Mutational Analysis of the Putative Receptor-Binding Domain of Moloney Murine Leukemia Virus Glycoprotein gp70

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The entry of Moloney murine leukemia virus (MoMuLV) to murine cells is mediated by the binding of its envelope glycoprotein gp70 to its receptor, the cationic amino acid transporter MCAT-1. The binding property of the envelope protein lies mainly in the N-terminal half of the protein. To identify essential residues involved in the binding of gp70 to its receptor, we have mutated amino acids within the putative receptor-binding domain of MoMuLV gp70. Changes in the residues P94 and W100 resulted in lower viral titers in comparison to the wild-type virions. Single, double, or triple point mutations involving the residue W100 make the envelope protein severely defective in binding to its receptor. Binding studies and cell fusion experiments with murine XC cells suggested that the residue W100 might play an important role in the process of infection by making contact between gp70 and its receptor.

INTRODUCTION

Attachment of MuLV virions to the surface of the cells occurs through the specific interaction between the envelope glycoprotein and its cellular receptor (Albritton et al., 1989). The envelope glycoprotein complex of replication-competent MuLV is composed of two polypeptides. One is the glycosylated external hydrophilic polypeptide, termed surface glycoprotein gp70 (SU), and the other is the membrane-spanning, hydrophobic transmembrane protein p15E (TM). Both of these polypeptides are synthesized from the same spliced RNA and the precursor protein (gp85) is proteolytically cleaved during the assembly process to give rise to both SU and TM proteins.

Murine leukemia viruses (MuLVs) are classified as ecotropic (infecting only mouse cells) (Horowitz and Risser, 1985), amphotropic (infecting both murine and human cells) (Hartley and Rowe, 1975), xenotropic (infecting only cells other than mouse cells), polytropic (mink cell focus forming virus) (Evans and Cloyd, 1984), and the 10A1 type (having an expanded host range and binding both amphotropic and GALV receptors) (Ott and Rein, 1992; Rein and Schultz, 1984). The receptor for the ecotropic MuLV has been identified as a cationic amino acid transporter (CAT-1) with 14 membrane-spanning regions (Albritton et al., 1989). The Moloney murine leukemia virus (MoMuLV) is an ecotropic virus, the entry of which is mediated by MCAT-1 in murine cells.

The MoMuLV envelope protein has two hypervariable regions, VRA and VRB, that contribute to receptor recognition (Battini et al., 1995; Morgan et al., 1993; Ott and Rein, 1992). It has been suggested that the amino acids 81–88 might be responsible for the direct contact between MoMuLV gp70 and its receptor (MacKrell et al., 1996). The residues responsible for receptor recognition have been mapped to the first potential disulfide-linked loop within the VRA region of the ecotropic gp70 (Bae et al., 1997; McDougall et al., 1994). The expression of a truncated protein containing the amino-terminal portion of the Friend MuLV gp70 was reported to establish superinfection interference, consistent with binding and down-regulation of the receptor expression (Battini et al., 1995). The N-terminal 236 amino acids of Friend MuLV gp70, when expressed using the baculovirus expression system, has been found to bind to its receptor MCAT-1 with the same affinity as the wild-type protein (Davey et al., 1997). Other studies have identified the regions of gp70 involved in its expression or stability (Felkner and Roth, 1992), fusion activity (Jones and Risser, 1993; Skov and Andersen, 1993), and receptor-binding ability (Bae et al., 1997; MacKrell et al., 1996).

Arginine residues at positions 83 and 95 in MoMuLV gp70 have been reported to be involved in receptor interaction and mutations at these sites abolish the binding of gp70 to its receptor (Bae et al., 1997). The region of the receptor required for specific interaction with the ecotropic envelope protein lies in its third and/or fourth extracellular domain (Yoshimoto et al., 1993). Both ty-
Rosine 235 and glutamic acid 237 in the third extracellular loop of the receptor have been found to be important, and mutations of both these amino acids abolish the infection by ecotropic virus (Yoshimoto et al., 1993). There is a possibility (Malhotra et al., 1994) that the charged arginine residues 83 and 95 of MoMuLV gp70 bind to tyrosine 235 and glutamic acid 237 of the receptor and initiate the process of infection (Bae et al., 1997).

