NIH 3T3, XC, and 293T (293Tts/A1609 neo) cells (11) (obtained from D. Baltimore, Rockefeller University) were grown in Dulbecco's modified Eagle medium (GIBCO, Bethesda Research Laboratories) supplemented with 10% fetal calf serum (FCS; GIBCO, Bethesda Research Laboratories). The three-plasmid transient transfection method was used to produce a replication-incompetent virus (36). 293T cells were transfected by $CaPO_4$ transfection at 50 to 70% confluency with a total of 30 μg of DNA consisting of 10 μg of the envelope expression plasmid, 10 μg of Gag-Pol expression plasmid pHIT60 (36), and 10 μg of pHIT111 (36) for the *lacZ* expression or pRV172 for *luc* expression in a 10-cm-diameter dish. pRV172 has the *luc* gene from pGEM-luciferase (Promega) inserted into the polycloning site of LNCX (25). The next day, the $CaPO_4$ precipitates were removed and 10 ml of medium containing sodium butyrate at a final concentration of 10 mM was added. The cells were treated for 12 h,

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washed with phosphate-buffered saline (PBS), and refed with 5 ml of fresh medium. Viral supernatants were harvested 12 h later (48 h posttransfection) and filtered through 0.45-μm-pore-size filters.

NIH 3T3 cells were plated onto six-well culture dishes at a density of 2×10^5 per well on the day before infection. Supernatant (1 ml at appropriate dilutions of the original stock) was added to each well with 8 μg of Polybrene (Sigma) per ml and incubated for 8 to 10 h, and then 2 ml of fresh medium was added. Viral titer was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining as previously described (32).

Interference assay. Interference assays were performed to examine whether the soluble forms of the gp70 protein were capable of binding the ecotropic receptor. The culture supernatants from 293T cells transfected with each gp70 fragment plasmid were used. NIH 3T3 cells were seeded in a six-well plate on the day prior to use and incubated with each culture supernatant for 3 h at 37°C. After incubation, wild-type Mo-MLV carrying either the *lacZ* or *luc* gene was added with 8 μg of Polybrene per ml. Competition for infection was determined by counting X-Gal-positive cells or by measuring luciferase activity.

Analysis of luciferase activity. At 48 h postinterference and -transduction, the transduced NIH 3T3 cells were washed with PBS and harvested in 500 μl of luciferase lysis buffer. A 50-μl portion of the 500 μl of NIH 3T3 cell lysate was analyzed for luciferase activity by dilution to 250 μl in 25 mM Tris phosphate (pH 7.75) in a luminometer cuvette with 4 μl of 40 mM ATP and assayed by injection of 100 μl of 2 mM luciferin (Promega) in a Lumac M2010 Biocounter. The light units per second were recorded.

Cocultivation fusion assay. A 6-cm-diameter dish of 70% confluent 293T cells was transfected with 20 μg of either wild-type or mutant envelope expression plasmids by overnight $CaPO_4$ transfection and treated with sodium butyrate on the following day as described above. On the next day, the 293T cells were harvested and cocultivated with 10^6 XC cells on 6-cm-diameter dishes. At 24 h postcoculture, the cells were stained with 1% methylene blue in cold methanol prior to being photographed.

Biochemical analysis of virus proteins by Western blotting. Cell lysates were in PBS containing 1% Nonidet P-40 (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma), and virus particles were pelleted from supernatants. Samples were subjected to sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Sigma) in 25 mM Tris–200 mM glycine–20% methanol for 60 min at a constant voltage of 100 V. The filter was first blocked overnight at 4°C with agitation in PBS containing 5% (wt/vol) milk powder and 0.1% (vol/vol) Tween 20. The envelope proteins and gp70 fragments were detected by using a 1:3,000 dilution of an antibody raised against Rauscher leukemia virus (RLV) gp69/71 (Quality Biotech Inc., Camden, N.J.). The Gag protein was detected with an anti-Gag rabbit polyclonal antibody (Serotec) at a dilution of 1:1,000. The filters were washed three times in PBS–0.1% Tween 20 and subsequently incubated for 1 h with horseradish peroxidase-conjugated anti-goat (Sigma) or anti-rabbit (Sigma) immunoglobulin used at a dilution of 1:2,000 or 1:1,000, respectively. Specific proteins were visualized by using the enhanced-chemiluminescence detection system (Amersham).

^{35}S metabolic labeling and immunoprecipitation. 293T cells were transfected by $CaPO_4$ with the plasmid encoding the gp70 fragments. Two days after transfection, cells were labeled with Tran^{35}S-label ([^{35}S]Met, [^{35}S]Cys; 100 μCi/ml; ICN) for 1 h and chased for various intervals in medium containing a 500-fold excess of unlabeled amino acids. Cell extracts and culture supernatants were collected at each time point and precleared three times with agarose conjugated with normal goat serum (Sigma), goat immunoglobulin-conjugated agarose (Sigma), and protein G beads (Pharmacia). Anti-RLV gp69/71 antibody was used for detection of gp70 fragments, and immune complexes were precipitated by using protein G beads. After being washed, immunoprecipitates were subjected to SDS-PAGE followed by fluorography.

