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Sequences Determining the pH Dependence of Viral Entry Are Distinct from the Host Range-Determining Region of the Murine Ecotropic and Amphotropic Retrovirus Envelope Proteins

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The entry of ecotropic and amphotropic murine leukemia retroviruses (MuLV) into cells was investigated by using viral vector particles carrying chimeric amphotropic-ecotropic envelope glycoproteins on their surface. Chimeras were made by joining, at or near the polyproline hinge, the N-terminal portion of the amphotropic (4070A) gp70 onto the C-terminal portion of the ecotropic (Moloney) gp70 and p15E (constructs AE2 and AE4) or vice versa (AE12). Transduction efficiency of the constructs was tested on target cells that either have only ecotropic receptors (CHO-2 and CHO-11 cells), only amphotropic receptors (mink lung fibroblasts and Cos 1 cells), or both types of receptors (NIH 3T3 cells). The assay made use of the fact that the mechanism for viral entry of ecotropic viruses is pH dependent while that of amphotropic viruses is pH independent. Treatment of target cells with NH_4Cl, which prevents the reduction of pH within endosomes, reduced the titers of viral particles bearing the C-terminal moiety from the ecotropic envelope but did not reduce the titers of particles which had a C-terminal moiety from the amphotropic envelope. In addition, in contrast to other low-pH-dependent enveloped viruses, brief acid treatment did not allow surface-bound viruses to bypass the NH_4Cl block. The results indicate that the pH dependence of viral entry is a property of the sequences C terminal to the polyproline hinge.

Retroviral transduction efficiency reflects the success of several events, including binding, fusion, reverse transcription, integration, and expression. In this study, we focused on the viral entry event of ecotropic and amphotropic type C murine leukemia retroviruses (MuLV). The virus envelope of MuLV is composed of two proteins, gp70 and p15E, which are processed from a precursor envelope protein (16). Sequence comparison and mutational analysis indicate that the transmembrane protein p15E carries a viral fusion factor at its amino-terminal end (4). The membrane surface glycoprotein gp70 carries the receptor binding domain at its amino-terminal end within the first 160 amino acids (1, 9), while much of the carboxylic end of gp70 is thought to interact with p15E (1, 16).

Five subgroups of type C MuLV have been reported (11). Of these, the ecotropic MuLV infects rodent cells only, while the amphotropic MuLV infects a wide range of species. The C-terminal halves of both the gp70 and p15E sequences of these two subgroups are highly homologous, while the N-terminal half, carrying the receptor binding domain, has only short areas of homology.

Similar to other enveloped viruses (7), MuLV is assumed to penetrate its host cell by a membrane fusion event. Amphotropic virus particles penetrate their target cells in a pH-independent mechanism by fusion with the cell's outer membrane as described for Sendai virus, human immunodeficiency virus, simian immunodeficiency virus, and human T-cell lymphoma-leukemia virus (6, 7, 14). In contrast, the ecotropic particles penetrate host cells in a pH-dependent mechanism by fusing with the endosomal membranes as described for influenza virus, vesicular stomatitis virus, and Semliki Forest virus (6, 7, 12). The low-pH environment in the endosome induces a conformational change in the envelope glycoprotein of these latter viruses which exposes and triggers the fusion factor (6, 12). An elegant model for influenza virus has recently been described (2).

We have begun to study the binding and entry of MuLV. Using recombinant DNA techniques, we have exchanged the sequences encoding the receptor binding domain of one MuLV subgroup for that of another (e.g., amphotropic-ecotropic switches). The chimeric constructs were made in or near the polyproline hinge, and they had the host range dictated by their receptor binding domain (9). We now use these amphotropic-ecotropic chimeras to study the entry function of these viruses. Using the lysosomotropic agent NH_4Cl to inhibit the acidification of endosomes, we found that viral entry is dependent on the structure of the envelope C-terminal to the polyproline hinge. Thus, in contrast to parental pH-dependent ecotropic MuLV and pH-independent amphotropic MuLV, we were able to construct chimeric viruses that behave as pH-dependent amphotropic MuLV or as pH-independent ecotropic MuLV.

MATERIALS AND METHODS

Envelope glycoprotein expression plasmids. pMLV-K, a plasmid encoding an infectious ecotropic Moloney MuLV envelope glycoprotein, was obtained from Sandra Ruscetti and has been described previously (8). pAM-MLV, a plasmid encoding the amphotropic MuLV (strain 4070A) envelope glycoprotein, was obtained from Dusty Miller and has been described elsewhere (11).