From computer modeling and structural studies on Friend MuLV's receptor-binding domain the region of amino acids 85–102 that forms the hydrophobic pocket and a charged ridge has been predicted to form the binding pocket between the envelope and its receptor (Fass et al., 1997).

Taking the results from previous biochemical and structural studies, we have chosen a region of the MoMuLV envelope from amino acids 89 to 100 for mutagenesis analysis to identify important residues that play a key role(s) in the envelope–receptor interaction. Different mutations in the envelope protein are schematically represented in Fig. 1. Six different single point mutations, three double mutations, and one triple mutation were created in the region spanning amino acids 89–100 to elucidate the importance of amino acids in the putative receptor-binding domain of MoMuLV gp70.

RESULTS

Expression of viral proteins

The level of expression of proteins in transfected cell extracts and envelope incorporation into viral particles were checked by Western blot analysis. Gag expression was checked in the cell extracts and the viral supernatants as depicted from the Western blots (Fig. 2). The expression of Gag suggested that virions were produced from the cells transfected with WT as well as mutant envelopes. In all cases, viruses also processed the Gag precursor into individual components and this characteristic was unaffected by mutations in the envelope protein. All the mutants and the WT envelopes were expressed in transfected 293T cells. In all cases the presence of envelope proteins in viral particles was unaffected except in P94S and P94S/W100T, where low levels of envelope incorporation were observed (see Fig. 2).

Role of envelope mutants in the process of infection

The initial characterization of envelope mutations was done by testing the viral supernatants from transfected 293T cells for their ability to transduce murine NIH 3T3 fibroblasts. Transduction efficiency was measured by counting the number of lacZ-positive blue cells. For control experiments, the wild-type Moloney MuLV envelope plasmid (pHIT 123) was used. For a negative control, cells were mock transfected with MuLV gag-pol plasmid (pHIT 60) and vector genome with lacZ (pHIT 111) without any envelope. The result of the infection assay is given in Table 1. Mutations in amino acids other than 94 and 100 did not have any effect on the infection of virions on murine fibroblasts. In the case where amino acid 94 (proline) is changed to serine, a three-log reduction in the titer was observed. Changing the amino acid from tryptophan to threonine at position 100 also resulted in a hundredfold reduction in viral titer.

In one of the double mutants (pRV 737) where both amino acids 91 and 100 were mutated, a thousandfold reduced viral titer was obtained. Double mutations at positions 93 and 100 or positions 94 and 100 (pRV 738 and pRV 739) completely abolished viral infectivity. A similar pattern of infectivity was also observed when the triple mutant (involving amino acids 89, 93, and 100, pRV 740) was used in the cotransfection experiments. A reverse transcriptase assay with virions carrying mutations at their envelopes showed that equal quantities of virus
particles were produced from all transfected dishes (data not shown).

The reduction in viral titer due to single, double, or triple mutations in the putative receptor-binding region could have resulted either due to low binding efficiency of the envelope protein to its receptor or due to any change(s) in the postbinding events. In order to test these possibilities, we carried out superinfection and binding analyses.

Superinfection with wild-type MoMuLV particles

Superinfecting NIH 3T3 cells with wild-type virions gave us an indication of the initial receptor-binding abilities of mutant envelopes, particularly those mutations that caused a decrease in viral titer. In mutant glycoproteins that retained binding ability, we expected no change in viral titer upon superinfection. However, an increase in titer upon superinfection could suggest a loss in binding ability. Results of the superinfection assay are given in Table 1.