Direct binding assay by fluorescence-activated cell sorter analysis. After trypsinization with a trypsin-EDTA solution (Sigma) and washing with PBS containing 0.2% FCS and 0.05% sodium azide (washing solution), NIH 3T3 cells were incubated with 1 ml of each virus supernatant for 1 h at 4°C. Following incubation, cells were collected and washed three times with cold washing solution. Cells were incubated with anti-RLV gp69/71 antibody for 1 h at 4°C and then washed three times. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat immunoglobulin (Sigma) was used as the secondary antibody, and analysis was done with a flow cytometer (FACScan; Becton-Dickinson).

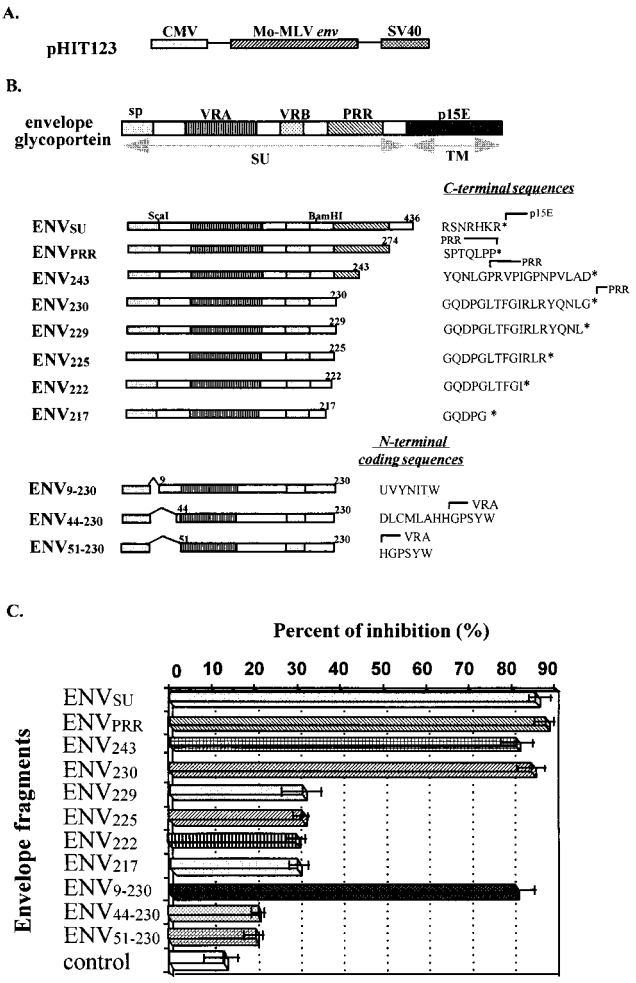


FIG. 1. Expression vectors encoding full-length gp70 or truncated gp70 fragments and functional analysis of the gp70 fragments. (A) Schematic diagram of envelope expression vector pHIT123 (36). (B) Schematic diagrams of a full-length Mo-MLV envelope protein and of truncated envelope fragments indicating VRA, VRB, the PRR, the SU protein, and TM protein p15E. sp, signal peptide. The amino acids shown on the right indicate the exact start (N terminus) or termination (C terminus) point of each protein, according to the published coding sequences of Mo-MLV (33). Each envelope fragment was generated by inserting a stop codon (*) in the end of each construct. N-terminal truncations were generated by the PCR technique, and each envelope fragment was ligated with the authentic Mo-MLV signal sequence (*Sca*I site). (C) Assays of interference induced in NIH 3T3 cells by culture supernatants. Mouse NIH 3T3 cells were incubated with 1 ml of the culture supernatant from 293T cells transfected with each construct and then transduced with the Mo-MLV pseudotype of a helper-free retrovirus (approximately 10^5 LFU added) produced by the three-plasmid transient expression system (36). The Mo-MLV retroviral vectors carried the β-galactosidase or luciferase coding gene as a reporter. Relative reductions in numbers of X-Gal-positive cells or luciferase activity are shown as percentages. Averaged results of five independent experiments are shown. Culture supernatants from pGWIHG transfectants were used as a negative control (control). The errors observed over five experiments are shown as error bars. CMV, cytomegalovirus promoter; SV40, simian virus 40 *ori*.

RESULTS

Construction of gp70 fragments and analysis of interference capacity. We used the pHIT123 expression vector (36) (Fig. 1A) containing the complete *env* gene as a template to generate soluble gp70 fragments. A full-length fragment was produced by placing a stop codon (TAG) just downstream of the cleavage site between gp70 and p15E to produce a soluble gp70 fragment containing the entire SU protein. In addition, a series of truncations were produced (Fig. 1B). The construct designated ENV_{243} is analogous to the Fr-MLV fragment which was reported previously (5, 15), and three of the fragments, ENV_{9-230}, ENV_{44-230}, and ENV_{51-230} (Fig. 1B), were designed to assess the role of N-terminal residues in receptor binding. In previous reports (15, 26), it has been shown that large deletions at the N terminus caused the envelope protein to lose its ability to bind to the ecotropic receptor.