Genes for the envelope glycoproteins were incorporated into a bacterial plasmid (pBluescript KS+; Stratagene) and regulated by the cytomegalovirus large promoter and the simian virus 40 polyadenylation signal as previously described (9). The plasmids encoding the envelope glycoproteins for the

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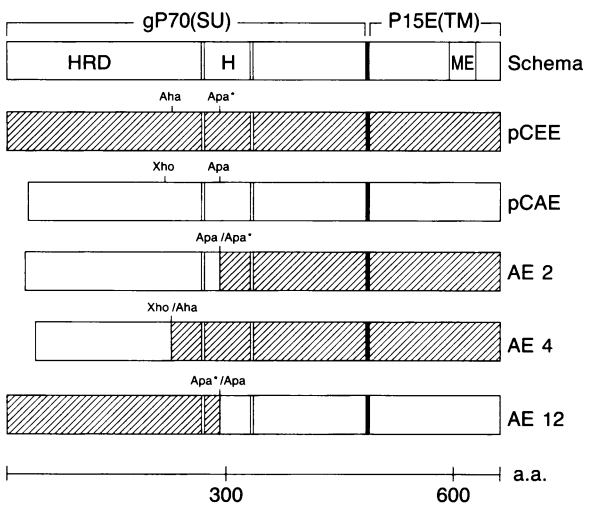

FIG. 1. Schematic structures of the ecotropic, amphotropic, and chimeric MuLV envelope glycoproteins. Construction of the plasmids carrying the different native and chimeric envelope glycoproteins is described in Materials and Methods and in reference 9. Ecotropic sequences are hatched; amphotropic sequences are unhatched. H, hinge, the hypervariable polyproline domain; SU, surface envelope glycoprotein; TM, transmembrane envelope glycoprotein; ME, membrane anchor domain; a.a., amino acids.

ecotropic and amphotropic subgroups were named pCEE and pCAE, respectively (9).

To facilitate production of chimeric envelopes, site-directed mutagenesis (Oligonucleotide Directed In-Vitro Mutagenesis System, version 2; Amersham) was used to create homologous restriction sites as described previously (9). Chimeras were made by switching an N-terminal portion (i.e., the host range domain [HRD]) of the ecotropic envelope glycoprotein for the amphotropic HRD to obtain AE2 (with the join in the polyproline hinge) and AE4 (with the join several dozen amino acids N terminal to the polyproline hinge) or switching an N-terminal portion of the amphotropic envelope for the ecotropic HRD to obtain AE12 (with the join in the polyproline hinge) (9). The envelope glycoprotein constructs are illustrated schematically in Fig. 1.

Cells. NIH 3T3 cells, mink lung fibroblasts, Cos 1 cells, GPL cells (an envelope glycoprotein-deficient 3T3 cell line containing the Gag and Pol proteins as well as the LNL6 retroviral vector, which encodes a neomycin resistance [Neo^r] gene [9]) and GPNZ cells (an envelope glycoprotein-deficient 3T3 cell line like GPL but containing the LBgSN retroviral vector, which encodes a β-galactosidase [β-Gal] gene as well as the Neo^r gene [9]) were maintained in Dulbecco's modified minimal essential medium (Biofluids) supplemented with 10% fetal calf serum (HyClone) and 2 mM glutamine (D_{10} medium). CHO-K1 cells (ATCC CCL 61) and CHO cells expressing the ecotropic receptor (CHO-2 and CHO-11) were described previously (5). These cells were cultured in alpha minimal essential medium containing 10% fetal calf serum and 2 mM glutamine (A_{10} medium) (5).

Transfection, transduction, titration, and β-Gal stain. DNA (30 μg) was transfected into GPL or GPNZ cells (5×10^5 cells in a 100-mm-diameter petri dish) by the calcium phosphate precipitate method (3). The medium was changed after 24 h, and at 48 h posttransfection the supernatant was collected and either used immediately or stored at −70°C. Supernatants were exposed to a low-pH environment (pH 5.0) by reducing the pH as previously described (10). Following 30 s or 10 min at 37°C (see footnotes to the tables), the pH was neutralized as described previously (7, 10).