In all cases where a loss of viral titer had been observed earlier (with P94S, W100T, and all double and triple mutants), wild-type titers were restored upon superinfection. This suggests that the mutant envelopes that caused a decrease in titer were deficient in binding to their receptors. Further direct-binding assays by FACS analysis helped us to confirm the binding deficiency of mutant envelopes to their receptors compared to the wild-type virions.

Binding studies with mutant envelopes

FACS analysis was performed with polyclonal anti-gp70 antibody to check the efficiencies of the virions that bear mutant envelopes to bind to the cellular receptors. As seen in Fig. 3, virions bearing mutant envelope T90V, S91A, and T93V bind to NIH 3T3 cells with nearly the same efficiency as the wild-type virions. At the same time, virions carrying mutations at amino acids L89P, L92P, and T94S resulted in a reduced binding compared to the wild-type virions (see Fig. 3). Interestingly, this binding is not strictly reflected in the viral infectivity.

FIG. 2. (A, B) Analysis of viral proteins by Western blot analysis. Virus particles (V) were produced by three plasmid cotransfections. pHIT 123 WT MoMuLV particles and the rest of the numbers represent particles bearing mutations at corresponding amino acids in the MoMuLV envelope protein. Both cell extracts (C) and viral particles (V) were probed with goat anti-gp69/71 polyclonal antisera (gp85env and gp70env) and with monoclonal anti-p30 (capsid) antibody (p65gag and p30gag).
Binding to NIH3T3 cells could not be determined using virions bearing envelopes with a mutation at amino acid 100. In this case, there is a possibility that the binding was so poor that it was below the level of detection by FACS analysis. For viruses bearing envelopes with either double or triple mutations, very poor or barely detectable binding was observed (see Fig. 3B).

As the FACS analysis represented here is not quantitative, no direct correlation between the results of the titer and the binding can be established. However, taking transduction and superinfection data in conjunction with the FACS analysis, it can be concluded that the mutations in the envelope had altered their binding abilities, resulting in lower infection efficiencies.

Syncytia-forming ability of mutant envelopes

The syncytia-forming capacity of ecotropic MuLV viruses is attributed to the fusion ability of its envelope protein (Rowe et al., 1970). Syncytia are large, multinucleated structures in which the cell membranes are fused together with the nuclei at the center. It has been shown previously that Moloney MuLVs form syncytia on XC cells (Jones and Risser, 1993; Ragheb and Anderson, 1994). In order to test the syncytia-forming abilities of the mutant envelope proteins, 293T cells were transfected with the wild-type or individual mutant envelope plasmids (without any gag-pol or vector genome) and XC cells were seeded onto the same disc 24 h posttransfection. The cells were stained with methylene blue to observe the syncytia and later photographed.

As shown in Fig. 4, all the mutant envelopes bearing single point mutations produced syncytia. However, the degree of syncytia formation varied in different mutant envelopes. In envelopes with either double or triple mutations, the ability of gp70 to form syncytia was greatly reduced.

Results from the syncytium assays suggested that the mutant envelopes that bound poorly to the receptors fused to XC cells very inefficiently and formed fewer syncytia on the cells. Similarly, the envelopes with double and triple mutations that failed to bind to their cellular receptors could not fuse to the XC cells at all.

DISCUSSION

The N-terminal 230 amino acids of MoMuLV gp70 are important for binding to its cellular receptor and for the