The gp70 fragments were expressed in 293T cells transiently

transfected with the appropriate plasmids. To visualize the expression of the gp70 fragments, Western blot analysis of cell lysates was performed as described in Materials and Methods. All constructions produced, in transfected cells, the predicted size of protein in approximately equal quantities (Fig. 2A), showing that any differences are not due to differential expression. The cell culture supernatants were then used in interference assays (Fig. 1C) to examine whether the gp70 fragments produced were able to bind to the ecotropic receptor and thereby inhibit transduction by ecotropic retroviral vector particles (14, 16). As expected, the full-length gp70 moiety, ENV_{SU}, strongly inhibited transduction (Fig. 1C). To examine whether interference efficiencies were proportional to the amount of plasmid used in the transfection, interference assays were performed with various amounts of supernatant. Interference efficiencies varied linearly with the supernatant volumes used (data not shown). ENV_{243} also inhibited transduction as efficiently as the full-length fragment, and this is in agreement with Fr-MLV data (15). The data in Fig. 1C show that the first 230 amino acids, ENV_{230}, are necessary for maximum competition. This construction, ENV_{230}, excludes all of the PRR sequences and therefore demonstrates that the PRR of the ecotropic envelope protein does not contribute to receptor binding. Interestingly, with ENV_{229}, which contains the first 229 amino acids of the SU protein, inhibition of transduction was substantially reduced (Fig. 1C) and this difference was still observed when five times as much ENV_{229} as ENV_{230} culture supernatant was used (data not shown). This implies that amino acid 230, glycine, may be important for binding of the ecotropic receptor.

Because this observation differed from that of Yu et al. (43), we sought to demonstrate that there was no difference in cellular processing and stability between the amino acid 1 to 230 and 1 to 229 proteins. We had already demonstrated that there was no difference in expression level (Fig. 2A). To ensure that there was no difference in turnover or rate of secretion of the gp70 amino acid 1 to 230 and 1 to 229 fragments, a label-chase experiment was performed with ENV_{230} and ENV_{229}. 293T cells were transfected with either ENV_{230} or ENV_{229} and labeled with 100 μCi of Tran^{35}S-label per ml for 1 h. The system was then chased for various time intervals with an excess of unlabeled amino acids (Fig. 2B). Cell extracts and supernatants (Fig. 2B, a and b) at each time point were incubated with an anti-RLV gp69/71 antibody (Quality Biotech Inc.), and immune complexes were precipitated. Figure 2B, a, shows that the gp70 bands for ENV_{230} and ENV_{229} decreased during the chase, suggesting that both proteins were expressed in transfected cells. The gp70 fragments were detected in culture supernatants in both cases and remained in the media for at least 8 h (Fig. 2B, b). This shows that the difference between ENV_{230} and ENV_{229} is not due to a difference in expression, secretion, or stability.

Of the three N-terminal truncations, only ENV_{9-230} efficiently inhibited gene delivery by the ecotropic virus (Fig. 1C). This result suggests that the first eight amino acids are not required for receptor binding, and it reduces further the definition of the size of the minimal continuous receptor-binding region. The interference effect of ENV_{9-230} is specific to the ecotropic virus, as it does not inhibit transduction by an amphotropic (4070A) virus (data not shown). Furthermore, the interference efficiency shows a clear dose response (data not shown). Taken together, these results imply that the gp70 fragment expressing amino acids 9 to 230 binds specifically to the ecotropic receptor and blocks further virus entry.

Identification of residues important for receptor binding. We have shown that amino acids 9 to 230 of the Mo-MLV SU protein are enough for competition for the receptor. Previous reports (5, 22, 26) have suggested that VRA amino acids 51 to 130 contain the primary determinants for receptor choice (5) and that the first disulfide loop, consisting of amino acids 46 to 95 of Mo-MLV gp70, interacts with the ecotropic receptor (22). Furthermore, Morgan et al. (26) showed that in amphotropic-ecotropic hybrids the first 88 amino acids of Mo-MLV gp70 are sufficient for some degree of receptor interaction. Therefore, taken together, these various reports suggest that amino acids 51 to 88 of gp70 may be the key region for receptor interaction and merit further analysis. In addition to this region, we have evaluated the role of amino acids 89 to 95 because this sequence is restricted to ecotropic gp70 (Fig. 3). The ecotropic receptor (EcoR) is a multipass membrane protein that transports basic amino acids (20, 39), and mutational analysis of this protein has identified the third extracellular domain as central to the interaction with the virus envelope. In particular, Tyr-235 and Glu-237 are critical for the interaction (1, 2, 42), and it has been implied (24, 34) that a positively charged amino acid(s) in the envelope is a key contact point(s). With these findings in mind, we compared the VRA sequences of several different MLV subgroups, focusing on the first disulfide loop of gp70, and found two positively charged residues, R83 and R95 (Fig. 3), that are highly conserved in the ecotropic envelope proteins (data not shown). Mutations of these residues were introduced by site-directed mutagenesis to produce 11 mutant *env* genes, which were then evaluated for