TABLE 1. Binding to target cells of viral particles carrying different chimeric envelope glycoproteins[a]

Envelope construct	Binding to indicated target cells				
	3T3	Mink	CHO-2	CHO-11	CHO
None	−	−	−	−	−
pCAE	+	+	−	−	−
pCEE	+	−	+	+	−
AE2	+	+	−	−	−
AE4	+	+	−	−	−
AE12	+	−	+	+	−

[a] The different constructs were transfected into GPL cells, the GPL cells were incubated, and the supernatants were collected as described in Materials and Methods. All other experimental conditions, such as addition of the supernatants to the target cells and immunostaining procedures, were performed as previously described (9). Flow cytometric analyses were performed by FAST SYSTEM Inc., Gaithersburg, Md.

pH dependence of transduction was studied as follows. Cells were plated (2.5×10^4 cells in a 30-mm-diameter well) in 2 ml of D_{10} medium. After 18 to 24 h, the medium was replaced with 0.5 ml of either D_{10} or D_{10} containing 50 mM NH_4Cl, a lysosomotropic agent, and incubated for 30 min at 37°C. The medium was then replaced with 0.5 ml of viral supernatant, with or without 50 mM NH_4Cl, in the absence of Polybrene. Following 2 h at 37°C, the supernatant was replaced with 2 ml of either D_{10} or D_{10} containing 50 mM NH_4Cl, and after an additional 2-h incubation at 37°C, the cells were washed with 5 ml of D_{10} and incubated overnight in 2 ml of D_{10}. Polybrene was eliminated from the transduction medium in order to simulate the natural conditions of viral infection.

At 18 h posttransduction, the cells were selected for Neo^r with D_{10} containing 0.8 mg of G418 per ml for 12 to 14 days. Colonies were scored by microscopic counting and by methylene blue staining as described previously (9). For estimation of β-Gal-positive cells, the medium was replaced 18 h posttransduction with fresh D_{10}, and following 48 h at 37°C, the cells were stained as previously described (13). In similar experiments performed with CHO cells, A_{10} medium was used (5).

Fluorescence-activated cell sorter analysis was carried out as described in reference 9.

RESULTS AND DISCUSSION

Host range of the chimeric amphotropic-ecotropic envelope constructs. To identify the host range for the chimeric ecotropic-amphotropic constructs, binding and gene transfer (i.e., transduction) studies were performed with the parental and chimeric glycoprotein envelope constructs shown in Fig. 1.

Introduction of a plasmid encoding either parental (pCEE or pCAE) or a chimeric (AE2, AE4, or AE12) envelope glycoprotein gene into GPL (or GPNZ) cells yielded viral particles capable of transferring the Neo^r (or β-Gal and Neo^r) gene(s) to target 3T3 cells. Flow cytometry analysis revealed that binding of particles carrying the chimeric envelope glycoproteins to target cells was determined by their N-terminal portion (Table 1 and reference 9).

pCEE and the chimera AE12 (in which the ecotropic N-terminal half of gp70 was transferred to the appropriately truncated amphotropic gp70) promote specific binding to the ecotropic receptor expressed on the surface of 3T3, CHO-2,

TABLE 2. pH dependence of viral entry as measured by β-Gal gene transfer[a]

Plasmid	Relative β-Gal titer (%)[b]					
	3T3 cells			Cos 1 cells		
	NT	pH	NH_4Cl	NT	pH	NH_4Cl
pCEE	100	90	10	<1	<1	<1
pCAE	100	95	90	100	90	90
AE2	100	90	15	100	90	15
AE4	100	90	20	100	95	20
AE12	100	85	90	<1	<1	<1

[a] Transfection of the envelope glycoprotein plasmids into GPNZ cells, collection of supernatants, and treatment of supernatants in low pH (pH 5.0, 10 min) were performed as described in Materials and Methods. Viral supernatants (1 ml) were incubated with the target cells in the absence of Polybrene (2.5×10^5 cells in a 30-mm-diameter well), with or without 50 mM NH_4Cl, and the cells were stained for β-gal as described in Materials and Methods.

[b] Calculated by using the titer of each untreated sample as 100% in the same system of cells and construct. NT, cells and supernatants were not treated; pH, supernatants were pretreated in low pH (pH 5.0, 10 min); NH_4Cl, supernatants were incubated with the target cells in the presence of NH_4Cl.