<table>
<thead>
<tr>
<th>Envelope used</th>
<th>Titer* (CFU/ml) on NIH 3T3 cells</th>
<th>Titer* (CFU/ml) after superinfection on NIH 3T3 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHIT 123</td>
<td>1.3 x 10^6</td>
<td>2.08 x 10^6</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRV 730 L89P</td>
<td>9.6 x 10^5</td>
<td>3.7 x 10^6</td>
</tr>
<tr>
<td>pRV 731 T90V</td>
<td>1.4 x 10^6</td>
<td>4.12 x 10^6</td>
</tr>
<tr>
<td>pRV 732 S91A</td>
<td>2.2 x 10^6</td>
<td>3.03 x 10^6</td>
</tr>
<tr>
<td>pRV 733 L92P</td>
<td>1.2 x 10^6</td>
<td>3.6 x 10^6</td>
</tr>
<tr>
<td>pRV 734 T93V</td>
<td>1.2 x 10^6</td>
<td>3.34 x 10^6</td>
</tr>
<tr>
<td>pRV 735 P94S</td>
<td>9.6 x 10^3</td>
<td>2.87 x 10^5</td>
</tr>
<tr>
<td>pRV 736 W100T</td>
<td>9.6 x 10^4</td>
<td>2.06 x 10^5</td>
</tr>
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<td>pRV 737 S91A/W100T</td>
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<td>8.9 x 10^5</td>
</tr>
<tr>
<td>pRV 738 T93V/W100T</td>
<td>0</td>
<td>1.1 x 10^6</td>
</tr>
<tr>
<td>pRV 739 P94S/W100T</td>
<td>0</td>
<td>7.9 x 10^5</td>
</tr>
<tr>
<td>pRV 740 L89P/T93V/W100T</td>
<td>0</td>
<td>6.7 x 10^5</td>
</tr>
<tr>
<td>Mock</td>
<td>4.3 x 10^6</td>
<td></td>
</tr>
</tbody>
</table>

* Titers represented are the mean ± SD of the replicated determinants from a representative experiment of a total of three performed.

† The same cells were reinfected after viral supernatants bearing mutant envelopes were removed and infected with wild-type virions with polybrene.
process of infectivity (Bae et al., 1997; Battini et al., 1995). Morgan et al. (1993) have identified a region between amino acids 81 and 88 that plays a critical role in binding of the SU to its receptor. A single point mutation in aspartate 84 and double mutations in arginines 83 and 95 in MoMuLV gp70 have been reported to yield

FIG. 3. Direct binding assay on NIH 3T3 cells by FACS analysis. X-axis represents the log of fluorescence, the Y-axis represents the cell number for each histogram, and the numbers represent the corresponding amino acid numbers in the envelope gp70 that were mutated (see text for details). (A) Studies with virions bearing envelopes with single mutations; faint histograms represent negative controls (virions carrying no envelopes), dark histograms represent virions bearing envelopes with mutations, and histograms with solid border lines represent the WT MoMuLV particles. (B) Studies with virions bearing envelopes with double and triple mutations, dark histograms represent negative controls (virions bearing no envelopes), histograms with dark border lines represent WT MoMuLV virions, and the histograms with faint border lines represent virions with envelopes with different double and triple mutations.
noninfectious particles (Bae et al., 1997; MacKrell et al., 1996).

To identify amino acids in SU that interact with its receptor, we have made mutations in the putative-receptor-binding domain of MoMuLV gp70. A region from amino acids 89 to 100 was chosen for the above study. The effect of various envelope mutations studied is summarized in Table 2. None of the point mutations in the envelope protein abolished viral infectivity completely. Mutations in amino acids 94 and 100 resulted in a thousand- and hundredfold reduction in viral titer, respectively (Table 1). The reduction in viral titer in P94S might have been a result of a low level of envelope incorporation into particles (see Fig. 2A). Double or triple mutants involving amino acid 100 resulted in loss of viral infectivity as no blue cells were detected in the lacZ transduction assay.