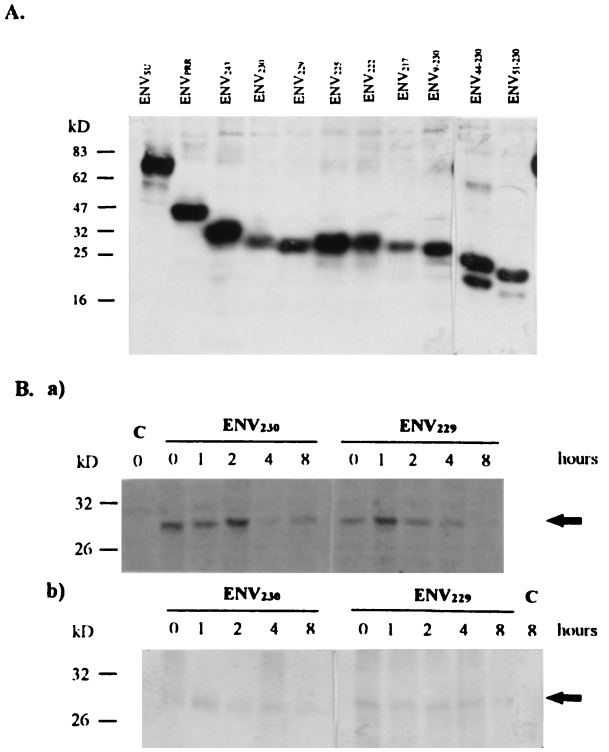


FIG. 2. Expression of truncated gp70 fragments. (A) Western blot analysis of 293T cell extracts (10 μg of total protein, respectively) transfected with each gene construct. The gp70 fragments were detected by immunoblotting with a goat anti-RLV gp69/71 antibody (Quality Biotech Inc.). (B) Detection of envelope protein fragments by a pulse-chase experiment with cells expressing the ENV_{230} and ENV_{229} envelope fragments. Cell extracts (a) and culture supernatants (b) from untransfected control cells (lanes C) or cells transfected with ENV_{230} or ENV_{229} were immunoprecipitated with a goat anti-RLV gp69/71 antibody before analysis by SDS-PAGE. The gp70 fragments are indicated by arrows on the right. Molecular size markers are indicated on the left in kilodaltons.

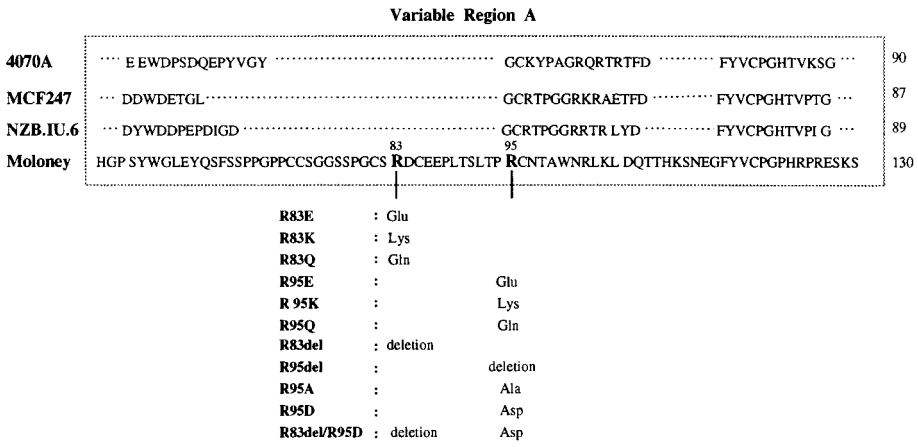


FIG. 3. Multiple sequence alignment of VRA in gp70s. Amino acids of VRA in SU proteins from the following viruses are shown: amphotropic MLV 4070A, polytropic MLV MCF247, xenotropic MLV NZB.IU.6, and ecotropic Mo-MLV. The standard one-letter code is used for amino acids. Two arginine residues (in boldface letters) in Mo-MLV gp70 were chosen for mutagenesis analysis, and the indicated amino acids were deleted or replaced as shown.

expression, incorporation into virus, and ability to function as mediators of viral entry (Fig. 3).

Figure 4 shows the expression and incorporation into virus particles of the 11 mutants. *gag-pol* expression is approximately the same in all cases, and this is reiterated by the reverse transcriptase (RT) activities shown in Table 1. *env* expression is also approximately the same in all of the mutants and the wild type. Interestingly, incorporation of the mutated gp70 molecules into virus particles is not equal across all of the mutants. In particular, R95E, R95Q, R95del, and R95A are all virtually absent from the virus particles. Not surprisingly, when these proteins were used in a standard procedure for making transducing vector particles, there was no gene transfer (Table 1). These data suggest that R95 is important for gp70 incorporation into virus, although the residue can tolerate some mutations, such as R95K and R95D, in which incorporation was the same as that of the wild type. When there is normal incorporation, as with R95K and R95D, the mutations have no effect on gene transfer. R83 was completely insensitive to the changes that we made both in terms of incorporation and function. These data suggested that single changes in this pair of arginine residues could not disrupt receptor binding and entry. Interestingly, however, a double mutant comprising R83del and R95D, which had no effect individually, was deficient in mediating transduction (Table 1). Expression and incorporation into particles were indistinguishable from those of the wild type (Fig. 4), suggesting that this double mutant was defective in receptor binding or entry. A direct binding assay revealed that the inability to transduce was caused by failure of the mutant envelope to bind to the ecotropic receptor (Fig. 5C). We concluded, therefore, that the arginine residues are partially redundant but essential for efficient interaction with