TABLE 3. Effect of NH_4Cl incubation and/or low-pH treatment on viral particles[a]

Expt	Plasmid	Relative Neo^r titer (%)[b]			
		Untreated samples		Low-pH-treated samples	
		NT	NH_4Cl	NT	NH_4Cl
1	pCEE	100 ± 15	15 ± 5	70	10
	pCAE	100 ± 15	90 ± 10	75	70
	AE2	100 ± 20	12 ± 7	80	9
	AE4	100 ± 17	15 ± 5	90	7
	AE12	100 ± 15	90 ± 10	75	75
2	pCAE	100 ± 12	90 ± 10	85	80
	AE2	100 ± 15	15 ± 5	85	8
	AE4	100 ± 14	17 ± 8	75	8

[a] Transfection of the envelope glycoprotein plasmids into GPL cells, collection of supernatants, treatment with a pulse of low-pH environment (pH 5.0, 30 s), addition of the supernatant to the target cells (3T3 [experiment 1] or mink [experiment 2] cells) in the absence or presence of NH_4Cl, and estimation of Neo^r titers were performed as described in Materials and Methods. For the experiments involving low-pH-treated samples, the supernatants were incubated with the cells for 1 h at 4°C prior to the low-pH pulse in order to allow the viral particles to bind to the target cells.

[b] Calculated by using the titer of each untreated sample on untreated cells as 100% in the same system of cells and construct. The 100% values were between 8×10^2 and 3×10^3. The results for untreated samples are the means of at least five experiments. The results for low-pH-treated samples are the means of two experiments. NT, the samples were not incubated with NH_4Cl; NH_4Cl, the samples were incubated with NH_4Cl.

and CHO-11 cells. Similarly, pCAE and the chimeras AE2 and AE4 bind specifically to the amphotropic receptor on 3T3 and mink cells (Table 1). The titers for the chimeras on their target cells were similar to those obtained for the parental envelope glycoprotein plasmids (i.e., approximately 2×10^4 G418-resistant CFU/ml with Polybrene and about 10^3 without polybrene) (9).

Effect of NH_4Cl and low-pH environment on viral penetration of the chimeras. The chimeric MuLV envelope glycoproteins were used to determine regions of the envelope involved in viral entry. Introducing the envelope plasmids into GPNZ cells yields retroviral particles that can transfer the β-Gal and Neo^r genes into target cells carrying the appropriate receptor. Since all of the parental and chimeric particles studied were derived from the same producer cell line, they contained the same Gag and Pol proteins. Therefore, the ability of different viral particles to carry out gene transfer into different cells was primarily dependent on virus binding and penetration (virus-induced membrane fusion) and not on uncoating, reverse transcriptase activity, or integration.

It has been reported that unlike influenza virus, vesicular stomatitis virus, and Semliki Forest virus, the ecotropic envelope protein does not lose its fusogenic activity after a long exposure to a low pH (pH 5.0) (6, 7, 10). We first investigated whether our vector viral particles are resistant to incubation at low pH. Evaluation of the β-Gal titers upon use of each of the envelope glycoprotein constructs transduced into either 3T3 or Cos 1 cells demonstrated that the viral particles are not inactivated by a low-pH environment (Table 2).

Next, we investigated the effect of the lysosomotropic agent NH_4Cl on the penetration of these viral constructs. NH_4Cl can be used to inhibit the acidification of endosomes. Incubation of target cells with NH_4Cl raises the pH of the endosomal environment, thereby inhibiting the entry of those viruses that use a low-pH environment in endosomes as a mechanism of penetrance. The Moloney ecotropic MuLV is such a virus. As expected, NH_4Cl blocked the entry of the ecotropic pCEE-based particles into 3T3 cells, while amphotropic pCAE-based particles, whose entry is pH independent, were not affected by NH_4Cl with either 3T3 or Cos 1 cells (Table 2). NH_4Cl blocked the penetration of particles based on AE2 and AE4 (which have an amphotropic N-terminal gp70 moiety and an ecotropic C-terminal moiety) into both 3T3 and Cos 1 cells. Thus, although AE2 and AE4 bind like amphotropic virions, their entry is pH dependent like that of ecotropic virions. In addition, the entry of viral particles based on AE12, which binds like ecotropic virions, is pH independent like that of amphotropic particles (Table 2). Therefore, while the N-terminal portion of gp70 contains the receptor binding domain and consequently determines the host range of the virus, the C-terminal portion together with p15E is responsible for the mechanism of viral entry. Our attempts to define more precisely the C-terminal moiety responsible for fusion were unsuccessful, since initial efforts to make an amphotropic-ecotropic chimera in the last third of gp70 or in p15E have resulted in noninfectious virions.