In this study, the only parameter changed in the infection process was the envelope protein. Hence it was thought that the less or noninfective nature of the virions bearing the mutant envelopes could have resulted from a loss of binding between the mutant envelopes and their cell surface receptors or a defective process of fusion and/or postfusion steps. Here, we have been able to discriminate among mutations in MoMuLV gp70 that result in a range of effects using transduction and FACS-binding assays. From all the point mutations created, only two mutant envelopes (P94S and W100T) resulted in a loss of viral titer, whereas the double and triple mutants involving these two residues abolished the viral infectivity completely. It is likely that multiple interactions exist between the amino acids of the envelope and that of the receptor. Disruption of any individual interaction by amino acid mutations might produce changes in affinity that are too small to be observed in a virus-based transduction assay. Envelope proteins bearing mutations in amino acids 89, 92, and 94 bind to the receptors with less efficiency than the wild-type virions. Interestingly, the binding studies using these above mutant envelopes are not reflected by viral titers. Viral particles with less than wild-type affinity might still have sufficient opportunity to bind to a cell to allow transduction. Mutation in amino acid W100 produces an envelope that retains very little binding affinity to its receptor. There is a possibility that W100 provides a point of contact between gp70 and its receptor. The fact that all mutants involving amino acid W100 significantly reduced or abolished viral infectivity provides evidence for this hypothesis. Recently Davey et al. (1999) have shown that W102 in Friend MuLV gp70 plays an important role in receptor interaction that further substantiates our result regarding the important role of W100 in Moloney gp70. From the above data, we would like to propose that the process of binding might not be a limiting factor for achieving wild-type titers in our functional assays. A similar result was reported by MacKrell et al. (1996), who found that certain mutations in
FIG. 4. Cell fusion (syncytia) on murine XC cells by different mutant and wild-type envelope proteins. A–K represent mutant envelopes pRV 730–pRV 740 (see text for details), L-WT MoMuLV gp70, and M-mock transfected cells.
amino acids 83, 86, and 87 produced wild-type titers but the binding to cellular receptors was far too low in comparison to the wild-type particles.

The incorporation of gp70 in viral particles was not affected by mutations except when amino acid 94 was changed where a significant loss of gp70 expression was observed. The viral titer was not reduced to the extent of the gp70 incorporation in mutant particles involving this amino acid. This might be due to the fact that any change in proline 94 altered the structure of the region between R83 and R95, thereby altering its antigenicity.

In all gp70 mutations, titers were restored to wild-type levels upon superinfection with WT virions. There is a possibility that mutant envelope proteins could bind to the receptor far less effectively, i.e., less tightly than their wild-type counterparts. This could mean that the mutant proteins, due to their low affinity for receptor binding, were easily displaced at the time of reinfection, thereby giving easy access to the wild-type envelope proteins. Hence, we are tempted to conclude that all the receptor molecules expressed on the cell surface were utilized by the wild-type proteins that could not be used by the mutant proteins due to their low binding affinity. Further structural analysis and binding studies between purified mutant envelope proteins and their receptors would resolve this issue.
MATERIALS AND METHODS

Construction of envelope mutants

Mutations were introduced in the wild-type envelope gene (pHIT 123, (Soneoka et al., 1995) by using a recombinant PCR technique (see Fig. 5). An external primer, 5′ CCG GAA TTC CGG ATG GCG CGT TCA ACG CTC 3′, was designed to bear an EcoRI site (underlined) that has complementarity with the 5′ end of the wild-type envelope gene. The other external primer used was 5′ TCT GAG TAT CCG GCT GTG AAC ACC TCA 3′, which has a BamHI site (lowercase letters). The sequences of the internal primers used for creating different envelope mutants were as follows: L89P forward, 5′ CCA GAG ACT GCG A A GA A CC T CC A AC C TC C AC C 3′; L89P reverse, 5′ GCA CCG AGG GGT GAG GGA GGT TAA AGG GCC 3′; T90V forward, 5′ AGA GAC TGC GAA GAA CCT TTA ACC CTC TCC ACC CC 3′; T90V reverse, 5′ GTA GAG TAC TGC GAA GAA CCT TTA ACC CTC TCC ACC CC 3′; S91A forward, 5′ GAC TGC GAA GAA CCT TTA ACC CTC TCC ACC CC 3′; S91A reverse, 5′ AGT GTT GCA CCG AGG GGT GGG GGA GGT TAA AGG GCC 3′; L92P forward, 5′ TGC GAA GAA CCT TTA ACC TCC CCC ACC CCT CGG TAC 3′; L92P reverse, 5′ GGG AGT GGT GCA CCG AGG GGT GGG GGA GGT TAA AGG GCC 3′; T93V forward, 5′ GAA GAC TGC GAA GAA CCT TTA ACC CTC TCC ACC CC 3′; T93V reverse, 5′ GAT GCA CCG AGG GGT GGG GGA GGT TAA AGG GCC 3′; P94S forward, 5′ GAC TGC GAA GAA CCT TTA ACC TCC CCC ACC CCT CGG TAC 3′; P94S reverse, 5′ GTC GAA GAC TGC GAA GAA CCT TTA ACC CTC TCC ACC CC 3′; W100T forward, 5′ ACC CCT TTA ACC CTC TCC ACC CCT CGG TAC 3′; W100T reverse, 5′ AGA GAC TGC GAA GAA CCT TTA ACC CTC TCC ACC CC 3′.