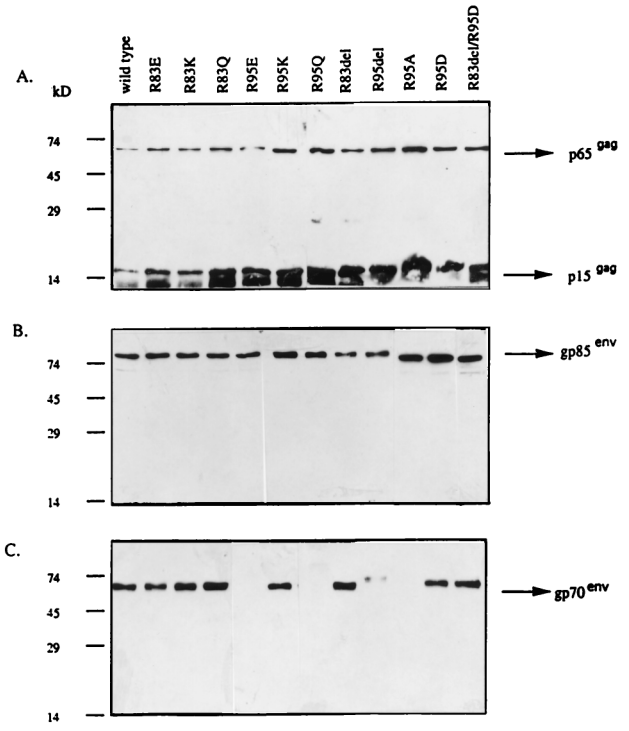


FIG. 4. Western blot analysis of transfected cells (B) and pelleted viral particles (A and C) containing the wild-type envelope protein or mutant envelope proteins. Filter A was probed with an anti-MLV Gag serum, and B and C were probed with an anti-gp70 antibody. MLV gene products are indicated on the right, and apparent molecular sizes are shown on the left in kilodaltons (kD).

TABLE 1. Properties of arginine mutants

env mutant tested	Titer (LFU/ml) on NIH 3T3 cells	RT activity	Western blot analysis[a]	
			SU	INC
pHIT123	5×10^6	+++	+++	+++
R83E	2×10^6	+++	+++	+++
R83K	1.8×10^6	+++	+++	+++
R83Q	1.5×10^6	+++	+++	+++
R95E	$<<10^2$	+++	±	±
R95K	2×10^6	+++	+++	+++
R95Q	$<<10^2$	+++	±	±
R83del	6×10^5	+++	+++	+++
R95del	$<<10^2$	+++	±	±
R95A	$<<10^2$	+++	±	±
R95D	5×10^5	+++	+++	+++
R83del/R95D	0	+++	+++	+++

[a] A summary of Fig. 4 is shown. The presence of the SU protein in the virus pellet and its incorporation (INC) into the virus are shown. ±, hardly detectable; +++, wild-type signal.

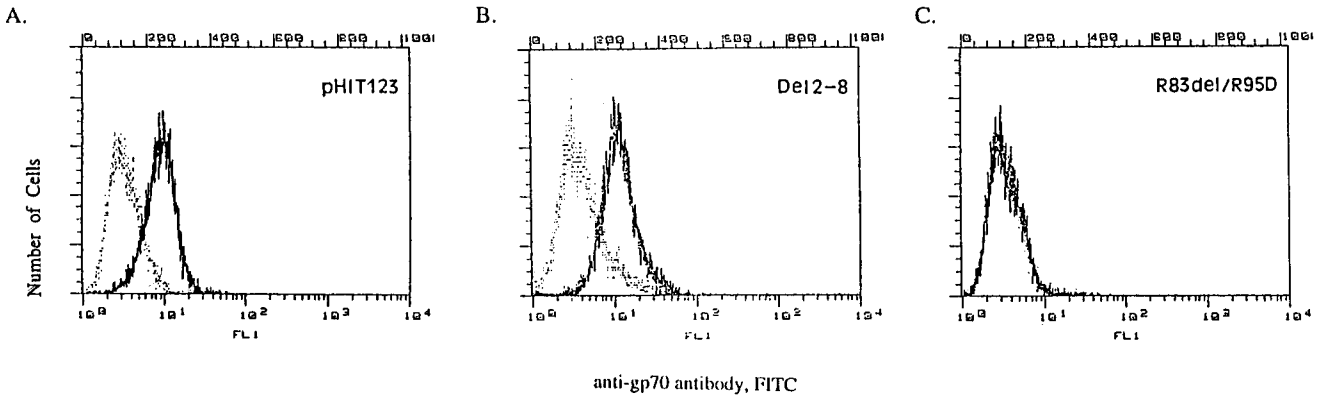


FIG. 5. NIH 3T3 cell direct binding assay. Virus supernatants containing the wild-type envelope protein (A) or mutant envelope proteins (B and C) were incubated with NIH 3T3 cells, and an anti-RLV gp69/71 antibody was used to analyze the binding capabilities. Background fluorescence is shown as dashed lines. Cells were incubated in Dulbecco modified Eagle medium supplemented with 10% FCS. Binding assay results are shown as solid lines. The *x* axis is log green fluorescence intensity, and the *y* axis shows the relative cell number for each histogram.

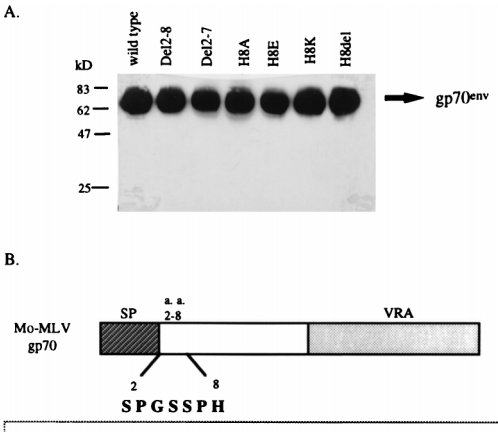
the ecotropic receptor. This supports the notion that charge interactions are an important feature of Mo-MLV binding (24).