We also investigated the effect of low pH on the penetration of viral particles already bound to target cells. Previous studies have indicated that brief exposure of influenza virus, vesicular stomatitis virus, or Semliki Forest virus to a low-pH environment, after the particles have already bound to the cells, is sufficient to trigger fusogenic activity of these viruses and induce fusion with the outer membrane of their respective target cells in a process that is not inhibited by lysosomotropic agents (15).

The effect of a low-pH environment on the triggering of parental and chimeric virions is presented in Table 3. GPL cells were chosen as a preproducer cell line since the Neo^r titers obtained with these cells are higher than the β-Gal titers obtained by using GPNZ cells. Only a small decrease in the Neo^r titer was observed for parental or chimeric virions which were preincubated at low pH (pH 5.0, 30 s) when transduction took place in the absence of NH_4Cl (Table 3). Incubation in the presence of NH_4Cl, after the low-pH pulse, has no influence on pCAE-based or AE12-based virions (which have amphotropic C-terminal moieties) but significantly inhibits the transduction efficiency of virions based on pCEE, AE2, or AE4 (which have ecotropic C-terminal moieties) (Table 3). Thus, a low-pH pulse after the virus is bound to the target cells does not eliminate the need for a low-pH environment in the

TABLE 4. Effect of NH_4Cl incubation and/or low-pH treatment on the entry of pCEE-based and AE12-based viral particles into CHO-2 and CHO-11 cells[a]

Cells	NH_4Cl	Relative Neo^r titer (%)[b]					
		pCEE particles			AE12 particles		
		NT	pH	pH pulse	NT	pH	pH pulse
3T3	−	100	90	75	100	87	75
	+	17	10	5	90	85	70
CHO-2	−	100	95	90	100	92	80
	+	25	20	13	95	90	77
CHO-11	−	100	95	90	100	100	86
	+	27	21	17	100	95	83
CHO	−	<1	<1	<1	<1	<1	<1
	+	<1	<1	<1	<1	<1	<1

[a] Transfection of constructs pCEE and AE12 into GPL cells, collection of supernatants, addition of the untreated supernatants (NT) or low-pH-treated (pH 5.0, 10 min) supernatants (pH) to the target cells, treatment with a low-pH pulse (pH 5.0, 30 s, after a 1-h incubation of supernatant with cells at 4°C) (pH pulse), and incubation with 50 mM NH_4Cl were performed as described in Materials and Methods and Tables 2 and 3, footnotes *a*.

[b] Estimated as described in Table 3, footnote *b*, as a mean of two experiments. The 100% values were between 1×10^3 and 3×10^3 CFU/ml for 3T3 cells and CHO-2 cells and between 8×10^3 and 2×10^4 CFU/ml for CHO-11 cells.

endosomes. These results indicate that the fusogenic activity of the ecotropic envelope glycoprotein is not triggered by a low-pH environment.

Entry of pCEE and AE12 into CHO cells. The chimeric AE12 viral particle was shown to be a pH-independent ecotropic MuLV when 3T3 cells were used as the target (Tables 1 and 2). To demonstrate that these novel viral particles utilize the same receptor as the pH-dependent ecotropic parent does, we studied the penetration of viral particles carrying the chimeric envelope, AE12, or the ecotropic Moloney MuLV envelope, pCEE, into nonpermissive rodent CHO cells (which have neither ecotropic nor amphotropic receptors) and into CHO cells that express only the ecotropic receptor (CHO-2 and CHO-11) (5).

While neither pCEE-based nor AE12-based viral particles bound to the control CHO cells under the conditions specified above, they did bind to CHO-2 and CHO-11 cells (Table 1). Likewise, similar Neo^r titers were observed when 3T3, CHO-2, or CHO-11 cells were transduced in the absence of NH_4Cl with either pCEE-based or AE12-based particles, while no transduction was observed when control CHO cells were used (Table 4).

When AE12-based viral particles were tested for pH dependence of transduction into CHO-2 and CHO-11 cells, the results were similar to those obtained for 3T3 cells (Table 4). Penetration of pCEE-based particles into CHO-2 and CHO-11 cells was inhibited by NH_4Cl, while no effect of the reagent was found when AE12-based particles were used. Thus, AE12-based virions do appear to use the same receptor as the parental ecotropic virus does. Finally, as with 3T3, Cos 1, and mink cells, little or no effect of low pH (short or long exposure) was found for either pCEE-based or AE12-based virions with CHO-2 or CHO-11 cells (Table 4).

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