Both primary and secondary amplification reactions were carried out using Pfu turbo polymerase (Stratagene) at an annealing temperature of 60°C. The secondary PCR products were digested with EcoRI and BamHI, inserted into pBluescript KS(+) (Stratagene) cloning vectors, and sequenced. The final fragments were assembled in a mammalian expression vector that contains the cytomegalovirus promoter and the simian virus 40 origin of replication (Soneoka et al., 1995). The envelope plasmids bearing point mutations were named pRV 730–pRV 736.

The double and triple mutant envelope plasmids were constructed by using the plasmid containing the point mutations as templates in the amplification reactions. The plasmid bearing a mutation at one amino acid was used as template and sets of oligonucleotides bearing a mutation at another amino acid were used as primers. The sequences of double and triple mutants were con-

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TABLE 2

Summary of the Effect of Envelope Mutations on Their Expression in Virions, Viral Infectivity, Binding to Cellular Receptors, and Fusion to XC Cells

<table>
<thead>
<tr>
<th>Envelope used</th>
<th>Titer (CFU/ml)*</th>
<th>gp70 expression in virions*</th>
<th>Binding to NIH 3T3 cells*</th>
<th>Fusion to XC cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHIT 123 WT</td>
<td>1.3 × 10⁵</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>pRV 730</td>
<td>9.6 × 10⁵</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>pRV 731 T90V</td>
<td>1.4 × 10⁵</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>pRV 732 S91A</td>
<td>1.2 × 10⁵</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>pRV 733 L92P</td>
<td>1.2 × 10⁵</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
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<tr>
<td>pRV 734 T93V</td>
<td>9.6 × 10³</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pRV 735 P94S</td>
<td>9.6 × 10⁴</td>
<td>++++</td>
<td>±</td>
<td>+</td>
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<tr>
<td>pRV 736 W100T</td>
<td>1.2 × 10³</td>
<td>++++</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>pRV 737 S91A/W100T</td>
<td>0</td>
<td>++++</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>pRV 738 T93V/W100T</td>
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<td>pRV 740 L89P/P94S/W100T</td>
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<td>Mock</td>
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<td>−</td>
<td>−</td>
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</tr>
</tbody>
</table>

* Titers represented are the mean ± SD of the replicated determinants from a representative experiment of a total of three performed.

* The effect of envelope mutations was compared to the virions carrying WT envelope proteins (++++).
firmed by DNA sequencing. The envelope plasmids bearing double and triple mutations were named pRV 737–pRV740.