Detailed analysis of the amino terminus. As described above, the first eight amino acids of the SU protein gp70 are dispensable for receptor binding. However, in other studies we have observed that the replacement of these residues with peptides such as FLA16 or fibronectin-derived sequences disrupts 3T3 cell infectivity (3a). This suggests that the first eight amino acids are important for a function of gp70 other than receptor binding. To characterize the role of these residues, we introduced mutations into this region. Results obtained with six of these are shown in Fig. 5 to 7. All six mutant envelope proteins were expressed and incorporated into virus particles with efficiencies comparable to that of the wild type. However, their abilities to mediate gene transfer in our standard transduction assay differed considerably. Both deletions Del2-8 and Del2-7 bound the receptor as efficiently as did the wild type (Fig. 5A and B and data not shown), but Del2-8 was completely defective in transduction and Del2-7 showed only 1% of the wild-type activity. This confirmed that this region is important for some postbinding event and identified H8 as a possible key residue. To investigate this further, we analyzed the mutations at residue H8, which is conserved among all MLV subgroups (data not shown). Surprisingly, although all of the H8 mutants bound to the receptor as efficiently as did the wild type (data not shown), subtle changes in this residue caused total loss of infectivity (Fig. 6B), confirming that H8 is critical for postbinding functions of gp70.

As the defect in transduction by the mutant envelope proteins shown in Fig. 6 reflected a block in a step after receptor binding, we performed XC cell cocultivation fusion assays with the mutant envelope proteins. XC cells have been used widely to study the fusion abilities of different ecotropic envelope proteins (18, 26, 29). The results obtained with our mutant envelope proteins are shown in Fig. 7. There was a complete correlation between the transduction levels with the mutant gp70 proteins and their fusion abilities. The mutants which showed no infectivity (Fig. 6B, less than 10 *lacZ*-positive forming units (LFU)/ml) in NIH 3T3 cells could not form syncytia between transfected 293T and XC cells (Fig. 7). The Del2-7 mutant showed approximately 5% of the fusion activity of the wild type (Fig. 6B and 7B and D). We concluded, therefore, that the amino terminus of gp70 is crucial for fusion and virus entry and within this region H8 is critical.

DISCUSSION

We have shown that the minimal continuous receptor-binding region of gp70 resides within amino acids 9 to 230. This is the smallest continuous fragment described to date, and it may be useful in further structural studies by X-ray diffraction or nuclear magnetic resonance analysis. This fragment contains VRA and VRB but lacks all of the PRR, supporting the notion that the PRR is not required for simple binding.



Constructs	Mutations	Titer	RT (X 10^4 cpm)	Fusion activity (%)
pHIT123	wild type	5 x 10^6	1.3	100
Del 2-8	deletion a.a. 2-8	< 10	2.1	< 0.1
Del 2-7	deletion a.a. 2-7	4 x 10^4	1.2	5
H8A	His 8 --> Ala	< 10	1.6	< 0.1
H8E	His 8 --> Glu	< 10	2.1	< 0.1
H8K	His 8 --> Lys	< 10	2.0	< 0.1
H8 del	His 8 --> deletion	< 10	2.9	< 0.1

FIG. 6. Expression of the SU protein in virus particles and functional analysis. (A) Virus particles generated by three-plasmid cotransfection were pelleted and analyzed by Western blot analysis. Each virus contained either the wild-type or a mutant envelope protein and was probed with an anti-gp70 antibody. Molecular sizes are shown at the left in kilodaltons (kD). The arrow indicates the gp70 SU protein. (B) Mutations in the N terminus of the envelope protein, relative infectivities, and RT activities. Titers were analyzed by determining *lacZ* CFU per milliliter of viral supernatant on NIH 3T3 cells. Relative fusion activities were determined by counting cells containing more than five nuclei. SP, signal peptide; a. a., amino acids.