Cell lines, transfection, production of viruses, and transduction assay

NIH 3T3 (Anderson, 1979), XC (Rowe et al., 1970), and 293T cells (DuBridge et al., 1987) (obtained from Professor David Baltimore, Rockefeller University) were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum and appropriate antibiotics. Virus was produced by three-plasmid cotransfection by the CaPO₄ method as described previously (Soneoka et al., 1995) with 10 μg each of envelope expression plasmid, Gag-Pol expression plasmid pHIT 60 (Soneoka et al., 1995), and retroviral lacZ expression plasmid pHIT 111 (Soneoka et al., 1995). The cells were treated with sodium butyrate the next day at a final concentration of 10 mM and left for 12 h. Finally the cells were washed with phosphate-buffered saline (PBS) and refed with 5 ml of fresh medium. Viral supernatants were collected 12 h later and filtered through 0.45-μm-pore-size filters for functional analysis.

NIH 3T3 cells were seeded on six-well culture dishes at a density of 2 × 10⁵ per well the day before the transduction assay. The transduction assay was performed by infecting cells with 1 ml of viral supernatants (or with appropriate dilutions) with 8 μg of polybrene per milliliter for 4 h before 2 ml of fresh medium was added to each well. Viral titer was determined by counting the number of X-Gal-positive cells after staining as described previously (Soneoka et al., 1995).

Virus superinfection assay

NIH 3T3 cells were infected with virus carrying each of the mutant envelope proteins for 3 h at 37°C. Then the cells were superinfected with viruses carrying wild-type envelope proteins with 8 μg of polybrene per milliliter for 4 h before 2 ml of fresh medium was added to each well. Viral titer was determined by counting the number of X-Gal-positive cells after staining as described previously (Soneoka et al., 1995).

Fusion assay

293T cells were grown to 70% confluence on 6-cm-diameter dishes and transfected with either individual mutant or wild-type envelope expression plasmid by CaPO₄ transfection and treated with sodium butyrate. The next day the cells were harvested and cocultivated with 10⁶ XC cells on 6-cm-diameter dishes. Cell fusion was observed 24 h later with methylene blue staining and was photographed.

Protein analysis by Western blotting

Transfected cells were washed in PBS and harvested in REPA buffer (Maniatis et al., 1989) with 1 mM phenylmethylsulfonyl fluoride (Sigma). Total protein expressed in the cell was quantified by using the BCA reagent (BCA Inc., USA). Protein samples were prepared for both cell extracts and viral supernatants before being analyzed on the SDS–polyacrylamide gel. Equal quantities of proteins from the cell extracts and equal amounts of viral supernatants were loaded onto the SDS–polyacrylamide gel (with both the stacking and the resolving gels) along with the molecular weight markers. The particles were centrifuged at 16,000 g for 30 min before being used for Western blot analysis. Then the gel was transferred to the Immobilon (Millipore) membrane and blocked overnight at 4°C with 5% defatted milk containing 0.1% Tween 20 (Sigma). The blots were probed with goat anti-gp69/71 primary antibody (Quality Biotech Inc., Camden, NJ) at 1:3000 dilutions in the blocking solution for 1 h at RT. After being probed, the blots were washed thrice with 0.5% milk containing 0.01% Tween 20 for 10 min each. Then the blots were probed with the peroxidase-conjugated secondary antibody (Vector Laboratories) at 1:1000 dilutions for 1 h at RT. The blots were washed for 20 min twice in the same washing solution as above followed by a wash in PBS containing 0.01% Tween 20. The blots were finally washed in PBS before being developed using the ECL kit (Amersham).

Binding studies with fluorescence-activated cell sorter

NIH 3T3 cells (10⁶ in number) were trypsinized with trypsin–EDTA solution (Sigma) and washed with wash buffer containing PBS, 0.2% fetal calf serum, and 0.05% sodium azide. Following washing, the cells were incubated with 1 ml of virus supernatants at 4°C for 1 h and then the cell pellet was treated with goat anti-gp69/71 primary antibody (Quality Biotech Inc.) for 1 h at 4°C. Subsequently the cells were washed three times with cold wash buffer and treated with the secondary rabbit anti-goat FITC-conjugated antibody (Vector Laboratories). Finally the cells were washed twice with cold wash buffer and used to study the fluorescence using FACS-can (Becton Dickinson).

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