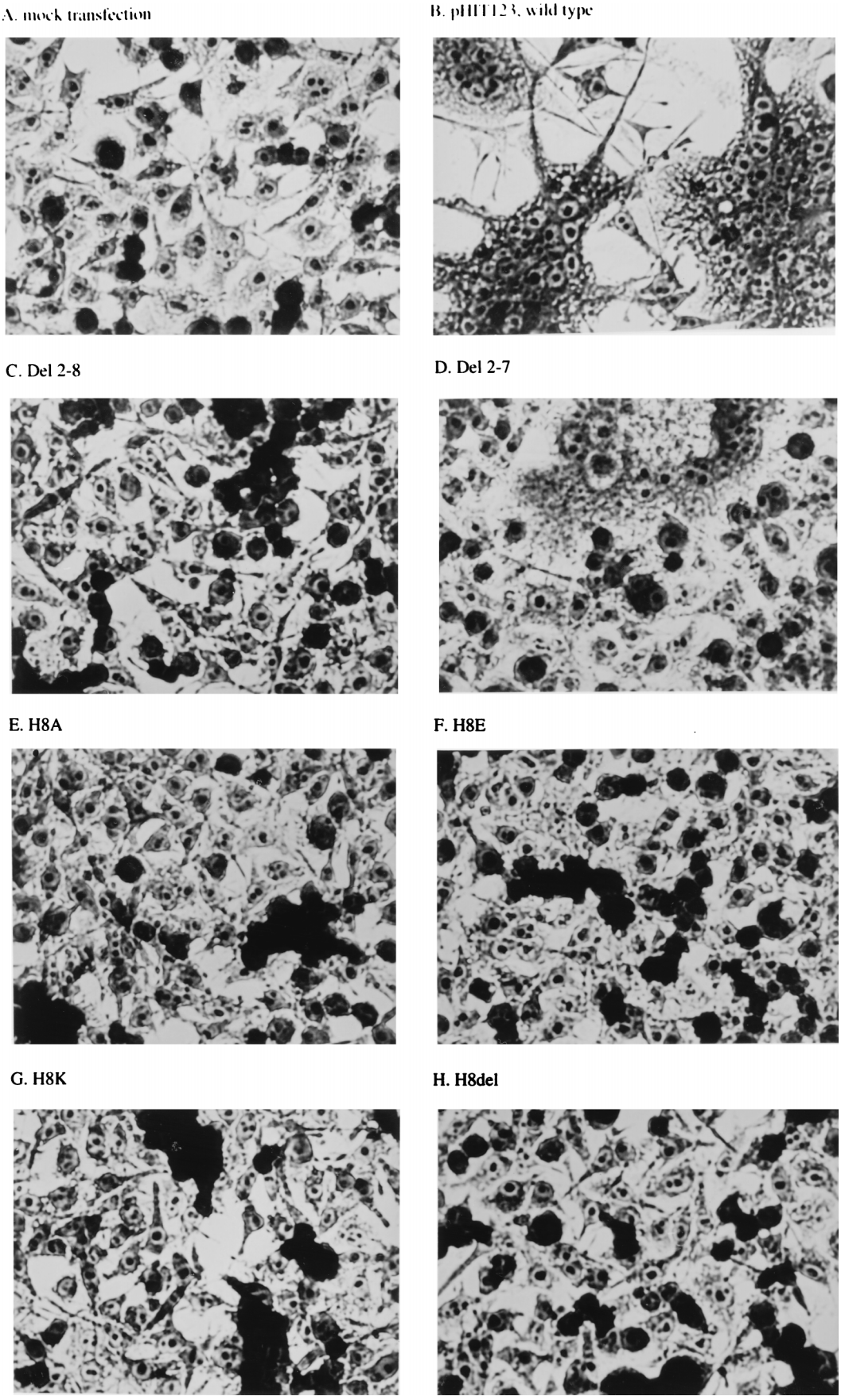


FIG. 7. Ecotropic envelope protein-mediated syncytium formation on XC cells. 293T cells were transfected with an envelope protein expression vector encoding the wild-type protein (B) or mutant (C to H) envelope proteins and cocultivated 48 h later with XC cells. Objective magnification, ×84.

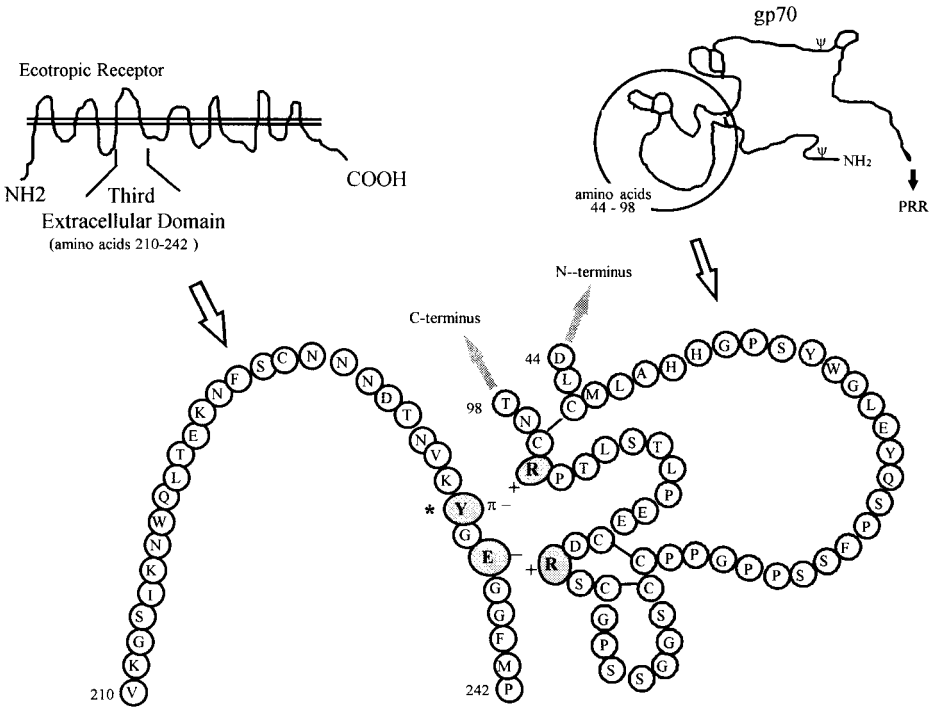


FIG. 8. Proposed working model of the interaction between Mo-MLV SU protein and the ecotropic receptor. The amino acid sequences of the third extracellular domain of EcoR and the first disulfide-constrained loop of Mo-MLV gp70 are shown in the single-letter code. The speculated interacting residues are shown as gray ellipses. Intracellular disulfide links of the envelope protein are based on a report by Linder et al. (22). The two arginine residues in gp70 are located close to cysteine residues, and therefore the structure exposing the two residues may be relatively rigid. Furthermore, Tyr-235 and Glu-237 in EcoR lie near the plasma membranes at the C terminus of the third extracellular domain with a predicted extended conformation (Program Garnier; data not shown). As a result, the interaction between gp70 and EcoR most likely occurs through Tyr-235–Arg-95 and/or Glu-237–Arg-83. As the pK of the hydroxyl group of Tyr is 10.0 and virus infections were carried out at pH 7.4, the Tyr-235–Arg-95 interaction may be via the pi electrons in the aromatic ring of Tyr-235 (*), as demonstrated in the acetylcholine-synthetic receptor interaction (10). Steric hindrance by the bulk of gp70 will most likely prevent interactions in the other configuration (Tyr-235–Arg-83 and/or Glu-237–Arg-95).

It has been shown previously (43) that the first 229 amino acids of Mo-MLV gp70 produced in a baculovirus system could bind the ecotropic receptor. In contrast, our system reproducibly showed that the first 229 amino acids could not compete for virus entry as well as the constructs expressing the first 230 amino acids or amino acids 9 to 230 (Fig. 1). These data suggest that amino acid 230 is critical but that its requirement differs in different expression systems, perhaps because of differences in glycosylation. Certainly, it has been demonstrated that Gag protein release differs between insect and mammalian cells (6). Alternatively, as Yu et al. (43) used a direct binding assay without competion for transduction with the wild-type virus, the discrepancy may result from the difference between the experimental systems used.

Within amino acids 9 to 230, we identified two positively charged amino acids, R83 and R95, that are important in receptor interaction. Both lie in the first disulfide-constrained loop (22) of VRA, an important domain for receptor choice (3a, 4). These residues are conserved in ecotropic envelope proteins, and we speculated that they interact with two negatively charged amino acids, Tyr-235 and Glu-237, on EcoR in a way similar to the interaction between acetylcholine and its receptor (21). Interestingly, gp70 was functionally sensitive, in terms of incorporation into virus, to mutations at R95 but was relatively insensitive to changes at R83. This might be because R95 exists in a more rigid environment between P94 and C96 (38). In addition, to exclude the possibility that the lack of infectivity of the R83del/R95D mutant was due to a synergistic effect of the amino acid changes at both sites, an additional R83E/R95D double mutant was created and analyzed. This mutant showed the same phenotype as the R83del/R95D mutant (data not shown). These results support the notion that R95 and R83 are functionally redundant for receptor binding, as a single arginine residue is sufficient for interaction with the receptor. These results match the observation that a single mutation of Tyr-235 or Glu-237 in EcoR does not affect its function as a viral receptor (42). However, when both residues Tyr-235 and Glu-237 were changed, no infection by the ecotropic virus was detected (42). Accordingly, as suggested in Fig. 8, the interaction between the ecotropic envelope protein and EcoR may occur through Tyr-235–Arg-95 and Glu-237–Arg-83 interaction. This also supports the notion that the first disulfide-constrained loop of the ecotropic envelope protein, which is more complicated than that of polytropic gp70, determines the ecotropic host range (22).

A surprising observation from these studies is that the first eight amino acids of the mature gp70 protein are essential for fusion and virus entry. Within this region, a particularly important residue is H8. This is conserved among all MLVs and some other retroviruses, such as a gibbon ape leukemia virus SEATO and feline leukemia virus types A, B, and C (4). Recently, we constructed the amphotropic counterpart of Del2-8, which has the first six amino acids (Met-Ala-Glu-Ser-Pro-His) deleted. As expected, vectors bearing the deletion mutant envelope protein could not transduce NIH 3T3 or HeLa cells. This supports the idea that the importance of this region extends to other subtypes.

The mechanism of action of this sequence is not known, but it must act in concert with the fusogenic sequence at the amino terminus of TM protein p15E (3, 12, 30). In this context, it is interesting that X-ray diffraction data show that the first 27 amino acids of influenza virus HA1 are tightly associated with

HA2 (7), suggesting that they have a role in preserving the functional integrity of the fusogenic protein. It is conceivable that the amino terminus of gp70 folds back and is intimately associated with p15E and that that association is critical for fusogenic activity.

The requirement for the amino terminus of gp70 for fusion may also bear on recent attempts to achieve retargeting of MLV vectors by adding targeting molecules to gp70. In almost all of these cases, gene transfer titers are low, despite demonstrable binding to the new receptor, and gene transfer requires incorporation of the wild-type ecotropic envelope protein for any functional activity at all. Most of these hybrids have been made by inserting or adding ligands within or close to the first eight amino acids of gp70 (9, 31, 35, 37). It is quite possible that the poor results obtained to date are because the essential fusion region is disrupted. Addition of wild-type envelope proteins may restore fusion activity not by providing p15E, which is already present, but by providing the N terminus of gp70. If this is the case, it may be possible to achieve the same result with fragments of gp70 or gp70-derived peptides. Alternatively, new chimeric gp70 molecules should be constructed without disrupting the N terminus.

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